Dynamics of cyanobacteria and cyanotoxins in Kranji Reservoir

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NANYANG
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**DYNAMICS OF CYANOBACTERIA AND
CYANOTOXINS IN KRANJI RESERVOIR**

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**SCHOOL OF CIVIL & ENVIRONMENTAL
ENGINEERING
NANYANG TECHNOLOGICAL UNIVERSITY
2012**

DYNAMICS OF CYANOBACTERIA AND CYANOTOXINS IN KRANJI RESERVOIR

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School of Civil & Environmental Engineering

A thesis submitted to the Nanyang Technological University
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ABSTRACT

Mass occurrences of toxic cyanobacterial blooms have been reported world-wide in eutrophic waters. Due to rapid urbanization, the reservoirs in Singapore will be exposed to pollution. Therefore, it is important to assess toxic cyanobacteria species in local reservoirs to ensure a safe drinking water supply. While publications on the occurrences and characterizations of toxigenic cyanobacteria are rapidly increasing, most of the studies were done in temperate or cold climate regions. To date, there are only limited reports on cyanobacterial blooms in South East Asian region. With high and uniform temperature throughout the year, cyanobacterial blooms in the tropics could have distinct behaviors compared to those in the temperate regions as responses to local climatic condition.

The temporal variations in cyanobacteria and microcystin production in a tropical reservoir, Kranji Reservoir in Singapore, were studied using molecular techniques and enzyme-linked immunosorbent assay (ELISA) respectively. Water samples were collected monthly from February 2008 to August 2009. Results from PCR and qPCR showed that *Microcystis* was the major microcystin producer. *Anabaena* spp. were found to coexist with the *Microcystis* spp. with Spearman's rho coefficient, $r_s = 0.498$ ($P < 0.001$). The average concentrations of *Microcystis* and *Anabaena* (equivalent to 16S rRNA) were 4.16×10^6 gene copies/mL and 4.47×10^4 gene copies/mL respectively. The average percentage of toxigenic *Microcystis* spp. was 55.92 % (SD 21.1 %), whereas no *Anabaena*-specific microcystin producing gene was detected. Microcystin-producing genes *mcyB* and *mcyD* were detected in all samples, together with the detections of *mcyA* and *mcyE* genes in 98 % and 96 % of the samples, respectively. However, a low detection rate was observed for *mcyC*. Due to the combined effects of high temperature, light and nutrient conditions in the tropics, the *Microcystis* cell concentrations were higher and more uniform compared to other *Microcystis* blooms reported in subtropical and temperate regions. Furthermore, the proportion of toxigenic *Microcystis* was considerably high and more stable throughout the year. Principle component analysis and multiple linear

regression analysis showed that total nitrogen and total phosphorus were positively correlated with the abundances of total *Microcystis* and toxigenic *Microcystis*. In addition, total nitrogen, pH and dissolved oxygen were positively correlated with the microcystin concentration. The total microcystin concentration measured using an ELISA kit ranged from below the detection limit to 14.4 µg/L with a mean concentration of 2.66 ± 2.80 µg/L, which is higher than those reported by local water agency, PUB, using LC-MSMS detection on 4 microcystin variants. This suggests a more thorough survey of natural toxin variants in local waters is needed.

The dynamics and genetic variation of cyanobacterial population in Kranji Reservoir were also examined. Quarterly samples were analyzed from February 2008 to August 2009 to reveal the genetic diversity of cyanobacterial community and *Microcystis* species based on the 16S-23S rRNA internal transcribed spacer (ITS). Clustering of the sequences show that *Microcystis*, *Anabaena/Aphanizomenon*, *Synechococcus* and *Oscillatoria* were the dominant genera in the reservoir, consistent with the morphological identification results by PUB. The *Microcystis* ITS phylogenetic tree can be divided into 4 clusters where 50% of the sequenced clones were grouped in Cluster II, which was shown to contain only microcystin producers. Temporal changes of ITS genotypes were observed but there was no spatial genetic difference between samples. Partial *mcyA* and *mcyH* gene sequences confirmed that *Microcystis* species was the sole microcystin producer in the reservoir.

Variability in the *mcyH* sequence encoding the putative ATP-binding cassette (ABC) transporter found in the microcystin synthesis genes cluster was also examined. It was found that the *mcyH* gene has greater variability in comparison with the *mcyA* gene. The nucleotide similarity in all sequenced clones was 0.988 ± 0.006 , corresponding to 43 acid amino variations in its 420 peptide sequence. Results from this study suggest that the *mcyH* sequence can be used as an alternative tool to evaluate the phylogeny of toxigenic *Microcystis* species.

Isolation and culturing efforts were conducted to obtain indigenous cyanobacterial species from the Kranji Reservoir. Two *Anabaena* spp., a *Limnothrix* sp., a *Synechococcus* sp. and two toxic *Microcystis* spp. were successfully isolated using a combination of culture medium enrichment, direct isolation and agar isolation methods. One toxic *Microcystis* sp. was further purified with the sulphide selection gradients method to attain axenic culture suitable for further experiment.

The effects of different light spectra on *Microcystis* growth rate and toxin production were investigated using the local axenic *Microcystis* strain. Four *Microcystis* culture batches were prepared and grown under white, red, green and blue lights for 14 days. The cell number and cell quotas for microcystin (Q_{MCYST}), chlorophyll-a (Q_{chl}), phycocyanin (Q_{PC}) and microcystin synthesis *mcyE* gene (Q_{mcyE}) were measured from the exponential to stationary phases. During exponential phase, the blue light microcosm demonstrated significantly lower specific growth rate while microcystin content per cell was enhanced under red light irradiance. A positive correlation was found between the specific cell growth rate and microcystin production rate. Q_{chl} turned out to be the most suitable parameter in estimating Q_{MCYST} . These results suggest that microcystin is possible a constitutive metabolite, and its quota in cells is closely associated with the growth rate and chl-a quota. Data from the controlled experiment showed that light quality/spectra have positive impacts on the growth and toxin synthesis of *Microcystis* species. Results of this study support the hypothesis that photosynthesis may be involved in the modulation of microcystin production.

PUBLICATIONS

Peer-reviewed publication

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Te, Shu Harn, Gin, Karina Yew-Hoong and Thompson, Janelle R. (2011) *Genetic diversity of cyanobacterial blooms in a tropical reservoir using the 16S-23S rRNA internal transcribed spacer and mcyH gene*. 14th International symposium on Microcystis studies in Asia, 5 – 7 April 2011, Wuhan, China. (Poster presentation)

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LIST OF SYMBOLS / ABBREVIATIONS

ABC transporter	ATP-binding cassette transporter
ADDA	3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid
ANOVA	analysis of variance
APC	allophycocyanin
APHA	American Public Health Association
ATP	adenosine-5'-triphosphate
BLAST	basic local alignment search tool
bp	base pair
BSA	bovine serum albumin
CCL	contaminant candidate list
CE	capillary electrophoresis
Chl-a	chlorophyll-a
cpc	c-phycocyanin
C _t	threshold cycle
df	degrees of freedom
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DO	dissolved oxygen
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionization
HPLC	high performance liquid chromatography
IPTG	isopropyl β -D-1-thiogalactopyranoside
ITS	internal transcribed spacer
KMO	Kaiser-Meyer-Olkin
KW test	Kruskal-Wallis test
LB	lysogeny broth

LC-MS/MS	liquid chromatography/mass spectrometry/mass spectrometry
LD ₅₀	lethal dose 50
LPS	lipopolysaccharides
MALDI-TOF	matrix assisted laser desorption ionization time of flight
MLR	multiple linear regression
MW	molecular weight
N	nitrogen
NaPi	sodium phosphate
NBD	nucleotide binding domain
NIES	National Institute for Environmental Studies
NMR	nuclear magnetic resonance
NMT	N-methyltransferase
NOAEL	no observed adverse effect level
NRPS	non-ribosomal peptide synthetases
P	phosphorus
PAR	photosynthetically active radiation
PC	phycocyanin
PCA	principal component analysis
PCC	Pasteur Culture Collection
PC-IGS	phycocyanin intergenic spacer
PCR	polymerase chain reaction
PDA	photodiode-array
PE	phycoerythrin
pgp	P-glycoprotein
PKS	polyketide synthases
PPIA	protein phosphatase inhibition assay
PS	peptide synthetases
PSI/II	photosystem I/II
PUB	Public Utilities Board
qPCR	quantitative real-time PCR
rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphism

RNA	ribonucleic acid
<i>rpo</i>	RNA polymerase
rRNA	ribosomal RNA
r_s	Spearman's coefficient
RT-PCR	reverse transcriptase PCR
RuBisCO	Ribulose-1,5-bisphosphate carboxylase oxygenase
SOC	super optimal broth with catabolite repression
SPE	solid phase extraction
Taqman	Taq Polymerase + PacMan
TDS	total dissolved solid
T_m	melting temperature
TMD	transmembrane domain
TN	total nitrogen
TOC	total organic carbon
TP	total phosphorus
tRNA	transfer RNA
TSI	trophic state index
TSS	suspended solid
USEPA	United States Environmental Protection Agency
UV	ultraviolet
Vis	visible light
WHO	World Health Organization

CHAPTER 1

Introduction

1.1 Toxic Cyanobacteria and Water-related Health Problems

Cyanobacteria, also known as blue green algae or cyanophyceae, are gram-negative prokaryotes containing chlorophyll-a and phycobilin pigments, which provide the characteristic blue-green color for many species. As photosynthetic prokaryotes evolved from ancient times, cyanobacteria have been important primary producers in marine and freshwater environments. They play significant roles in the balance of many biogeochemical processes including carbon, nitrogen and sulfur cycles (Hayes *et al.*, 2007). However, massive proliferation of cyanobacteria in eutrophic waters due to global environmental change and human activities deteriorate water quality for drinking and recreational purposes, and lead to ecological problems (Duan *et al.*, 2009). Harmful cyanobacterial blooms have been recognized as an emerging issue causing human health problems in the late twentieth century, especially for communities which depend on surface waters as drinking water resources (Bartram *et al.*, 1999b; Ouellette and Wilhelm, 2003). Based on data collected over 30 years, it has been shown that harmful cyanobacterial incidences have increased by 10-fold since the 1960's (Carmichael, 2008).

Major cyanobacterial genera known for their toxin producing abilities are *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Lyngbya*, *Microcystis*, *Nodularia*, *Nostoc* and *Planktothrix* (Kardinaal and Visser, 2005). Toxic cyanobacterial blooms associated with animal deaths or water resource contaminations have been documented globally (Figueiredo *et al.*, 2004). In severe incidents, cyanotoxin contamination can cause human poisonings and deaths. In 1979, the "Palm Island Disease" in Australia that caused 146 clinical cases with hepatitis-like symptoms was a result of *Cylindrospermopsis raciborskii* bloom lysis in a reservoir (Kuiper-

Goodman et al., 1999; Schembri et al., 2001). For the infamous “Caruaru Syndrome” in Brazil, 76 patients died after they were treated with dialysis water polluted by microcystin and 52 of the deaths could be attributed by the syndrome (Carmichael et al., 2001). There is also the possibility of trace levels of toxins in drinking water which may accumulate in human or animal tissue over time: in 2007, a drinking water crisis took place when a prolonged *Microcystis* bloom occurred in Lake Taihu, China. Two million people had no access to safe drinking water supply for at least a week (Qin et al., 2010).

As a preventive step to protect the public from adverse health effects, WHO adopted a guideline value of 1 µg/L for microcystin-LR in drinking water in 1998. It is based on a tolerable daily intake of 0.04 µg microcystin per kg body weight (WHO, 1998). Following this, several countries have developed regulations or guidelines for cyanobacteria or cyanotoxins in drinking water. For example, in 2005, the United State Environmental protection agency (USEPA) announced cyanobacteria as one of the microbial contaminants in the second Drinking Water Contaminant Candidate List (CCL2); four years later, the third Contaminant Candidate List (CCL3) has added cyanotoxins as chemical contaminants to be regulated in the future (<http://water.epa.gov/scitech/drinkingwater/dws/ccl/index.cfm>). In Brazil, a mandatory standard of 1 µg/L for microcystin has been implemented (Burch, 2008).

1.2 Research on Harmful Cyanobacterial Bloom: The Current State

In addition to traditional microscopic identification and counting methods, molecular detection methods have gained popularity in recent years by providing rapid and sensitive analysis for detecting and quantifying cyanobacteria. Discoveries of genes coding for toxin production and toxin biosynthesis pathways enable the design of specific PCR (Polymerase Chain Reaction) primers to detect potential cyanotoxin producers (Neilan et al., 1999). Cyanobacteria can produce cylindrospermopsins, microcystins, nodularins (hepatotoxins), anatoxins and

saxitoxins (neurotoxins) as secondary metabolites by using peptide synthetases (PS) and polyketide synthases (PKS) (Hutchinson and Fujii, 1995; Schembri et al., 2001). The detection of different PKS and PS genes are used to demonstrate the abilities to produce toxin in genera *Cylindrospermopsis*, *Anabaena*, *Microcystis*, *Nostoc* and *Planktothrix* (Dittmann and Borner, 2005; McElhiney and Lawton, 2005; Schembri et al., 2001).

Molecular methods have been successfully applied to a diverse range of environmental samples. The primer sets used in PCR amplifications usually target DNA fragments from cyanobacterial 16S-rDNA, RNA polymerase gene, c-phycocyanin BA, species-specific genes, microcystin gene cluster, PS genes and/or PKS genes (Bittencourt-Oliveira, 2003; Fergusson and Saint, 2000a; Oberholster et al., 2006; Rinta-Kanto et al., 2005; Schembri et al., 2001; Tillett et al., 2001; Vaitomaa et al., 2003; Valerio et al., 2005). Molecular techniques are not only useful for revealing the presence of target genes, but they are also efficient tools that can differentiate cyanobacterial species to genotype level. Genetic variability of different cyanobacterial species can be compared based on specific gene sequences or intergenic spacer sequences. Phylogenetic analyses on these DNA sequences are capable of showing the evolutionary pathways of these bacteria (Bozarth et al., 2010; Briand et al., 2009; Iteman et al., 2000; Neilan et al., 1995; Nishizawa et al., 2007; Otsuka et al., 1999; Sabart et al., 2009; Tillett et al., 2001).

Many studies have been done to investigate the occurrence, chemical properties and effects of cyanotoxins. However, the function and regulation of toxins and the reasons why they are produced are still inconclusive (Osswald et al., 2007). Understanding correlations between toxin production and ecological/environmental factors help to reveal the physiological function of cyanotoxins. A number of such studies focused on the conditions that regulate and stimulate the production of cyanotoxins. Both field and laboratory studies showed that correlations exist between toxin content and environmental factors such as nutrients (nitrogen, phosphorus, iron), light and temperature (Long et al., 2001; Rapala et al., 1997) and turbulence (Carmichael, 2008). However, the results are sometimes

conflicting (Kardinaal and Visser, 2005), thus making generalizations between toxin synthesis and environmental factors difficult. These findings still do not explain the tremendous toxin level variability in cyanobacterial blooms, which can differ by five orders of magnitude (Kardinaal and Visser, 2005). It is now suggested that the huge difference could be due to the successive replacement of toxic and non-toxic strains in the genera (Carrillo et al., 2003; Haande et al., 2007; Kardinaal and Visser, 2005). This is an area where molecular approaches could be used to study the dynamics of toxic and non-toxic strains within a cyanobacterial bloom.

1.3 Research Aims, Questions and Objectives

In the past, water treatment plants in Singapore typically withdrew water from reservoirs that were situated in protected catchments. However, with rapid economic development and urban expansion, these catchments are coming under increasing urbanized pressure and in fact, the newer catchments are completely exposed to densely populated urbanized environments. Under such conditions and given the high light and temperature conditions of the tropics, eutrophication is common in these water bodies. In many cases, the reservoirs in Singapore are dominated by cyanobacteria.

The best way to control harmful effects from cyanobacteria and their toxins on human and animals is to prevent the formation of blooms. Singapore is a heavily urbanized city and categorized as a “water-stressed” country. The constructed reservoirs are not only important as water resources but also serve as the safety barriers for the water reclamation program in the country. Maintaining good water quality within these reservoirs is therefore an important objective. Thus, characterizing and understanding the occurrence and dynamics of cyanobacteria and their cyanotoxins in our tropical water bodies is necessary for the management of water resources. Despite the great interest in cyanobacteria, research on tropical harmful cyanobacterial blooms is relatively limited.

This thesis aims to provide comprehensive data on the surveillance of cyanobacterial blooms in a Singapore reservoir. Information produced by this study is important for the understanding of cyanobacteria and cyanotoxin (microcystin) occurrence and dynamics in an urbanized tropical environment. To achieve this, the following research questions are addressed:

1. (a) How does a tropical cyanobacterial bloom fluctuate in a eutrophic reservoir?
- (b) What are the major dominant cyanobacterial genera in Kranji Reservoir? Are these cyanobacterial genera able to produce toxic compounds?
- (c) Do cyanobacteria and cyanotoxin correlated well with environmental variables?
2. (a) Are there spatial and temporal genetic variations in tropical cyanobacterial bloom?
- (b) Do the cyanobacterial species from Singapore waters have different genetic makeup compared to those in other regions or temperate countries?
3. (a) How does the cyanotoxin quota change in cells?
- (b) Does different light quality influence toxin production?

To answer these research questions, the objectives of this study are as follows:

1. To develop and optimize molecular methods for the detections and quantification of local cyanobacterial species.
2. To study the dynamics of toxic cyanobacterial blooms, assess the cyanotoxins concentrations and investigate the correlation between environmental factors and the abundance of toxic cyanobacteria in Kranji Reservoir.

3. To examine the genetic diversity of natural cyanobacterial populations in Kranji Reservoir.
4. To isolate indigenous cyanobacterial species so that the mechanism of cyanotoxin production can be studied.
5. To examine the toxin quotas of *Microcystis* species at different growth stages.
6. To study the effect of the wavelength of light on the growth rate and toxin production of a local *Microcystis* species.

1.4 Thesis Structure

This thesis is organized into 6 chapters. Chapter 2 is a detailed literature review on the occurrence, detection, quantification and characterization of cyanobacterial blooms reported worldwide, focusing on toxic *Microcystis* species. Toxin producing behaviour in response to different growth factors is also discussed. Chapter 3 describes an occurrence study of cyanobacterial blooms in Kranji Reservoir and the correlations of cell abundance and toxin with various environmental variables. Chapter 4 discusses the genetic diversity of cyanobacterial and *Microcystis* populations in Kranji Reservoir and the isolation of indigenous cyanobacterial species. Based on the field data and isolates collected in Chapters 3 and 4, a further in-depth study was conducted under laboratory controlled conditions to examine the response of cyanobacteria to specific environmental factors. Based on recent literature, there is evidence to suggest that the synthesis of cyanotoxin, microcystin, is light-dependent. However, it is hard to access the effect of light on microcystin *in-situ* because (i) light intensity and wavelength profile change throughout the day and (ii) the vertical migration ability of cyanobacteria via buoyancy regulation affects the light irradiance received by the cells. Therefore, a controlled experiment was carried out in Chapter 5, focusing on the effects of different wavelengths of light on the growth and toxin synthesis of a local *Microcystis* isolate and evaluating the changes in the toxin quotas of cells. Finally,

Chapter 6 provides important conclusions of this thesis and some recommendations for future study.

CHAPTER 2

Literature Review

2.1 Cyanobacteria

Cyanobacteria are important primary producers which contribute to the bioenergetic foundation of higher trophic levels in marine, brackish and freshwater environments (Bartram et al., 1999b; Ehrenreich et al., 2005). Due to their plant-like photosynthetic ability and similar size to algae, they are still referred to as blue-green algae. However, the intracellular organization of cyanobacteria, i.e. cell wall structure containing peptidoglycan, no membrane-bound organelles and protein synthesis mechanism are of prokaryotic type. They are therefore, also classified as bacteria (Mur et al., 1999; Rantala, 2007).

Cyanobacteria, classified as oxygenic phototrophs, are among the oldest forms of life on earth. Their photosynthetic capability appears to be responsible for the production of primitive oxygen on earth (Osswald et al., 2007) and thus they are important for the evolution of life forms. Cyanobacteria need only water, carbon dioxide, simple inorganic substances and light to survive (Mur et al., 1999). They can be found everywhere in all kinds of habitats including Arctic and Antarctic lakes, hot springs, acidic soils, snow, ice, rock and deserts (Kaebernick and Neilan, 2001; Mur et al., 1999; Rantala, 2007) due to their abilities to endure infertile environments and extreme thermal conditions. These abilities suggest the early organisms on earth were thermophilic and thus able to survive in oceans heated by volcanoes and hot springs (Mur et al., 1999).

Cyanobacteria form symbiotic relationships with plants, fungi and animals (Mur et al., 1999). It is believed that endosymbiosis between a protist and cyanobacterium caused the formation of a membrane-bound organelle, the chloroplast (Rantala, 2007). Common forms of cyanobacteria are unicellular,

multicellular filamentous or colonial with cell sizes varying from ≤ 2 μ m to 40 μ m in diameter (Kaebernick and Neilan, 2001). Unicellular cyanobacteria, for example order *Chroococcales*, appear as single cells and reproduce by binary fission (Mur et al., 1999). They can also aggregate together by secreting a slimy matrix during the formation of colonies. Multicellular cyanobacterial species contain a chain of cells, called a trichome. This trichome can be straight or coiled and consist of essentially identical cells (*Oscillatoriales* order) or cells differentiated into heterocysts (*Nostocales* and *Stigonematales* orders). All cyanobacteria reproduce asexually (Mur et al., 1999).

A cyanobacterial bloom event can produce different secondary metabolites including peptides, alkaloids, polyketides and polysaccharides which may be toxic to other bacteria, lower and higher eukaryotes (Borner and Dittmann, 2005; Hisbergues et al., 2003). Blooms also cause unpleasant odour and taste, hypoxia, fish kills and alter the nutrient cycle food web dynamics (Paerl and Fulton-III, 2006). Release of cyanotoxins into a water body associated with drinking water or recreational purposes poses a threat to the environments as well as to humans and livestock (Bartram et al., 1999a). Therefore, enormous efforts were focused on the detection, control and removal of cyanotoxins in past decades (Pearson and Neilan, 2008).

2.2 Occurrence and Ecology of Cyanobacteria

Harmful cyanobacterial blooms have been present for centuries in eutrophic inland, estuarine and coastal waters (Paerl and Fulton-III, 2006). The occurrence of a certain genus and species is likely to be influenced by the regional chemistry and local climatic conditions. For example, *Cylindrospermopsis* spp. is usually found in tropical regions but not in temperate regions; *Anabaena* blooms have been commonly reported in temperate regions; while *Microcystis* has been widely detected in both tropical and temperate regions (WHO, 1998). In temperate countries, cyanobacterial diversity decreases in the warm periods and usually ends

up with the rapid dominance of a sole species, usually *Microcystis*, *Planktothrix* or *Anabaena* (Osswald et al., 2007). Cyanobacterial blooms usually occur in water bodies that are shallow, containing large amounts of inorganic nutrients such as nitrogen (N) and phosphorus (P), with lower molar ratio of total and soluble N to P (<15), a water temperature between 15 to 30°C and pH between 6 and 9 (Oberholster et al., 2004; Paerl and Fulton-III, 2006; WHO, 1998).

Cyanobacteria have some special adaptations which allow them to outcompete other phytoplankton present in the water. They have higher affinity for nitrogen and phosphorus compared to other phytoplanktonic organisms that enable them to thrive better under nutrient limiting conditions (Msagati et al., 2006). The nitrogen-fixing ability is a fundamental metabolic process for some species of cyanobacteria such as *Anabaena*, *Nostoc*, *Calothrix* and *Oscillatoria*. It gives these species the simplest nutritional requirement and reproductive success under nitrogen limited conditions (Mur et al., 1999). Chlorophyll-a is the major pigment for light harvesting in cyanobacterial cells. Sometimes, pigments such as phycocyanin (blue protein) and phycoerythrin (red protein) which allow harvesting of green, yellow and orange light can also be found. These pigments, together with chlorophyll-a, provide efficient light energy absorption which cannot be found in other phytoplankton species (Mur et al., 1999).

Many cyanobacterial species possess gas vesicles. These gas vesicles make them buoyant by giving them an overall lower density than that of water (Msagati et al., 2006). By regulating the buoyancy, they can actively seek water depths with optimal growth conditions (WHO, 2003a). It was also found that the production of gas vesicles decreased under high light intensity, suggesting vesicles might aid in shielding the cells from excess sunlight (Waaland et al., 1971). Cell buoyancy is mainly controlled by carbohydrate production from the photosynthetic process. This mechanism will break down in the absence of carbon dioxide. Despite no photosynthesis at night, cells can still adjust their buoyancy by reducing their carbohydrate content through respiration (WHO, 1998). This unique feature

provides strategic advantages to cyanobacteria over other plankton species in the water (Oberholster et al., 2004).

The higher optimum temperature for most cyanobacteria (20 – 25°C) compared to other green algae and diatoms, explains why cyanobacterial blooms form during summer in temperate water bodies and subside in winter when temperature is low (Mur et al., 1999; Oberholster et al., 2004). Studies also found that temperature correlates to toxin variants produced. At temperatures below 25°C, *Anabaena* sp. produces microcystin-LR, whereas higher temperature favours microcystin-RR production (Msagati et al., 2006). Turbulent and high flowing water is unfavorable for the growth of cyanobacteria as it is hard to maintain their position in the water column. Although heavy rain falls disturb the hydraulic stability of water bodies creating unfavourable conditions for cyanobacteria, it can result in runoff with high nutrient content, which encourages the formation of blooms (WHO, 1998).

2.3 The Cyanotoxin – Microcystin

Globally, around 60% of the cyanobacterial samples investigated contain toxins (WHO, 2003a). Toxic cyanobacterial blooms are most frequently associated with the cyclic peptide toxin, microcystin (Sivonen and Jones, 1999), and the most common bloom forming genus is *Microcystis* (WHO, 2003a). *Microcystis* populations have been described in water bodies ranging from small ponds to large lakes, distributed all over the world (Visser et al., 2005). Their success in such diverse water bodies has been attributed to the following reasons: (1) high nutrient loadings; (2) increase in pH and decrease in carbon dioxide; (3) interaction between the light intensity and stability of the water column; (4) cell tolerance to low dissolved oxygen concentration and low redox potential which affects the availability of sulphur, iron, and other metals; (5) cell size (colonies) and toxicity reduce the grazing pressure from zooplankton; and (6) low N:P ratio (Visser et al., 2005).

Cyanotoxins can be categorized into three major groups according to their chemical structures, namely cyclic peptides, alkaloids and lipopolysaccharides (LPS) (Sivonen and Jones, 1999). Meanwhile, classification based on the toxicological actions divides the cyanotoxins into hepatotoxins (microcystins, nodularins, and cylindrospermopsins), neurotoxins (anatoxin-a, anatoxin-a(s), saxitoxin and neosaxitoxin), tumor promoters (microcystins and LPS) and dermatotoxins (lyngbyatoxin A, apysiatoxins and LPS) (Msagati et al., 2006).

2.3.1 Chemical structure, toxicity and mechanism of action

The cyclic peptide toxins of the microcystins and nodularin family are the most commonly found cyanobacterial toxins in freshwater and brackish water blooms (Sivonen and Jones, 1999). They contain either five (nodularins) or seven (microcystins) amino acids with two ends joined and condensed to form cyclic peptides. Microcystins consist of more than 80 structural variants which share a general structure of cyclo-(D-alanine¹-X²-D-MeAsp³-Z⁴-ADDA⁵-D-glutamate⁶-Mdha⁷) (McElhiney and Lawton, 2005; Sivonen and Jones, 1999) as shown in

Figure 2.1 General structure of heptapeptides microcystins (Sivonen and Jones, 1999).

Microcystin consists of three D-amino acids (alanine¹, erythro- β -methyloaspartic acid³, γ -linked glutamic acid⁶), N-methyldehydroalanine (Mdha⁷), two L-amino acids variable (X² and Z⁴) and the amino acid, ADDA⁵. Although structural differences can arise in all seven amino acids, microcystin variants are usually characterized according to their methylation pattern and two L-amino acids, X and Z, at positions two and four (McElhiney and Lawton, 2005). The ADDA (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, is the most unusual part in microcystin molecules (Sivonen and Jones, 1999). In solution, the ADDA glutamate parts for both microcystins and nodularins have similar shapes.

Most of the microcystins are soluble in water and unable to penetrate lipid membrane of cells but can enter the cells through membrane transporters. Therefore, its target organ in mammals is mostly the liver (Sivonen and Jones, 1999). Studies have found the ADDA region is crucial for the interaction with the protein phosphatases and thus, determines the toxicity of the toxin molecules (Sivonen and Jones, 1999). Separating the ADDA component from the cyclic peptide will eliminate toxicity of both components (IACR, 2010). Microcystins inhibit the serine/threonine protein phosphatases 1 and 2A of eukaryotic cells (MacKintosh et al., 1990) which serve as important regulators to maintain homeostasis of the cell (Cohen, 1989). Protein phosphatases are enzymes which remove the phosphate groups from their substrates in the post-translation modification. Inhibitions of these enzymes cause higher phosphorylation of target protein such as tumour suppresser proteins which results in excessive cell proliferation and tumour formation (Kuiper-Goodman et al., 1999). The effect in liver cells is the hyper-phosphorylation of cytoskeleton filaments that leads to cell disruption (Kuiper-Goodman et al., 1999). The inhibitory effect of microcystins on protein phosphatase 2A can be neutralized by anti microcystins/polyclonal antibodies (Lin and Chu, 1994).

Table 2.1 gives a summary of the toxicities of microcystin variants through the intraperitoneal route in mice. The LD₅₀ for microcystin-LR in mice ranges from 25 to 150 μ g per kg body weight (bw) with an average of 50 μ g/kg bw (Kuiper-

Goodman et al., 1999). There is no evidence to show the hydrolysis of microcystins by peptidases in the stomach, therefore microcystins can pass through the intestinal barrier and be absorbed by the body (Kuiper-Goodman et al., 1999). A total daily intake of 0.04µg microcystin-LR/kg bw/day was calculated by applying a safety factor of 1,000 on the no observed adverse effect level (NOAEL). Based on a 60kg adult consuming 2 litres of water daily and assuming 80% of microcystins exposure from drinking-water, the resulting WHO guideline value for microcystin-LR has been set at 1 µg/L in drinking-water (WHO, 1998).

Table 2.1 Reported LD₅₀ on microcystins variants (Sivonen and Jones, 1999).

Microcystin	Molecular Weight	LD ₅₀ µg/kg bw*	Cyanobacteria
Microcystin-LA	909	50	<i>M. aeruginosa</i> , <i>M. viridis</i>
Microcystin-AR	952	250	<i>Microcystis spp.</i>
Microcystin-LR	994	50	<i>M. aeruginosa</i> , <i>M. viridis</i> , <i>A. flos-aquae</i>
Microcystin-LY	1,001	90	<i>M. aeruginosa</i>
Microcystin-RR	1,037	600	<i>M. aeruginosa</i> , <i>M. viridis</i> , <i>Anabaena spp.</i>
Microcystin-YR	1,044	70	<i>M. aeruginosa</i> , <i>M. viridis</i>

* bw: body weight

Pure cyclic microcystins are stable and resistant to hydrolysis or oxidation at pH 7. They can remain unspoiled after boiling and persist in natural water for months or years in dark conditions (Sivonen and Jones, 1999). A 90% breakdown by hydrolysis at pH 1 needs around 10 weeks, while at pH 9 it needs about 12 weeks. Microcystins are degraded in natural water but a lag phase is usually preceded before significant degradation takes place. If a lag phase is present, the half-life for microcystin is about 9 -10 days or even longer up to 21 days (WHO, 1998). In full sunlight, microcystin undergoes photochemical degradation and isomerisation. The breakdown process will be enhanced by cell pigments in water (Tsuji et al., 1994). In the presence of sunlight and pigment, 90% removal of

microcystin takes two to six weeks. A faster breakdown has been reported in the presence of humic substances in field water (Sivonen and Jones, 1999).

2.3.2 Toxicology, contamination and ecological impacts

Livestock deaths as a result from drinking cyanobacterial scum water have been reported in the Mediterranean, continental and temperate climate countries and mostly from South Africa (Falconer, 2005). The earliest reports in Africa by Steyn in 1943 and 1945 described thousands of cattle and sheep poisoned within 25-30 years. The Vaal Dam in South Africa, one of the major contaminated sites which was described as “green pea soap”, has caused deaths of horses, mules, donkeys, dogs, poultry and fish (Harding and Paxton, 2001). In the U.S. and Canada, a survey of cyanobacterial blooms in Minnesota has found that 87% of the blooms contained *Microcystis aeruginosa* and 82% (49 out of 60) tested samples were toxic (Falconer, 2005).

Fewer human poisoning cases due to drinking water have been reported compared to animal poisoning for two possibilities: bad taste and odour from contaminated water deter people from consumption; cyanotoxin poisonings were mistaken as gastrointestinal diseases because of similar illness symptoms (Falconer, 2005). However, human poisoning by microcystin can be devastating, as seen in the gastro-enteritis epidemic in Brazil which caused 88 deaths and approximately 2000 casualties in 1988 due to cyanobacteria contamination (*Anabaena* and *Microcystis*) at a water treatment plant (Falconer, 2005; Kuiper-Goodman et al., 1999). Another case in 1996 in Caruaru, Brazil, occurred where 76 patients died of acute liver failure after haemodialysis (Carmichael et al., 2001). It was later found that these patients were treated with dialysis water containing 19.5µg/L microcystins, equivalent to an intravenous injection of toxins into their bodies (Falconer, 2005).

Apart from acute toxic effects, chronic effects via contaminated food and drinking water are highly possible when long-term contact of toxin is inevitable (Zhang et al., 2009). In a recent study, microcystins were identified in human serum

from a group of fishermen at Lake Chaohu, China who had chronic exposure to contaminated water. The fishermen also showed symptoms of hepatocellular damage (Chen et al., 2009). Besides this, the high incidence of primary liver cancer in Haimen City and Fusui County in China, where residents have been drinking pond-ditch water, is considered to be related to microcystins in drinking water (Yu, 1995). Moreover, microcystins can also be transferred along the food chain, causing a potential risk to humans and high trophic level species through the consumption of contaminated aquatic products (Smith and Haney, 2006; Zhang et al., 2009).

Adverse effects on aquatic organisms were observed for algae, plants and zooplankton when exposed to microcystins (Wiegand and Pflugmacher, 2005). Long-term microcystin contact has been shown to reduce the growth, chlorophyll-a/b and carotenoids of aquatic macrophytes. Photosynthesis of several aquatic plants were inhibited up to 90% in the presence of microcystin (Pflugmacher, 2002). Furthermore, microcystins also influence respiration, growth, grazing, ingestion efficiency and mortality of zooplankton which feed on phytoplankton (Wiegand and Pflugmacher, 2005). In addition, physiological and economic impacts as well as human health risks when irrigation water is contaminated by cyanotoxins have also been highlighted. Aquatic and terrestrial plants and crops may suffer negative effects on seed germination rate, seedling growth rate, development, photosynthesis and pigment content (Chen et al., 2004; Järvenpää et al., 2007; Pflugmacher et al., 2006). These studies indicate cyanotoxins not only reduce the productivity and quality of agricultural products, but can also be transferred and bioaccumulated in crops and plants via dissolved toxin in irrigation water or deposition of cyanobacterial cell during watering (Codd et al., 1999; Saqrane and Oudra, 2009). Cyanotoxins absorbed on plant tissues contribute part of the exposure route for introduction of these toxins into the human food chain (Saqrane and Oudra, 2009).

2.3.3 Detection and quantification of cyanotoxins

A number of methods are available for quantifying cyanotoxins in a water sample and identifying types of variants which are present (McElhiney and Lawton, 2005). Accuracy and efficiency of a detection method are influenced by the pre-concentration and clean-up procedures. At low toxin concentration, water samples need to be pre-concentrated. Simultaneously, pre-concentration cleans up co-extracting compounds in samples which may interfere with the analysis. The Solid Phase Extraction (SPE) is the most widely used pre-concentration method (McElhiney and Lawton, 2005; Msagati et al., 2006). In SPE, the water sample is filtered through a cartridge to allow toxin absorption to the sorbent in the cartridge. The toxins will then be eluted from the cartridge using a polar organic solvent such as ethanol or methanol (Xie et al., 2005) and/or further concentrated by reducing the solvent volume through evaporation. Cyanotoxin can be detected and quantified using biological/biochemical screening methods which include mouse assays, enzyme linked immunosorbent assay (ELISA) and protein phosphatase inhibition assay (PPIA); or the physicochemical analyses such as high performance liquid chromatography (HPLC), capillary electrophoresis (CE) and nuclear magnetic resonance (NMR).

ELISA is one of the cyanotoxin screening methods employing antibodies generated against the target toxins. This simple yet specific assay was developed either using monoclonal or polyclonal antibodies and requires minimum sample processing (McElhiney and Lawton, 2005). Several commercial ELISA kits are available including Abraxis LLC, USA; Wako Chemicals, Japan; EnviroLogix Inc., USA and Strategic Diagnostics Inc., USA (McElhiney and Lawton, 2005). The strength of the ELISA assays depends on the ability to recognize all toxic variants in the environmental sample. Research in the past has focused on improving the detection range of this assay (McElhiney and Lawton, 2005). Metcalf and colleagues (2000) have raised a polyclonal antibody in rabbits against a microcystin-LR conjugate. They managed to develop a highly sensitive and cross-reactive ELISA which was capable of detecting seven microcystin variants and the

structurally related nodularin below the WHO guideline for microcystin-LR (Metcalf et al, 2000). An alternative polyclonal antibody was later developed by raising antibody in sheep against the unique ADDA moiety present in microcystins and nodularins (Fischer et al., 2001). Directing the immune response to this common structural figure restricted the number of regions for antibody binding, thus giving higher reproducibility (McElhiney and Lawton, 2005). The antibody was able to recognize seven microcystins (microcystin-YR, LF, LR, RR, LW, 3-desmethyl-microcystin-RR and LR) and nodularin below 0.1µg/L and gave good performance in screening tap water and river water and blood samples without sample pre-treatment (McElhiney and Lawton, 2005). This ELISA technique was commercialized by Abraxis LLC with a detection limit between 0.15 – 5µg/L of microcystin. However, ELISA may not accurately estimate the toxicity of a sample because the degree of toxin binding to antibody has no relevance to the degree of toxicity. Some less toxic variants have higher affinity for the antibody and vice versa (Nicholson et al., 2007). Research evaluating the cross-reactivity of a number of microcystin variants using ELISA technique showed poor correlation between reactivity and acute toxicity (An and Carmichael, 1994; Rapala et al., 2002).

The protein phosphatase inhibition assay (PPIA) measures the inhibitory effect of microcystins on the release of phosphate groups from phosphorylated protein substrates. This method has been widely developed for screening microcystin activity in water and yields relatively quick results (McElhiney and Lawton, 2005; WHO, 1998). PPIA can be performed in a variety of sample matrices and without sample pre-concentration. It is sufficient to detect microcystin levels below the guideline value set by WHO (Rapala et al., 2002). Assays have been developed using two protein phosphatases, Type 1 and Type 2A, but the reaction of Type 1 enzyme is 50 times less sensitive than the Type 2A enzyme (McElhiney and Lawton, 2005). However, PPIA does not show the same sensitivity to all microcystin variants (McElhiney and Lawton, 2005). This is because these variants have different degrees of toxicity which are usually proportional to their inhibition of protein phosphatase activity (Nicholson et al., 2007). In addition, the reaction

may indicate the presence of other environmental protein phosphatase inhibitors too such as calyculin A and okadaic acid (Rapala et al., 2002; WHO, 1998).

HPLC combined with other detectors including UV/Vis absorbance, photodiode array (PDA), mass spectrometry (LC/MS), fast atom bombardment (FAB), electrospray ionization (ESI-MS) and Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) can be employed for cyanotoxin detection (McElhiney and Lawton, 2005). The full-range chromatographic separation of microcystin variants is usually accomplished using a reversed phase C18 packed column, amide C16 column, internal surface reversed phase column or ion exchange column as the stationary phase (Msagati et al., 2006); while methanol, acetonitrile, ammonium acetate/acetonitrile or water/acetonitrile acidified with formic acid or TFA is used for the mobile phase (McElhiney and Lawton, 2005; Sangolkar et al., 2006). Both UV/Vis and PDA detectors detect microcystin by measuring absorption at 238 nm or at 222 nm in the presence of tryptophan (eg. microcystin-LW) (Lawton et al., 1994; Sangolkar et al., 2006). A standard method to extract and detect microcystin-LR, RR and YR has been established by the International Organization for Standardization (ISO:20179, 2005). However, distinguishing toxin variants is difficult unless standards are available since most of the microcystins have similar absorption profiles between 200 and 300 nm. In addition, plastic containers and materials should be avoided for HPLC analysis as the plastic additives have been found to have similar absorbance at 238 nm (Msagati et al., 2006). Confirmation by mass spectrometry is currently the best solution for peptide cyanotoxins since individual toxins produce characteristic ions in their mass spectra (Lawton et al., 1994).

One of the most extensively used technologies in monitoring and identification of cyanotoxins is HPLC-ESI-MS. EMI-MS produces a protonated ion $[M + H]^+$ or divalent ion $[M + 2H]^+$, providing molecular weight (MW) information which can confirm the identification from HPLC-PDA (McElhiney and Lawton, 2005; Zhang et al., 2004). In detecting unknown cyanotoxins variants in water sample, in source or tandem mass spectrometry (MS/MS) fragmentation is very

useful without the mass data (McElhiney and Lawton, 2005). Despite its sensitivity and accuracy, HPLC usually necessitates laborious preparation and method development, as well as expensive machinery and the availability of toxin standards (Msagati et al., 2006; Pearson and Neilan, 2008).

2.4 Molecular Biology of Cyanobacteria and Cyanotoxin Production

Since the microcystin synthesis gene cluster was identified in 1996, molecular techniques have provided new insights into cyanotoxin research. Firstly, differentiation of toxic and non-toxic cyanobacterial strains, which is impossible under microscope, is now achievable with molecular probes (Dittmann et al., 1997; Furukawa et al., 2006; Meißner et al., 1996; Neilan et al., 2008). Next, interesting information have been obtained when the genetic techniques were applied, revealing: 1) the distribution of toxin-producing strains in natural environments; 2) toxin gene expression and regulation; and 3) the evolutionary pathway and inheritance of toxin-producing genes (Kurmayer and Christiansen, 2009). Today, molecular approaches have become the more popular tools to analyze toxin-producing cyanobacteria because they are rapid, sensitive and highly specific by targeting genes of interest (Ouellette and Wilhelm, 2003).

2.4.1 Biosynthesis of microcystins

The freshwater planktons which produce microcystins are *Microcystis*, *Planktothrix*, *Nostoc*, *Anabaena* and *Anabaenopsis*. Microcystin producing strains from terrestrial genera *Haplosiphon* and fresh-and-brackish genera *Arthrospira*, *Oscillatoria*, *Phormidium*, *Plectonema*, *Pseudanabaena*, *Synechococcus* and *Synechocystis* have also been reported (Chorus and Bartram, 1999; Kurmayer and Christiansen, 2009; Sivonen and Borner, 2008). Precursor studies on microcystin suggest a non-ribosomal enzyme-synthesis of this toxin, which utilizes large multifunctional modular enzymes, known as non-ribosomal peptide synthetases (NRPS). Each module of enzyme is responsible for the activation, modification

(thioesterification) and condensation (peptide elongation) of an individual amino acid (Borner and Dittmann, 2005; Christiansen et al., 2003; Nishizawa et al., 1999; Tillett et al., 2000). These modular enzymes consist of peptide synthetases (PS), polyketide synthetases (PKS), mixed PS and PKS and tailoring enzymes and are encoded by a microcystin synthesis gene cluster with 10 genes which spans about 55 kb (Borner and Dittmann, 2005; Rantala et al., 2004; Tillett et al., 2000). *Microcystis aeruginosa* PCC 7820 has been reported to synthesize microcystin-LR from L-methionine, L-phenylalanine, L-glutamic acid, acetate and pyruvate (Moore et al., 1991).

Since both toxic and non-toxic cyanobacteria contain NRPS, a genetic method by knock-out mutagenesis of NRPS was used to identify those genes required for toxin synthesis. Essentially, however, only a few cyanobacteria can survive the genetic amendment (Kurmayer and Christiansen, 2009). The microcystin gene cluster (*mcy* genes) was detected from an experiment that showed mutant cells for *Microcystis aeruginosa* PCC 7806 which had lost their toxin-producing ability (Borner and Dittmann, 2005). Applying similar methods, the *mcy* gene clusters for genus *Anabaena* (Rouhiainen et al., 2004) and genus *Planktothrix* (Christiansen et al., 2003) were also identified. In addition, NRPS and PKS responsible for nodularin (a hepatotoxin related to microcystin) synthesis was also found in the genus *Nodularia* (Borner and Dittmann, 2005).

The *mcy* gene clusters within two strains of *Microcystis aeruginosa* are similar but differ between the genera (Tillett et al., 2000). Gene transcriptions are controlled by two operons which are transcribed bidirectionally (Kaebernick et al., 2002). For *Microcystis*, upstream of the *mcy* gene cluster comprises seven genes: *mcyD* (polyketide synthases); *mcyE* and *G* (hybrid enzymes); *mcyF*, *I* and *J* (tailoring genes) and *mcyH* (putative ABC transporter). These enzymes encode for 45 out of the 48 steps needed for microcystin biosynthesis (Tillett et al., 2000).

Comparison of the structural organizations of the *mcy* gene clusters in genera *Microcystis*, *Anabaena*, *Planktothrix* and *Nodularia* (*nda* cluster) shows that

these four microcystin/nodularin-producing genera are not identical in cluster arrangement (Neilan et al., 2008). The enzyme order for the biosynthesis of microcystins starts with McyG, J, D and E (PKS or PKS & NRPS modules) and continues with McyA, B and C (NRPS modules). Gene transcriptions in *Anabaena* and *Nodularia* are co-linear with the predictive order of the catalytic process as shown in the above enzymatic steps of the toxin biosynthesis. However, this arrangement was not detected in genera *Microcystis* and *Planktothrix* (Dittmann and Borner, 2005; Neilan et al., 2008). In *Microcystis* and *Planktothrix*, the putative operon starts with *mcuD* and then *mcuE* and *G*. The positions of the tailoring genes (*mcuF*, *I* and *J*) also differ in the individual genera except in *Anabaena* and *Nodularia* which are highly identical (Borner and Dittmann, 2005). In *Planktothrix*, two missing tailoring genes (*mcuF* and *I*) have been replaced by additional *mcuT* which encodes for a thioesterase (Dittmann and Borner, 2005).

The *nda* cluster codes for nodularin production lacks two NRPS modules that are present in the *mcu* cluster. In *Nodularia*, the *ndaA* is a combination of *mcuA* and *mcuB* found in other genera (Borner and Dittmann, 2005). Two missing NRPS modules produce a smaller peptide chain with five amino acids compared to seven amino acids in microcystins. Despite this discrepancy, the cluster arrangement for toxin synthesis in *Anabaena* and *Nodularia* are the most similar as expected from their closely related phylogenetic relationship (Borner and Dittmann, 2005). Therefore, it is proposed that the nodularin cluster evolved from *Anabaena*'s *mcu* gene cluster (Moffitt and Neilan, 2004; Rantala et al., 2004).

The *mcu* clusters are highly similar between different genera. Most of the *mcu* genes (*mcuA*, *B*, *C*, *D*, *E*, *G*, *H* and *J*) are always present in all genera except for *mcuI*, *F* and *T* (Kurmayer and Christiansen, 2009). The occurrence of various microcystin variants raises the question as to whether or not many different *mcu* sequences or clusters are required for generating the toxin variants. One study showed that the insertional inactivation of the *mcu* gene cluster resulted in the transformation of *Microcystis* to the non-toxic state, such that each mutant clone was no longer capable of producing any kind of microcystins (Neilan et al., 1999).

This demonstrates that the single *mcy* gene cluster is responsible for the formation of all microcystin variants (Dittmann et al., 1997). It was suggested that the production of various microcystin variants was due to (1) less specific substrate specificity of the adenylation domains in the peptide synthetases (Borner and Dittmann, 2005) and (2) genetic variations in the microcystin synthesis genes (Mikalsen et al., 2003). Studies have shown that polymorphism of adenylation domains in the *mcyA2* and *mcyB1* gene modules of *Microcystis* strains resulted in different microcystin variant profiles (Allender et al., 2009; Mikalsen et al., 2003). Similarly, a study conducted on *Planktothrix* strains demonstrated that varied *mcyA* and *mcyB* genotypes correlated significantly with certain amino acid residues at positions 7 and 2 of microcystin as per Figure 2.1 (Kurmayer et al., 2005).

2.4.2 Evolutionary aspect of microcystin-producing gene cluster

The microcystin-producing cyanobacterial genera are only distantly related based on the phylogenetic tree of 16S-rRNA (Borner and Dittmann, 2005; Rantala et al., 2004). Both members which contain microcystin synthesis gene and those which do not usually coexist in a genus. Two hypotheses rose to explain this patchy distribution of the *mcy* gene cluster. The *mcy* gene cluster might have laterally transferred between the genera, species and strains. Alternatively, a vertical gene transfer was postulated where the *mcy* gene cluster evolved from common ancestral genes and all phylogenetically related cyanobacteria once had harboured this gene but slowly lost it through evolution (Dittmann and Borner, 2005).

The idea of total or partial lateral gene transfer of the *mcy* gene cluster is supported by the detection of transposases located downstream of the 3' end of *mcy/nda* clusters for *Microcystis* and *Nodularia* (Moffitt and Neilan, 2004; Tillett et al., 2000). It has been found that the transposable elements make up 11.8% of the entire genome of *Microcystis aeruginosa* NIES-843 (Kaneko et al., 2007). Disruptions of cyanobacterial gene sequences due to transposable elements have not only been reported for the gas vesicle gene and phycobilisome synthesis gene but also on *Planktothrix*'s *mcy* gene cluster, resulting in the lost of toxin producing

ability (Christiansen et al., 2006; Roberts, 2008). It was suggested that the lateral *mcy* genes transfer in *Microcystis* could be carried out with the type IV pilus system found in many bacteria as a DNA uptake apparatus (Kurmayer and Christiansen, 2009; Nakasugi et al., 2007).

Conversely, there are studies backing vertical transfer and gene loss as the reason for irregular distribution of the *mcy* cluster. A phylogenetic analysis of cyanobacterial housekeeping genes (16S rRNA and *rpoC1* gene) and microcystin genes (*mcyA*, *D* and *E*) showed coevolution of these genes by perfectly congruent data sets (Rantala et al., 2004). Incongruence indicates a role for horizontal gene transfer whereas congruence is consistent with descent through a common ancestor (Rantala et al., 2004). Therefore, microcystin synthetases genes appeared to be ancient. The loss of the *mcy* gene cluster was taken as an ongoing evolutionary process that might have started with function mutation (Rantala et al., 2004). A similar report focused on *Planktothrix* strains also supported the role of gene loss for the inactivation of the *mcy* cluster. For 25 non-toxic strains of *Planktothrix* tested in a study, *mcy* remnants were flanked by equal copies of transposable elements and the *mcyT* gene was frequently detected in most of the strains (Christiansen et al., 2006).

A high degree of *mcy* cluster structural similarity has been found in different *Microcystis* species, but is low in agreement with other genera. This suggested that lateral transfer of the *mcy* gene cluster is unlikely (Borner and Dittmann, 2005). By comparing the sequences of multiple housekeeping gene loci from a large number of toxic and non-toxic *Microcystis* species, Tanabe and colleagues identified that *mcy* gene was cluster clonal-dependent and occurred only in two clades of the phylogenetic branches (Tanabe et al., 2007). In short, these studies provided evidence on the vertical inheritance of the *mcy* cluster and simultaneously implied the role of horizontal gene transfer in the inactivation of partial *mcy* genes but not the exchange of complete toxin gene cluster (Kurmayer and Christiansen, 2009).

2.5 Molecular Detection of Cyanobacteria

Early molecular identification of cyanobacteria was based on the most widely used 16S rRNA in prokaryotes. Later, phylogenetic analysis showed that the 16S rRNA clusters always included both toxic and non toxic strains. Even the more diverse 16S-23S internal transcribed spacer (ITS) was unable to tell the difference between toxic and non-toxic cyanobacteria (Rantala, 2007). However, these gene fragments are still useful in determining the phylogenetic relationships of target species and help to reveal the evolutionary mechanism of toxic synthesis genes (Iteman et al., 2000; Neilan et al., 1997; Otsuka et al., 1999).

Since the discovery of toxin synthesis genes, followed by the full sequencing of the *mcy* gene cluster for genera *Microcystis*, *Anabaena*, *Planktothrix* and *Nodularia*, the adoption of molecular techniques to identify or quantify these genes in water samples have rapidly developed. Most of these techniques made use of the same strategy - nucleic acid amplification of specific toxin synthesis genes (Pearson and Neilan, 2008). Primer sets for microcystins producers mostly target the *mcyA*, *B*, *D*, *E* and *F* fragments. To identify toxic *Nodularia* in water, PS and PKS genes were used initially before the discovery of the nodularin synthesis genes (Moffitt and Neilan, 2004). Lately, primers based on the nodularin gene cluster (*nda*) are highly specific to toxic *Nodularia* and suitable for PCR and qPCR analyses (Koskeniemi et al., 2007). The putative cylindrospermopsin biosynthesis genes were first identified in *Aphanizomenon ovalisporum* and named as *aoaA*, *aoaB* and *aoaC* genes (Shalev-Alon et al., 2002). Later, Mihali et al. proposed a gene cluster and complete biosynthesis pathway for cylindrospermopsin involving 11 genes (*cyrA-cyrO*) encoding the amidinotransferase, PKS/NRPS, uracil ring, tailoring, transport, regulator and transposase (Mihali et al., 2008). For anatoxin production, a polyketide synthase, *ks2*, isolated from *Oscillatoria* was found to be well-correlated with 10 other anatoxin-a and homoanatoxin-a producing strains (Cadel-Six et al., 2009). Following that, a cluster containing eight putative anatoxin synthesis genes was identified from *ks2* (Méjean et al., 2009). These fundamental

studies enable more specific and accurate assay designs for detecting a diverse range of toxin producers in the future.

DNA-based approaches have also been widely used to explore the regulation and expression of toxin genes (Pearson and Neilan, 2008). Designed PCR/qPCR reactions can be general to toxic genes from different genera (Neilan et al., 1999; Rantala et al., 2004; Rinta-Kanto and Wilhelm, 2006; Schembri et al., 2001) or can be specific to a genus (Furukawa et al., 2006; Hotto et al., 2007; Vaitomaa et al., 2003). However, it is important to note that positive detection of a certain toxin gene does not necessarily indicate the presence of toxin production. This could be caused by non-toxic strains which have retained some toxic genes but have inactivated their toxin synthesis during the evolutionary process (Sivonen, 2008). Alternatively, it could also suggest that cyanotoxin production is not constitutive. This argument is supported by laboratory and field studies showing temporary or permanent loss of toxin production in some toxic species or population (Martins et al., 2004; Wood et al., 2011). Table 2.2 summarizes some studies on the detection of toxigenic cyanobacteria.

Table 2.2 Molecular detection and quantification of toxigenic cyanobacterial species among laboratory cultures and environmental samples.

Methods	Genera	Target gene	Results	Reference
Dot blot hybridization PCR	<i>Anabaena</i> , <i>Aphnizomenon</i> , <i>Lyngbya</i> , <i>Microcystis</i> , <i>Nodularia</i> , <i>Nostoc</i> , <i>Oscillatoria</i> , <i>Synechococcus</i>	Peptide synthetases and <i>mcyB</i>	All tested genera were positive for peptide synthetases sequence except for <i>Synechococcus</i> . <i>mcyB</i> was detected in all microcystin/nodularin producing <i>Anabaena</i> (3/5), <i>Microcystis</i> (10/10), <i>Nodularia</i> (4/4), <i>Nostoc</i> (1/1) and <i>Oscillatoria</i> (4/8) but undetected in non-toxic strains. One non-toxic <i>Lyngbya</i> and <i>Microcystis</i> were found to be positive for the <i>mcyB</i> gene.	(Neilan et al., 1999)
PCR Sequencing	<i>Anabaena</i>	<i>rpoC1</i> (RNA polymerase gene)	<i>Anabaena circinalis</i> specific primers were developed from <i>rpoC1</i> sequencing and tested successfully on 15 reference cultures and 4 environmental samples. <i>A. spiroides</i> was found to be positive with PCR and showed 100% identity to <i>A. circinalis</i> type II.	(Fergusson and Saint, 2000a)
PCR	<i>Cylindrospermopsis</i> , <i>Anabaena</i> , <i>Microcystis</i> , <i>Nodularia</i>	Polyketide synthases, Peptide synthetases	PKS and PS genes in <i>C. raciborskii</i> isolates demonstrated a direct link between the two genes and cylindrospermopsin production. Possession of the two was genes also linked. <i>Anabaena bergii</i> contained these genes and was found to produce cylindrospermopsin.	(Schembri et al., 2001)
PCR Sequencing	<i>Microcystis</i>	<i>mcyA</i> (N-methyltransferase)	NMT was present in laboratory cultures or field samples (18/37). Two non-toxic strains also possessed the NMT regions. Region downstream of the <i>mcyABC</i> cluster in all NMT positive strains contained an open reading frame with conserved distance from <i>mcyC</i> gene.	(Tillett et al., 2001)
PCR RFLP	<i>Microcystis</i>	<i>mcyB</i>	PCR products found in 76% of <i>M. aeruginosa</i> , 16% of <i>M. ichthyoblade</i> but none in <i>M. wesenbergii</i> . RFLP revealed high variability (1.6-38%) between restriction types but low variability (0.4-4%) in nucleotide sequences within each restriction type.	(Kurmayer et al., 2002)

Methods	Genera	Target gene	Results	Reference
PCR Sequencing	<i>Microcystis</i>	<i>mcyB</i>	18 out of 60 isolated cultures showed the presence of toxic genes. Positive toxin detection was accompanied by each positive PCR.	(Bittencourt-Oliveira, 2003)
qPCR (TaqMan)	<i>Microcystis</i>	<i>mcyB</i>	<i>mcyB</i> was used to indicate microcystin synthesis in Lake Wannsee. Detected <i>mcy</i> gene numbers were between 1 and 38% of the total <i>Microcystis</i> gene (Intergenic spacer region of phycocyanin).	(Kurmayer and Kutzenberger, 2003)
qPCR (SYBR green)	<i>Microcystis</i> , <i>Anabaena</i>	<i>mcyE</i> (genus specific)	Two designed primer sets were specifically used to detect toxic <i>Microcystis</i> strains (10/13) and <i>Anabaena</i> strains (7/14) but not for genera <i>Planktothrix</i> and <i>Nostoc</i> . Detection limit was 40-400 copies of <i>mcyE</i> genes per mL of lake samples (Finland).	(Vaitomaa et al., 2003)
PCR RFLP	<i>Microcystis</i> , <i>Anabaena</i> , <i>Planktothrix</i> , <i>Nostoc</i>	<i>mcyA</i> (condensation domain)	Target fragment was amplified from all microcystin-producing strains of the genera <i>Microcystis</i> (10/24), <i>Anabaena</i> (6/8), <i>Planktothrix</i> (8/11) and <i>Nostoc</i> (1/2). No product was obtained from any of the 19 non-hepatotoxic strains. RFLP analysis generated genus-specific fragments and allowed genus of the toxin producer to be identified.	(Hisbergues et al., 2003)
PCR Sequencing	<i>Nodularia</i>	Polyketide synthases, Peptide synthetases	PS and PKS gene fragments associated with nodularin synthesis were identified. Sequencing allowed characterization of the nodularin gene cluster (48kb).	(Moffitt and Neilan, 2004)
PCR	<i>Microcystis</i>	<i>mcyA</i> and <i>mcyB</i>	<i>mcyA</i> and <i>mcyB</i> were used as indicative regions for microcystin synthesis. >75% of <i>M. aeruginosa</i> and <i>M. botrys</i> colonies contained <i>mcy</i> genes. ≤20% of <i>M. ichthyoblade</i> and <i>M. viridis</i> gave positive <i>mcy</i> amplifications.	(Via-Ordorika et al., 2004)

Methods	Genera	Target gene	Results	Reference
PCR Sequencing	<i>Nodularia</i> , <i>Nostoc</i> , <i>Anabaena</i> , <i>Microcystis</i> , <i>Planktothrix</i>	<i>mcyA</i> , <i>mcyD</i> and <i>mcyE</i>	Toxic gene sequences detected in all tested strains of <i>Anabaena</i> , <i>Microcystis</i> , <i>Planktothrix</i> , <i>Nostoc</i> and <i>Nodularia</i> .	(Rantala et al., 2004)
PCR Sequencing	<i>Cylindrospermopsis</i>	Peptide synthetases and polyketide synthases	PS and PKS genes were used to differentiate toxigenic strains from non-toxic strains (cylindrospermopsin producers) in one strain of <i>Cylindrospermopsis</i> (1/11) and one strain of <i>Aphanizomenon</i> (1/2)	(Valerio et al., 2005)
PCR qPCR (TaqMan)	<i>Microcystis</i>	<i>mcyB</i> and <i>mcyD</i>	Presence of <i>Microcystis</i> and toxic genes (<i>mcyB</i> and <i>mcyD</i>) in Lake Erie were detected by multiplex PCR. qPCR detection limit for <i>mcyD</i> gene was 5000 gene copies per liter of lake water. Limitation was highlighted where microcystin-producing cyanobacteria was not detected by the probe used in the study.	(Rinta-Kanto et al., 2005)
PCR	<i>Microcystis</i> , <i>Planktothrix</i> , <i>Pseudoanabaena</i> , <i>Cylindrospermopsis</i>	<i>mcyB</i>	Presence of toxic species was confirmed by <i>mcyB</i> gene with the coexistence of microcystin-RR, -LR and -YR	(Anjos et al., 2006)
qPCR (SYBR green)	<i>Microcystis</i>	<i>mcyA</i> (genus specific)	Only microcystin-producing strains of the <i>Microcystis</i> species (9/21) were identified using the primer sets. Detection limit was 8.8 cells per reaction.	(Furukawa et al., 2006)
PCR RT-PCR qPCR (SYBR green)	<i>Microcystis</i>	<i>mcyA</i> and <i>mcyB</i>	<i>mcyA</i> and <i>mcyB</i> genes were identified in strains of overwintering colonies of benthic <i>Microcystis</i> and also confirmed the expression of toxicity by RT-PCR and qPCR. Study also indicated that zooplankton ingested toxic <i>Microcystis</i> by the presence of <i>mcy</i> gene in DNA and RNA isolated from <i>Daphnia</i> specimens.	(Oberholster et al., 2006)

Methods	Genera	Target gene	Results	Reference
PCR	<i>Anabaena</i> , <i>Microcystis</i> , <i>Planktothrix</i>	<i>mcyE</i> (genus-specific)	Microcystin potential was evaluated with genus-specific primer sets targeting on <i>mcyE</i> gene in 70 Finnish lakes. The microcystin-producing <i>Anabaena</i> , <i>Microcystis</i> and <i>Planktothrix</i> were found in 37%, 70% and 63% of the samples. Approximately two thirds of the samples contained more than one microcystin producer.	(Rantala et al., 2006)
PCR Sequencing	<i>Microcystis</i> , <i>Planktothrix</i>	<i>mcyA</i>	Alignment of <i>mcyA</i> DNA sequences helped to identify previously unknown toxic <i>Microcystis</i> and <i>Planktothrix</i> species.	(Rinta-Kanto and Wilhelm, 2006)
PCR	<i>Microcystis</i> , <i>Oscillatoria</i>	<i>mcyA</i> , <i>mcyB</i> and <i>mcyC</i>	Presence of the microcystin synthesis genes in the lysates of three morphospecies of <i>Microcystis</i> and species <i>O. tenuis</i> indicated their roles in microcystin production.	(El Herry et al., 2008)
PCR Sequencing	<i>Microcystis</i> , <i>Anabaena</i> , <i>Planktothrix</i>	<i>mcyA,B,D</i> , <i>Microcystis</i> , <i>mcyE</i> for <i>Anab</i> , <i>Plankth</i>	<i>mcyB</i> and <i>mcyD</i> were used as initial screening for microcystin potential (25 positive out of 107). <i>mcyA</i> amplicons were used for sequencing to indicate the genotypes. Genus-specific <i>mcyE</i> primers confirmed <i>Microcystis</i> as the dominant toxin producer.	(Hotto et al., 2007)
qPCR (SYBR green)	<i>Nodularia</i>	<i>ndaF</i>	The primer sets were tested positive only for toxic <i>Nodularia</i> strains (3/18). The detection limit was 15-75 copies gene per mL seawater. 114 out of 120 water samples collected from the Baltic Sea contained <i>ndaF</i> gene copy numbers above the detection limit.	(Koskenniemi et al., 2007)
Sequencing qPCR (TaqMan)	<i>Anabaena planktonica</i>	ITS1-L, 23SrRNA	36 cyanobacteria found in freshwater New Zealand waters were analyzed for ITS sequences. A specific qPCR was designed for <i>A. planktonica</i> , tested with environmental samples and showed good correlation with microscopic cell count.	(Rueckert et al., 2007)

Methods	Genera	Target gene	Results	Reference
qPCR (TaqMan) Sequencing	<i>Microcystis</i>	<i>mcyA</i> , <i>Microcystis</i> 16SrRNA and <i>mcyD</i>	<i>Microcystis</i> was the dominant <i>mcy</i> genotype in Oneida Lake, with toxigenic cells ranging from 0 to 37% of the total <i>Microcystis</i> population. Toxic genotypes were grouped into 2 sub-clades. Microcystin concentration was higher when members of both sub-clades present.	(Hotto et al., 2008)
qPCR (TaqMan)	<i>Cylindrospermopsis</i>	<i>rpoC1</i> , <i>aoaC</i> (<i>pks</i>)	Duplex qPCR was designed to target a cylindrospermopsin-specific sequence (<i>aoaC</i> , encodes for a polyketide synthase) and a <i>Cylindrospermopsis raciborskii</i> -specific sequence for RNA polymerase. Assay results matched the microscopic cell count and toxin production.	(Rasmussen et al., 2008)
qPCR (TaqMan)	<i>Planktothrix agardhii</i>	<i>mcyA</i> , PC gene	Toxic and non-toxic <i>Planktothrix</i> populations were studied for two years by applying qPCR to the <i>mcyA</i> and phycocyanin genes. Only 54% of the toxin variations could be explained by toxin producer density, showing the method was not satisfactory for bloom monitoring.	(Briand et al., 2008a)
PCR-DGGE qPCR (SYBR green)	<i>Microcystis</i>	<i>mcyA</i>	The diversity and abundance of microcystin producing genotypes in Lake Taihu were evaluated. Toxic genotypes were more diverse during the bloom season than the non-bloom season. The highest toxin concentration did not correspond to the highest <i>mcy</i> gene copy number.	(Ye et al., 2009)
qPCR (SYBR green)	Microcystin producers	<i>mcyD_{KS}</i> , <i>mcyD_{DH}</i>	The qPCR was used to track the microcystin producers in Missisquoi Bay with detection limits lower than that of HPLC and microscope.	(Fortin et al., 2010)

Methods	Genera	Target gene	Results	Reference
qPCR (TaqMan) DGGE	<i>Microcystis</i>	<i>cpcBA</i> , <i>mcyJ</i>	The potentially toxic <i>Microcystis</i> cells in a Korean reservoir were quantified using qPCR assays targeting on the genus-specific <i>cpcBA</i> gene and <i>mcyJ</i> gene. The DGGE profile revealed the appearance and disappearance of new genotypes following seasonal changes.	(Kim et al., 2010)
qPCR (TaqMan) Sequencing	<i>Microcystis aeruginosa</i>	PC gene, <i>mcyB</i> , ITS	<i>Microcystis aeruginosa</i> populations in several French freshwater systems were assessed. Microcystin-producing genotypes dominated most of the population with toxic cell percentages between 70 to 100%. No correlation was found between microcystin concentration and proportion of toxic cells.	(Sabart et al., 2010)
qPCR (Taqman)	<i>Microcystis</i>	Cyano and <i>Microcystis</i> 16S-rRNA, <i>mcyD</i>	Three qPCR assays for the detection of cyanobacteria, <i>Microcystis</i> and <i>mcyD</i> gene were developed based on the sequences found in San Francisco Estuary.	(Baxa et al., 2010)
qPCR (SYBR green)	<i>Anabaena circinalis</i>	<i>sxtA (pks)</i>	A qPCR method was developed for saxitoxin-producing <i>A. circinalis</i> based on the unique polyketide synthase <i>sxtA</i> . The method was tested on 13 samples collected from different aquatic systems in Australia. Toxin concentration was correlated with saxitoxin gene copy number.	(Al-Tebrineh et al., 2010)
qPCR (SYBR green)	All microcystin and nodularin-producing cyanobacteria	<i>mcyE</i> , <i>ndaF</i>	A qPCR assay targeting hepatotoxic cyanobacteria was made based on all known microcystin/nodularin producing genera. The assay was validated with freeze-dried environmental samples. It has a detection limit of 1000 cell/mL using whole cell samples.	(Al-Tebrineh et al., 2011)

The PCR method is the most extensively used approach for detecting potential toxin producers. This method is considered fast and relatively inexpensive as it requires only a PCR machine and gel-electrophoresis processing facilities (Sivonen, 2008). Two post-PCR analyses commonly applied are restriction fragment length polymorphism (RFLP) and DNA sequencing. For a RFLP, the PCR product will be cut at specific positions using restriction enzymes to yield DNA fragment profiles of different sizes (Ouellette and Wilhelm, 2003). These final fragments allow differentiation between toxic cyanobacterial genera (Rantala, 2007). Sequenced PCR products for a certain gene fragment can be used to infer the phylogeny and the identity of the cyanobacterial community in the water (Ouellette and Wilhelm, 2003).

The major advantage of qPCR is its quantification of the target DNA sequence. Another significant improvement by qPCR is the increased speed of analysis, mainly due to the omission of the post-PCR detection step such as gel-electrophoresis (Mackay et al., 2004). Also, the fluorescence-sensitive equipment permits earlier amplicon detection and shortens the amplification cycle numbers required. QPCR methods include the SYBR green assays and the Taq nuclease assays. Fluorescence will be generated from the binding of dye to double-stranded DNA (SYBR green) or from hydrolysis of a TaqMan probe (Taq nuclease assay), in which the TaqMan probe is considered to be more specific (Sivonen, 2008). Gene quantification is done by measuring the fluorescence intensity at each cycle. The cycle number where the fluorescence signal of the amplification reaction is above the background noise is used to infer the target gene number and is called the threshold cycle (C_t). Comparison of C_t values from external standards with known gene concentrations with C_t values from unknown samples gives the target gene concentration in an unknown sample (Mackay et al., 2004). However, qPCR has disadvantages involving the detection of fluorogenic chemistries and it also restricts multiplex amplifications (Mackay et al., 2004).

QPCR optimized for a variety of cyanobacterial targets is increasing rapidly as the popular method for environmental detection and eco-physiological studies

(Table 2.2). A number of qPCR assays have been developed to quantify cell numbers of genera *Anabaena*, *Microcystis*, *Planktothrix* and *Nodularia*. It is a powerful tool that describes the complexity of a bloom by giving quantitative figures. It provides information on the seasonal and spatial variations of the proportion of cyanotoxin-producers in a water system. In addition, qPCR techniques have been employed to determine the correlations between toxigenic genes and environmental factors (nutrients, water depth, toxin concentration, etc.) to produce new insights into factors which modulate toxin production (Pearson and Neilan, 2008; Sivonen, 2008).

2.6 The Effect of Environmental Factors on Cyanotoxin Production

Effects of environmental factors on cyanotoxin production have been studied in the field and in the laboratory. In laboratory culture, the growth conditions can be well-controlled, thus enabling the effects of single environmental factors on toxin production to be investigated. The most frequently examined parameters include culture age in batch cultures, temperature, pH, light, nitrogen, phosphorus and trace metals (Lukac and Aegerter, 1993; Sivonen, 1990; Westhuizen and Eloff, 1985). However, a general consensus of cyanotoxin regulation is hard to achieve because most of the regulatory experiments have not been standardized, and a proper comparison of results cannot be carried out (Neilan et al., 2008).

Nitrogen and phosphorus are essential nutrients for cyanobacterial growth. At high phosphorus concentrations, hepatotoxic strains produce more toxins but this is not valid for anatoxin-a producing cyanobacteria (Sivonen and Jones, 1999). Nitrogen-fixing cyanobacteria (*Anabaena*, *Aphanizomenon*, *Nodularia* and *Cylindrospermopsis*) showed a high level of toxin production when kept under nitrogen-free medium (Kaebernick and Neilan, 2001). However, non-nitrogen fixing species (*Microcystis* and *Oscillatoria*) were more toxic under nitrogen-rich conditions (Sivonen and Jones, 1999). Under phosphorus limiting conditions,

Microcystis sp. synthesized more microcystin-LR instead of RR (Kaebernick and Neilan, 2001). Results on the effect of iron have been contradictory. A positive relationship was found between iron uptake and microcystin production with increasing light intensity, suggesting that microcystin may be considered as an iron chelating compound (Utkilen and Gjølme, 1995). A conflicting observation was also found where low concentrations of iron led to higher microcystin production (Lukac and Aegerter, 1993), indicating the complex response of microcystin production among different cyanobacterial strains toward light, iron and other external factors.

Temperature could alter different toxin contents by a factor of two to three. In most studies, the optimum temperature for cyanobacteria was between 20 to 25°C. The toxin content decreased at low (10 °C) and high (30 °C) temperature (Kaebernick and Neilan, 2001; Sivonen and Jones, 1999; WHO, 1998). Temperature also correlated well with the toxin variants produced. *Anabaena* spp. produced more microcystin-LR at temperatures below 25 °C and synthesized more microcystin-RR at higher temperature (Rapala et al., 1997). Investigation of the pH effect showed that cells were more toxic when grown at high or low pH (Sivonen and Jones, 1999).

While the above results show impacts on toxin production by nutrients, temperature and trace metals, they could be the result of the indirect influence of nitrogen, phosphorus, iron and optimum temperature on cell growth (Neilan et al., 2008). In fact, the majority of studies showed that the highest toxin concentrations are reported under conditions which are optimum for cell growth (Kaebernick and Neilan, 2001). It was also found that the amount of microcystin in culture increased during logarithmic growth phase and reached the highest level in the late logarithmic phase; whereas for anatoxin-a, maximum toxin concentration was found at the logarithmic growth phase (Sivonen and Jones, 1999; WHO, 1998). In addition to this, Orr and Jones (1998) have hypothesized that the impacts of environmental factors on microcystin production might be explained by growth rate-driven production (Kardinaal and Visser, 2005). They proposed that there was

a direct linear correlation between cell division (growth rate) and microcystin production rates in all microcystin-producing cyanobacteria, regardless of the environmental factors that was limiting cell division (Orr and Jones, 1998). However, several recent studies have refuted this hypothesis by showing contradictory results, for example *Microcystis* PCC7803 grown under light-saturated condition has less microcystin content at maximum growth rate (Wiedner et al., 2003). Similar findings was also found by Tonk et al. (2009) where high light intensity / temperature increased growth rate but reduced microcystin concentration of *Microcystis* PCC 7806, while anabaenopeptin content in *Anabaena* 90 increased when the growth rate decreased (Tonk et al., 2009).

An alternative hypothesis suggests that microcystin production is related to the nitrogen availability in the environments. By showing microcystin was significantly positive-correlated with nitrogen uptake and content but negatively correlated with carbon fixation rate, phosphorus uptake and content, Downing et al. (2005) disagreed that microcystin content in cells was related to their growth rate. Their study suggested that nitrogen uptake and assimilation play important roles in the toxin synthesis, possibly responding to the carbon fixation of *Microcystis* cells, and the availability of nitrogen and phosphorus in the environment (Neilan et al., 2008). Two recent studies by Van de Waal et al. (2009, 2010) further support this hypothesis. In their controlled studies, changes in carbon-nitrogen stoichiometry and nitrogen availability were shown to affect the amino acid composition and production of different microcystin variants in *Microcystis aeruginosa* and *Planktothrix agardhii*. Therefore, manipulating the nitrogen and carbon dioxide concentrations in water might have implication for varying the toxicity of a bloom (Van de Waal et al., 2009; Van de Waal et al., 2010).

Generally, the cellular toxin content of different cyanobacteria varied about two to three folds under a variety of light or irradiance conditions (Sivonen and Jones, 1999; Wiedner et al., 2003). The microcystin content in *Microcystis aeruginosa* CYA 228/1 per unit protein increased when the irradiance increased from 20 to 75 $\mu\text{mol of photon m}^{-2}\text{s}^{-1}$ (Utkilen and Gjølme, 1992). Several recent

studies on *Microcystis* species have indicated positive effects of increased photosynthetically active radiation (PAR) on toxin production up to the point where the maximum growth rate was achieved. After that, continuous increases in light irradiance caused light saturation conditions in which microcystin content decreased with increasing intensity (Deblois and Juneau, 2010; Lyck, 2004; Wiedner et al., 2003). In contrast, *Planktothrix agardhii*, species which can reach maximum growth rates at low irradiance, has been reported to give higher toxin concentration per unit dry weight at lower irradiances (Sivonen, 1990).

It is suggested that microcystin production may not respond directly to the changes in light but is regulated as a physiological component related to the photosynthetic process. This hypothesis may explain the inconsistent results from previous light experiments. A study by Dittmann et al. (2001) on wild-type and mutant *Microcystis aeruginosa* PCC7806 discovered a significant increase of MrpA protein only in wild type cells. Since the MrpA protein is homologous to RhiA and B proteins in *Rhizobium* sp. which account for the quorum sensing mechanism, microcystin was thought to have a similar function for light-sensing in cells (Dittmann et al., 2001; Neilan et al., 2008). On the other hand, Deblois et al. found significant inverse correlations between the microcystin content and the photosystem II (PSII) redox state and between the microcystin content and the relative electron transport rate. Based on their results, they suggested that more work should be done to investigate microcystin involvement in photosynthesis (Deblois and Juneau, 2010).

The effects of light quality (colour/spectra) were also discussed in several studies. *Microcystis aeruginosa* toxicity was enhanced by red and green light compared with white light (Utkilen and Gjølme, 1992). By using RNase assay to measure the transcription of toxin synthesis genes (*mcyB* and *mcyD*), Kaebernick et al. (2000) observed a higher gene transcription when the *Microcystis* culture was incubated under red light for 2 hours, while the culture under blue light had reduced transcription (Kaebernick et al., 2000). Another study conducted on *Cylindrospermopsis raciborskii* demonstrated that light quality can affect the

circadian rhythm of saxitoxin and neosaxitoxin production (Carneiro et al., 2009). The circadian rhythm of toxin modulation is a clock-like mechanism that was shown to affect physiological activities for cultures grown under natural dark/light cycle (Briand et al., 2008a). Under blue light conditions, the periods with maximum saxitoxin and neosaxitoxin production were expanded; while under red light conditions the circadian rhythm for neosaxitoxin was lost (Carneiro et al., 2009).

Although environmental factors can induce toxin production within a factor of three or four, the difference is relatively small (Kardinaal and Visser, 2005). Toxin production among different cyanobacterial strains grown under identical conditions can vary by more than three orders of magnitude. This may explain the huge toxicity variation found in natural blooms, i.e. due to the waxing and waning of several cyanobacterial species/strains with different toxin quotas (Rantala, 2007; Sivonen and Jones, 1999).

2.7 Putative Function of Cyanobacterial Toxins

Toxin production of cyanobacteria is an energy-demanding process since both the replication of the larger genome containing PKS and NRPS genes, and biosynthesis of toxin metabolites require energy. Therefore, cyanotoxins should be advantageous to the survival of cells (Sivonen and Borner, 2008). Despite extensive efforts carried out to identify the physiological role of cyanotoxins, the regulation and reasons for their production are still unresolved (Osswald et al., 2007). However, some putative functions of cyanotoxins have been summarized in reviews by Kaebernick and Neilan (2001) and Wiegand and Pflugmacher (2005). The first step in understanding the functions of cyanotoxins is to study their effects upon the local ecosystem. The second step is to investigate the intracellular functions in maintaining the basal cell metabolic system.

2.7.1 Putative extracellular function

Fish kills during cyanobacterial blooms have been attributed to the oxygen depletion and the protein phosphatases 1 and 2A inhibitions by microcystins (Kaebernick and Neilan, 2001). Interaction of cyanotoxins with zooplankton, phytoplankton and bacteria also give insights to the role of cyanotoxins in the ecosystem. More importantly, the recent discovery of a putative ABC transporter in the microcystin gene cluster responsible for transporting metabolites out from the cell has supported the extracellular function of cyanotoxins.

Studies have shown evidence that cyanobacteria and their toxins influence the population structure of zooplankton and may create ecological advantages for cyanobacterial success (Kaebernick and Neilan, 2001). Cyanobacteria are poor food for zooplankton and are often being avoided if alternative food (usually phytoplankton) is available. *Daphnia* populations have been observed to decline during a bloom event when running out of alternative food. Some zooplankton (*Daphnia pulicaria*, *Daphnia pulex*) evolved to adapt to toxic cells and changed the population dynamics of zooplankton (Kaebernick and Neilan, 2001). *Daphnia* killed by ingesting toxic cyanobacterial cells has led to the hypothesis that cyanotoxins may be designed to reduce the grazing effect from zooplankton. However, this idea was disputed by evidence that *daphnia* showed no selection between feeding on toxic and non-toxic cyanobacterial cells (Rohrlack et al., 1999). In addition to this, phylogenetic studies on the microcystin synthesis cluster showed its presence earlier than the predators, rejecting the defensive role of toxins against the grazing effect (Rantala et al., 2004).

Research has found that *Anabaena* PCC7120 and *Nostoc commune* UTEX584 contain genes which encode for eukaryotic type Ser/Thy protein kinases and protein phosphatases. This suggests another possibility for the function of microcystins, as inhibitors against protein phosphatases of other cyanobacteria. Microcystins do not inhibit the protein phosphatase activity in *Microcystis spp.* (Kaebernick and Neilan, 2001).

Association of cyanobacteria and other microorganisms in water may provide an answer to the role of cyanotoxins. *Pseudomonas aeruginosa* is chemotactically attracted to *Anabaena* and mutually share the fixed N₂ available. Also, *Microcystis aeruginosa* has a greater CO₂ fixation rate when interacting with bacteria and protozoan grazers (Kaebernick and Neilan, 2001). On the other hand, cyanotoxins inhibit the growth of various species such as *Chlamydomonas*, *Haematococcus*, *Navicula* and *Cryptomonas*. These findings suggest that cyanotoxins are produced as a signal to attract those which are beneficial hosts while driving away antagonistic microbes and grazers (Kaebernick and Neilan, 2001).

On the other hand, a study on the biological function of microcystin has demonstrated that when *Microcystis* cells lyzed and released toxin, either mechanically or due to stressed conditions, the expression of the microcystin synthesis gene (*mcyB*) of remaining cells were greatly enhanced. From this observation, the authors proposed an info-chemical signaling role of microcystin (Schatz et al., 2007), in which their presence will be sensed as cells die inside the population and simultaneously trigger microcystin production in the rest of *Microcystis* cells for enhanced fitness (Schatz et al., 2007).

However, in an experiment studying the competition for light between toxic and non-toxic *Microcystis* strains, it was found that the non-toxic strain was a better competitor than the toxic strain. In light-limited chemostats, the toxic *Microcystis* strain lost in the competition with the non-toxic strain even though an initial advantage was given to the toxic strain. From their results, the authors suggest that microcystins might not be as ecologically important as allelopathic compounds (Kardinaal et al., 2007). However, contradictory results were demonstrated by Briand and colleagues, who compared the competitive advantages of microcystin by growing toxic and non-toxic *Planktothrix agardhii* together under various environmental conditions including temperature, light intensity and nitrate concentration (Briand et al., 2008b). Their data revealed a better competency of

microcystin producing genotypes to outcompete non-toxic genotypes under growth stressed conditions. However, toxic genotypes outweighed their benefit and lost the competition under favorable growth conditions. These findings suggest that environmental variables could be a factor controlling the dynamics of toxin and non-toxin producers in waters and eventually affecting the toxin biosynthesis rate (Briand et al., 2008b).

2.7.2 Putative intracellular function

Two important observations suggest cyanotoxins possess intracellular rather than extracellular functions: (1) most of the toxins produced remain in the cell and are located in the thylakoid and nucleoid areas, (2) a large portion of toxin is released only when cell lysis occurs as a result of detrimental growth factors or culture age (Kaebernick and Neilan, 2001; Shi et al., 1995; Young et al., 2005). As this research study is focused on microcystin, the following discussion will be limited to this toxin.

Classifying cyanotoxins as secondary metabolites implies that these compounds are unnecessary for primary metabolism in cyanobacteria. This is supported by the survival of genetically modified mutant cells which lost their toxin production abilities (Dittmann et al., 1997). However, cyanotoxins do share features of primary metabolites in that they are present at all stages of growth unlike other secondary metabolites where their synthesis is induced by stress factors (Sivonen and Borner, 2008). Some researchers have questioned the classification of cyanotoxins as secondary metabolites. Their argument is based on the observation that most secondary metabolites are triggered when growth ceases, whereas microcystins production increases with an increase in the growth rate (Kardinaal and Visser, 2005; Orr and Jones, 1998).

Microcystin content in cells has shown to be affected by environmental factors such as temperature, pH, light, nitrogen, phosphorus and iron (Kardinaal and Visser, 2005). Microcystin has a high affinity for cations such as iron, copper and

zinc, thus suggesting its putative role as an iron scavenging molecule (Utkilen and Gjølme, 1995). It may be used for binding extracellular ferrous ions under low iron availability. Intracellularly, microcystin may have a protective role by forming the iron-microcystin complex when ferrous ion concentration is high (Utkilen and Gjølme, 1995). The complex can be stored in cells and dissociated to form free ferrous ion when the iron concentration decreases (Kaebernick and Neilan, 2001).

Microcystins in the cell are mostly located in the thylakoid and nucleoid regions (Shi et al., 1995). It is believed that microcystin may be associated with light harvesting in the photosynthetic process and the chromatic adaptation mechanism (Deblois and Juneau, 2010; Dittmann and Borner, 2005; Kaebernick and Neilan, 2001). Genetic regulation studies have shown increased transcription of genes involved in microcystin production in high light conditions (Kaebernick et al., 2000).

2.8 Summary of the Literature Review

Since the *mcy* gene cluster is the longest known cyanotoxin synthesis gene, most of the occurrence studies focused on the detection and quantification of microcystins producing genera such as *Microcystis*, *Anabaena* and *Planktothrix*. However, these studies were mostly carried out in temperate climate regions. Relevant studies from tropical regions are comparatively limited. To date, cyanotoxin synthesis gene clusters have been identified for microcystin (*mcy*) nodularin (*nda*) and cylindrospermopsin (*cyr*). Putative gene clusters for anatoxin-a/homoanatoxin-a and saxitoxins are under investigation. The current detection of toxic genes is based on the sequences of these clusters. Earlier studies were focused on the molecular detection method of PCR but in recent years, qPCR have become popular. Less research has been done on the level of gene expression of toxigenic strains in environmental samples.

The most widely used biochemical toxin analyses are ELISA and PPIA. They are useful for the initial screening of toxicity in water samples. HPLC equipped with UV/Vis or PDA detectors are popular as the physicochemical method. The combination of HPLC-ESI-MS is able to quantify several toxin variants at the same time.

The effects of environmental factors on toxin production including temperature, pH, light, nitrogen, phosphorus and iron have been widely studied. However, different species will respond differently to the same environmental parameter. Moreover, individual cyanobacterial species/strains have their own toxin quota. Hence, in order to predict and control cyanotoxin production, it is necessary to understand the characteristics and optimum growth of indigenous cyanobacteria found in a particular water system.

The physiological function of cyanotoxins is not clear. Two types of functions are suggested, either intracellular or extracellular. Observation that cyanotoxins usually remain in the cells until cell lysis gives support to the intracellular function of cyanotoxins. Discovery of the gene encoding for a putative ABC transporter, however, suggests that cyanotoxins could have an extracellular function.

CHAPTER 3

Occurrence and Dynamics of Cyanobacterial Blooms in Kranji Reservoir

3.1 Introduction

Production of cyanotoxins by cyanobacteria poses a significant threat to the safety of water supplies (Ye et al., 2009). Hence, an understanding of factors which trigger blooms of cyanobacteria and their production of toxins is important to safeguard water resources. Field and laboratory studies on toxigenic cyanobacteria have indicated a wide range of physiochemical variables that can affect toxin production and the growth of different cyanobacterial genotypes (Briand et al., 2009; WHO, 1998). However, no single variable can fully describe the formation of blooms or the synthesis of toxins (Graham et al., 2004). In previous laboratory studies, different cyanobacterial strains showed varied responses to different environmental variables (Davis et al., 2009; Paerl and Huisman, 2008; Sivonen and Jones, 1999). Nevertheless, the tremendous variability in toxin level in cyanobacterial blooms is suggested to potentially be due to the successive replacement of toxigenic and non-toxigenic strains (Kardinaal and Visser, 2005).

Traditionally, microscopic identification has been used to monitor and characterize cyanobacterial blooms (Ouellette and Wilhelm, 2003) but this method cannot differentiate between the toxigenic and non-toxigenic genotypes. In recent years, this problem has been resolved by using molecular techniques such as PCR and quantitative real-time PCR (qPCR) with primers targeting toxin-producing genes (Neilan et al., 1999). The microcystin gene cluster which contains 10 genes, namely from *mcyA* to *mcyJ* (Dittmann and Borner, 2005) has been widely used to reveal microcystin-producing species in genera *Anabaena*, *Microcystis*, *Nostoc* and *Planktothrix*. Detection of cyanobacteria and their toxin-producing ability using molecular methods has been successfully applied to a diverse range of water

environments, although most studies centre on subtropical and temperate regions (Bittencourt-Oliveira, 2003; Fergusson and Saint, 2000b; Oberholster et al., 2006; Rinta-Kanto et al., 2005; Schembri et al., 2001; Tillett et al., 2001; Vaitomaa et al., 2003; Valerio et al., 2005). There are very few studies conducted in tropical waters and these have mainly focused on the detection of toxins and the presence/absence of toxigenic species using PCR (Anjos et al., 2006; Bittencourt-Oliveira, 2003). There is still a lack of quantitative data from sustained high temperature environments such as those found in equatorial regions.

In this chapter, the dynamics of toxigenic and non-toxigenic *Microcystis* spp. and *Anabaena* spp. in a tropical reservoir in Kranji reservoir was studied. Significant environmental variables which affect the population dynamics of these species and the concentration of microcystin were also examined. Kranji Reservoir is one of the reservoirs in Singapore utilized for drinking water and recreational activities, including sport, fishing and kayaking. The reservoir experiences cyanobacterial blooms throughout the year, dominated by *Microcystis* colonies. The reservoir water is treated using conventional water treatment technology and the potable water obtained conforms to WHO drinking water guidelines.

3.2 Materials and Methods

3.2.1 Sampling site

Singapore is a densely populated country located 1°N of the equator, and experiences a tropical climate, with typical Northeast and Southwest Monsoons and intermonsoon periods. Kranji Reservoir is situated in the northwestern part of Singapore and has a water surface area of about 2.8 km². The reservoir was formed by damming the mouth of the Kranji estuary, thus separating freshwater and seawater. The catchment has a mixed land use where 17.8% is residential, 5.4% of the area is agricultural, 0.2% is industrial and 76.6% is undeveloped land (Lo et al., 2008). There are three major tributaries flowing into the reservoir: Kangkar River, Tengah River and Peng Siang River. Typical of tropical rainforest climates, the

reservoir has high average temperature (29.41 ± 1.14 °C) and monthly rainfall (218.58 ± 97.60 mm) throughout the year (Zikun Xing, personal communication). It is a eutrophic reservoir with chlorophyll-a concentrations generally >12.2 µg/L (95th percentile). The upstream tributaries contribute external nutrient loadings of 71,740 kg TN/year and 12,816 kg TP/year from their catchment; while the estimated internal loadings are 1 to 5-fold of the external loadings (Gin and Gopalakrishnan, 2010). Earlier studies showed that *Microcystis* was the dominant genus in years 2008 and 2009 while other genera detected include *Anabaena*, *Anabaenopsis*, *Lyngbya*, *Merismopedia* and *Oscillatoria* (Public Utilities Board of Singapore, unpublished data).

3.2.2 Field sampling

A total of 18 sampling events were carried out monthly from the end of February 2008 to the end of October 2009. Three sampling locations were selected to represent different hydrological conditions: Station A is at the confluence of the main tributaries; Station B is located at one of the deepest points in the reservoir and Station C is situated within the Peng Siang tributary serving an agricultural/urban catchment (Figure 3.1). Samples were collected in triplicate at each station by placing three 1-litre sterile amber glass bottles 30 cm below the water surface until fully filled. All samples were kept in ice boxes ($\sim 4^{\circ}\text{C}$) and transported to the laboratory. The triplicates were well-mixed before further analysis.

Physical water quality parameters such as water temperature, salinity, conductivity and total dissolved solid (TDS) were measured in-situ using a YSI meter (EC 300). The dissolved oxygen (DO) was measured with a YSI model 52 (YSI probe 5739) and the water transparency was measured with a Secchi disc. Water pH values were determined at the laboratory with a Thermo pH meter (Orion 555A). Turbidity was measured by using a turbidity meter (Euteoh Instruments, TN-100). Total nitrogen (TN), total phosphorus (TP) and total suspended solid

(TSS) were analyzed according to the standard methods USEPA 353.2, APHA 4500-P (after persulfate digestion) and APHA 2540D respectively (APHA, 1995b).

Figure 3.1 Sampling stations and catchment area for the Kranji Reservoir. Symbol '★' denotes the rain gauge.

Phytoplankton biomass was estimated using chlorophyll-a concentration. Chlorophyll-a concentration was measured according to the standard methods APHA 10200H (APHA, 1995b) with modification. Briefly, 50-250 mL of water sample with 0.001% w/v of MgCO_3 added was filtered through a cellulose nitrate membrane. 10 mL of 90% acetone was added to the membrane, sonicated for 3 mins and incubated in the dark for 24 hours. The sample was then centrifuged at 2800 rpm for 15 min to obtain a clear supernatant. Chlorophyll-a extracted by acetone (supernatant) was measured spectrophotometrically (Backman Coulter, DU 640B). Monthly rainfalls were measured by a rain gauge located at the Kranji catchment area as indicated in Figure 3.1 (Zikun Xing, personal communication).

3.2.3 DNA extraction

Cyanobacterial cells were harvested on a glass microfibre filter (Whatman 934-AH) from 50 to 500 mL of water sample depending on the concentration of phytoplankton and frozen at $-20\text{ }^{\circ}\text{C}$. 54 samples (18 samples from each location) were processed for PCR and qPCR analyses. Cyanobacterial DNA was extracted twice from the same frozen filter by using a DNA purification kit (Wizard Genomic DNA Purification Kit, Promega) with some modifications in the cell disruption steps. Briefly, the filter was added with 600 μL of nuclei lysis solution (provided in the purification kit) and broken twice by mechanical beading at a speed of 42 for 8 s (Biospec Product Inc.) with 2 μm glass beads (Sigma-Aldrich). After incubation at $80\text{ }^{\circ}\text{C}$ for 5 mins, the supernatant was collected by centrifuging the mixture at 14,000 rpm for 1 min. The above steps were repeated to get a total supernatant volume of 1.2 mL. DNA was then extracted from the supernatant by using the DNA purification kit according to the manufacturer's instruction. The concentrations and purities of the extracted DNA were measured with a spectrophotometer at absorbance 260 nm and ratio 260 nm/280 nm (A_{260}/A_{280}) respectively.

3.2.4 Reference strains

Microcystin-producing strains *Microcystis aeruginosa* PCC7806 and *Anabaena flos-aquae* f. *lemmermannii* NIYA/CYA 83/1 (CSIRO Collection of Living Microalgae, Australia) were used as the reference strains in this study. Cyanobacterial cultures were maintained in MLA medium (Bolch and Blackburn, 1996) under continuous light source of 15 – 20 $\mu\text{mol}/\text{m}^2\cdot\text{s}$. The cultures were harvested at late exponential growth phase to extract their DNA as the positive controls for PCR and qPCR assays.

3.2.5 PCR

Six monoplex and one triplex (*Microcystis* 16S rRNA gene, *mcyB* and *mcyD*) PCR amplifications were performed by using primer sets developed in previous studies. These PCR assays target a cyanobacterial 16S rRNA gene (Nubel et al., 1997), a *Microcystis* 16S rRNA gene (Rinta-Kanto et al., 2005), an *Anabaena* 16S rRNA gene (Doblin et al., 2007), a *mcyA* gene (Hisbergues et al., 2003), four *Microcystis*-specific *mcy* genes (*Microcystis mcyB* and *mcyD* (Rinta-Kanto et al., 2005), *Microcystis mcyC* (Tillett et al., 2001) and *Microcystis mcyE* (Vaitomaa et al., 2003) and an *Anabaena*-specific *mcy* gene (*Anabaena mcyE*) (Vaitomaa et al., 2003). The assays were optimized to enable the detection of target genes from local water samples. All amplifications were performed in 50 μL reaction volume. The assays were optimized to enable the detection of target genes from local water samples.

Table 3.1 PCR and qPCR primer sets used in this study. Primer sets for qPCR assays are marked with asterisks (*).

Target gene/primers	Sequence (5'-3')	Reference
Cyanobacterial		
16S rRNA		
CYA106F	CGGACGGGTGAGTAACGCGTGA	(Nübel et al. 1997)
CYA781R(a)	GACTACTGGGGTATCTAATCCCATT	
CYA781R(b)	GACTACAGGGGTATCTAATCCCTTT	
<i>Microcystis</i> 16S rRNA*		
MICR 184F	GCCGCRAGGTGAAAMCTAA	(Rinta-Kanto et al., 2005)
MICR 431R	AATCCAAARACCTTCCTCCC	
<i>Anabaena</i> 16S rRNA*		
Ana573F	AGTGGAAACTACAAAGCTAGAGTT	(Doblin et al., 2007)
Ana780R	CTTGGGTCGATACGAGCT	
<i>mcyA</i>		
<i>mcyA</i> -Cd 1F	AAAATTAAAAGCCGTATCAAA	(Hisbergues et al., 2003)
<i>mcyA</i> -Cd 1R	AAAAGTGTTTTATTAGCGGCTCAT	
<i>Microcystis mcyB</i>		
<i>mcyB</i> 2959F	TGGGAAGATGTTCTTCAGGTATCCAA	(Rinta-Kanto et al., 2005)
<i>mcyB</i> 3278R	AGAGTGGAAACAATATGATAAGCTAC	
<i>Microcystis mcyC</i>		
UMF	CCTATCGTCGTATTTGGAGT	(Tillett et al., 2001)
MCYCF	ATCCCAAACGTCAGGAATTAAGAG	
<i>Microcystis mcyD</i>		
<i>mcyD</i> F2	GGTTCGCCTGGTCAAAGTAA	(Rinta-Kanto et al., 2005)
<i>mcyD</i> R2	CCTCGCTAAAGAAGGGTTGA	
<i>Microcystis mcyE*</i>		
<i>mcyE</i> -F2	GAAATTTGTGTAGAAGGTGC	(Vaitomaa et al., 2003)
Mic <i>mcyE</i> -R8	CAATGGGAGCATAACGAG	
<i>Anabaena mcyE</i>		
<i>mcyE</i> -F2	GAAATTTGTGTAGAAGGTGC	(Vaitomaa et al., 2003)
Ana <i>mcyE</i> -R12	CAATCTCGGTATAGCGGC	

Amplification profiles and reagents concentrations are summarized in Table 3.2. Each reaction contained 5 µL of 10×PCR buffer, 1.5 mM of MgCl₂, 0.2 µM of dNTPs, 0.5 µM of each forward and reverse primers, 1.25 units (U) of Taq polymerase (GoTaq® Flexi DNA Polymerase, Promega), and 5 µL of DNA template, except for the *Anabaena* 16S rRNA assay (2 mM of MgCl₂), triplex assay (0.4 µM of primers, 2.25 U of Taq polymerase) and *mcyA* assay (4.5 mM of MgCl₂, 0.3 µM of dNTPs, 1.5 U of Taq polymerase). Bovine serum albumin was added in all reactions to a final concentration of 300 ng/µL to enhance the amplification of environmental samples (Rinta-Kanto et al., 2005).

The amplification profile for *Anabaena* 16S rRNA contained 40 cycles of 30 s at 94 °C, 30 s at 60.5 °C and 1 min at 72 °C; the profile for *mcyC* consisted of 40 cycles of 10 s at 94 °C, 20 s at 56 °C and 1 min at 72 °C; and the profile for *Anabaena mcyE* used a touchdown programme described by Gobler et al. (2007). Other amplification profiles followed their original references. Each reaction batch included positive and negative controls (DNA-free water) to ensure the assays performance. Final PCR products were run on a 1.5% agarose gel in 1×Tris-Borate-EDTA stained with ethidium bromide for 1 hour at 90 volts. The gel was visualized under UV trans-illumination and photographed.

Table 3.2 Amplification profiles and the reagent mixes for PCR assays. The final reaction volume is 50µL.

Target gene	10x PCR buffer ^a (µL)	MgCl ₂ (mM)	dNTPs (µM)	Primers (µM)	Taq Polymerase ^a (U)	BSA ^b (g/L)	DNA (µL)	Amplification profile
Cyanobacterial 16S rRNA	5	1.5	0.2	0.5	1.25	0.3	5	35 cycle; 95°C (40s), 60°C (50s), 72 °C (50s)
<i>Anabaena</i> 16S rRNA	5	2	0.2	0.5	1.25	0.3	5	40 cycle; 94°C (30s), 60.5°C (30s), 72 °C (60s)
<i>Microcystis</i> 16S rRNA } <i>Microcystis mcyB</i> } <i>Microcystis mcyD</i> } [†]	5	1.5	0.2	0.4	2.25	0.3	5	50 cycle; 94°C (30s), 56°C (60s), 72 °C (30s)
<i>mcyA</i>	5	4.5	0.3	0.5	1.5	0.3	5	33 cycle; 94°C (30s), 59°C (30s), 72 °C (30s)
<i>Microcystis mcyC</i>	5	1.5	0.2	0.5	1.25	0.3	5	40 cycle; 94°C (10s), 56°C (20s), 72 °C (60s)

Table 3.2 Continued.

Target gene	10x PCR buffer ^a (μL)	MgCl ₂ (mM)	dNTPs (μM)	Primers (μM)	Taq Polymerase ^a (U)	BSA ^b (g/L)	DNA (μL)	Amplification profile
<i>Microcystis mcyE</i>	5	1.5	0.2	0.5	1.25	0.3	5	35 cycle; 94°C (30s), 60°C (30s), 72 °C (60s)
<i>Anabaena mcyE</i> ^{††}	5	1.5	0.2	0.5	1.25	0.3	5	20 cycle; 94°C (40s), 65°C (50s) ^c , 72 °C (50s) 17 cycles; 94°C (40s), 55°C (50s), 72 °C (50s)

[†] Target genes detected in the triplex PCR assay.

^{††} A touchdown programme described by Gobler et al. (Gobler et al., 2007).

^a PCR buffer and Taq polymerase were from the GoTaq® Flexi DNA Polymerase set (Promega).

^b Bovine serum albumin

^c The annealing temperature declines at 0.5°C per cycle.

3.2.6 Cloning of PCR products and standard curves for quantitative real-time PCR (qPCR)

Amplicons of *Microcystis* 16S rRNA, *Microcystis mcyE* and *Anabaena* 16S rRNA genes were cloned into the T Easy cloning vectors (Promega) and transformed in the JM109 *E.coli* cells using pGEM®-T Easy Vector System (Promega). For the preparation of ligation reaction, 5 µL of ligation buffer, 1 µL of pGEM-T Easy vector, 1 µL of T4 DNA ligase, 1-2 µL of purified PCR product were mixed. Water was added to the mixture to a final volume of 10 µL. Amount of PCR product (insert) included in the ligation reaction was calculated using the following equation with a 3:1 insert: vector molar ratio.

$$\text{Amount of insert (ng)} = \frac{\text{Amount of vector (ng)} \times \text{Size of insert (kb)}}{\text{Size of vector (kb)}} \times \frac{3}{1}$$

The reaction was incubated overnight at 4 °C before the transformation.

JM109 competent *E. coli* cells were used for the transformation of ligated DNA. 2 µl of each ligation reaction were gently mixed with 50 µL of competent cells. The mixture was incubated on ice for 20 min, followed by heat shock for 45-50 seconds in a water bath at exactly 42 °C. After the heat shock, the mixture was immediately returned to ice for 2 min. The mixture was added with 950 µl of SOC medium and incubated at 37 °C for 1.5 hours with shaking at 150 rpm. After the transformation, 100 µl of each *E.coli* culture was plated onto duplicate LB agar containing 100 µg/ml ampicillin, 20 µl of 50 mg/ml X-Gal and 100 µl of 100 mM IPTG. The agar plates were incubated at 37 °C for 16-24 hours until colonies appeared. Unsuccessful clones were distinguished by the development of blue-colour colonies.

Successful clones (white colonies) were randomly picked and grown overnight in 5 mL of LB broth with 100 µg/ml ampicillin. Plasmid DNA was extracted from cells by using QIAprep Spin Miniprep Kit (Qiagen, Germany) according to the manufacturer's instructions. All plasmids were linearized with restriction enzyme SacI (Promega) by 1 hour incubation at 37 °C following the

protocols. Linearized plasmids were cleaned with the Wizard SV Gel and PCR Clean-up System (Promega) to obtain purified DNA before running the standard curves.

Three different qPCR standard curves were established using plasmid DNA for the quantification of *Microcystis* 16S rRNA, *Anabaena* 16 S rRNA and *MicrocystismcyE* genes. Standard curves were obtained by amplifying samples containing $6 - 6 \times 10^6$ gene copies/ μL or $2.2 \times 10^{-5} - 22$ pg/ μL plasmid DNA with 10-fold serial dilutions in duplicate. The target gene copy number in a standard sample was calculated using the formula:

$$\text{Gene copy no.} = \frac{V_{\text{sample}}(\mu\text{L}) \times C_{\text{sample DNA}}\left(\frac{\text{g}}{\mu\text{L}}\right) \times N_A}{\text{cloned plasmid size} \times M.W._{dsDNA}}$$

where V_{sample} is sample volume in a qPCR reaction, $C_{\text{sample DNA}}$ is DNA concentration of sample, N_A is Avogadro's no. (6.022×10^{23} copies /mol), $M.W._{dsDNA}$ is molecular weight of double-stranded DNA (660 g/mol.bp) and cloned plasmid size is the sum of vector size (3015 bp) and the PCR product size. All standard curves were run at least three times.

The standard curve was built by plotting the average threshold values (C_T) against the gene copy numbers, where C_t value was reported as the fluorescence intensity due to the binding of SYBR Green I dye with double-stranded DNA. The amplification efficiencies for plasmid standard curves were determined with the following equation:

$$\text{Efficiency (\%)} = (10^{-1/S} - 1) \times 100$$

where S is the slope of standard curve. Reactions were run at least twice with duplicate samples to ensure reliability. The amplification efficiency was calculated as the average of all replicates.

3.2.7 QPCR assays

The concentrations of *Microcystis* spp. (equivalent to *Microcystis* 16S rRNA), toxigenic *Microcystis* spp. (equivalent to *Microcystis mcyE*) and *Anabaena*

spp. (equivalent to *Anabaena* 16S rRNA) were determined by qPCR with SYBR Green I. The assay for *Anabaena* spp. was developed based on the primers by Doblin et al. (2007) (Doblin et al., 2007). The qPCR assay for *Microcystis* spp. was modified from Rinta-Kanto et al. (2005) originally using a dual-label probe (Rinta-Kanto et al., 2005). Assay for the *Microcystis mcyE* gene was a modified version of Vaitomaa et al. (Vaitomaa et al., 2003).

Amplifications were carried out in 20- μ L capillaries (Roche Diagnostics). Each capillary contained 10 μ L of ABsolute QPCR SYBR Green Capillary Mix (ABgene Ltd), 700 nM of each forward and reverse primer and 5 μ L of DNA template. QPCR for *Microcystis* 16S rRNA consisted of 45 cycles of 15 s at 95 °C, 30 s at 56 °C and 20 s at 65 °C. The *Anabaena* 16S rRNA qPCR assay involved 45 cycles of 15 s at 95 °C, 30 s at 59 °C and 20 s at 72 °C while the protocol for *Microcystis mcyE* contained 45 cycles of 15 s at 95 °C, 30 s at 58 °C and 20 s at 72 °C. Amplifications were done in a Light-Cycler 2.0 (Roche Diagnostics). The heating rate was 20 °C/s except for the elongation step which was carried out at a rate of 2 °C/s. All samples were run in duplicate and the samples' C_t values were automatically calculated by software (LightCycler 4.05). Melting curve analysis was performed after amplification steps to confirm the correct qPCR product. Amount of target genes were determined by converting the samples' C_t values to gene copy numbers using the regression equations generated from standard curves.

3.2.8 Microcystin concentration

Chromatography detection (HPLC/HPLC-MS) is the most widely reported method for microcystin analysis (Harada et al., 1988; McElhiney and Lawton, 2005; Oh et al., 2001). However, concentrations of unknown toxins cannot be determined if the standards are unavailable (Msagati et al., 2006). To date, over 90 microcystin variants have been detected (Hotto et al., 2008) and it is unlikely that all microcystin variants can be quantified with HPLC. To ensure the measurement captured most variants in the sample, microcystin concentrations were determined by using a 96-well microcystin ELISA assay (Abraxis LLC, US). The assay allows

the detection of microcystins and nodularins (including microcystin-LR, YR, LF, LR, LW, DM-microcystin-LR, RR and nodularin) based on the competitive binding of microcystins, nodularins and their ADDA congeners (standards) to the antibodies immobilized on a well plate (Fischer et al., 2001). As the ADDA moiety is present in all microcystin congeners, the ELISA assay allows the measurement of total microcystin/nodularin in sample. Since no *Nodularia spp.* was detected in Kranji reservoir, the assay measured only the microcystin concentration. To measure the total microcystin concentration, 300 mL of water sample was sonicated without heat for 30 mins to release cell-bound microcystin. The ELISA quantifiable toxin range is 0.15 – 5 µg/L. Samples with concentrations higher than the upper detection limit were diluted and re-measured. All samples were technically duplicated.

3.2.9 Statistical analyses

Statistical analyses were carried out using PASW 18 software package (SPSS Inc.). A Principal Component Analysis (PCA) was performed to compare samples collected from different sampling locations. Prior to the PCA analysis, 14 environmental variables with complete data sets were accessed with correlation matrix, Kaiser-Meyer-Olkin Measure of Sampling Adequacy (KMO) and Bartlett's test of Sphericity to check the suitability of analysis. The environmental variables included limnological variables (TSS, turbidity, Secchi depth, pH, DO, TN, TP TDS and conductivity) and biological variables (chlorophyll-a, microcystin concentration, *Microcystis* 16S rRNA, *Anabaena* 16S rRNA and *Microcystis mcyE* gene copy numbers). The number of components to be retained was determined by Kaiser's criterion, scree test and Monte Carlo PCA for parallel analysis.

After the PCA, data from different locations were processed together to identify non-local specific trends. Correlation analysis was conducted to identify environmental variables which were significantly correlated with cyanobacteria abundance and microcystin concentration. This was followed by multiple linear regression (MLR) to check if the occurrence of cyanobacteria and microcystin could be explained by environmental variables. Three regression models were built

to predict changes of *Microcystis* spp. abundance (equivalent to *Microcystis* 16S rRNA), toxigenic *Microcystis* spp. abundance (equivalent to *Microcystis mcyE*) and microcystin concentration. The predictor variables used in MLR were those found to be significantly correlated with the response variables. However, *Microcystis* 16S rRNA gene copy number, *Microcystis mcyE* gene copy number, chlorophyll-a and microcystin concentration were not included in the models as they were assumed to be partially correlated with the same environmental variables (predictor variables) as the response variables (Rantala et al., 2006).

3.3 Results

3.3.1 PCR results

Results for the PCR assays are shown in Table 3.3. Cyanobacterial cells were detected consistently throughout the study period by using primers targeting the cyanobacteria-specific 16S rRNA gene. *Microcystis* and *Anabaena* 16S rRNA genes were detected in all samples except for the sample taken at Station C on September, 2009, where no *Anabaena* 16S rRNA gene was detected in the sample using conventional PCR. However, the qPCR assay was able to detect *Anabaena* 16S rRNA in the same sample with a concentration of 19.9 copies/mL. Similarly, one PCR-negative sample for *Microcystis mcyE* gene was positively amplified in the qPCR assay. Microcystin-producing *mcyB* and *mcyD* genes were detected in all samples, whereas *mcyE* and *mcyA* and *mcyC* genes were only detected in 98%, 96% and 43% of the samples respectively. All samples were negative for *Anabaena mcyE* gene, indicating that *Microcystis* was the main microcystin producer in the Kranji Reservoir.

Table 3.3 PCR-positive samples at sampling stations A, B and C. There were 18 samples analyzed at each station during the entire study period. The total number of samples is 54.

Sampling	Cyanobacterial	<i>Microcystis Anabaena</i>		<i>Microcystis</i>					<i>Anabaena</i>
Station	16S rRNA	16S rRNA	16S rRNA	<i>mcyA</i>	<i>mcyB</i>	<i>mcyC</i>	<i>mcyD</i>	<i>mcyE</i>	<i>mcyE</i>
A	18	18	18	18	18	7	18	18	0
B	18	18	18	18	18	6	18	18	0
C	18	18	17	16	18	10	18	17	0
Total	54	54	53	52	54	23	54	53	0

3.3.2 QPCR methods

All qPCR assays showed linear amplifications for sample concentrations between 30 and 3×10^7 gene copies per reaction. Therefore, the lower and upper assay detection limits were 6 and 6×10^6 gene copies per μL , respectively. However, sample detection limit depended on the sample volume processed. The largest sample volume in this study was one litre and the lowest sample detection limit was 600 gene copies per litre of water. Linear regression equations for *Microcystis* 16S rRNA, *Microcystis mcyE* and *Anabaena* 16S rRNA qPCR assays were: $y = -3.573x + 36.16$ ($R^2 = 0.995$, efficiency = 90.49%, $P < 0.0005$), $y = -3.706x + 37.47$ ($R^2 = 0.995$, efficiency = 86.14%, $P < 0.0005$) and $y = -3.713x + 37.39$ ($R^2 = 0.996$, efficiency = 85.92%, $P < 0.0005$) respectively (Figure 3.2), where y is the $\log_{10}$ of the gene copy number and x is the corresponding C_t value.

Figure 3.2 QPCR standard curves for *Microcystis* 16S rRNA gene, *Microcystis* mcyE gene and *Anabaena* 16S rRNA gene.

A common practice to assess the purity of a DNA sample is to measure the spectrophotometric absorbance of the sample at 260 nm (A₂₆₀) to that of 280 nm (A₂₈₀) (Held, 2001). For a nucleic acid sample with concentration 1 mg/mL, its extinction coefficients for 260 and 280 nm are 20 and 10, respectively. Using these coefficients, a pure DNA sample should have an A₂₆₀/A₂₈₀ ratio of 2 (Held, 2001). The A₂₆₀/A₂₈₀ ratios for all DNA templates were between 1.8 and 1.95, indicating relatively pure nucleic acid samples. However, it is also known that humic substances can interfere with quantification of DNA and RNA spectrophotometrically (Zipper et al., 2003). To avoid the effect of humic substances, all DNA samples were diluted 20 times prior to the PCR and qPCR analyses. Melting temperatures (T_m) of qPCR products obtained from the environmental samples were in agreement with the products amplified from the reference strains (Table 3.4). Similar melting temperatures showed that the qPCR products have correct length, thus, the correct gene fragments were amplified. Fluorescence due to the formation of primer-dimers in *Microcystis* 16S rRNA qPCR assay was distinguished by a T_m shown at 78.43±0.27 °C.

Table 3.4 Comparison of T_m values for qPCR products using reference strains and environmental samples.

Target Genes	qPCR Melting Temperatures (°C)		
	<i>Microcystis</i> 16S rRNA	<i>Microcystis</i> <i>mcyE</i>	<i>Anabaena</i> 16S rRNA
Plasmid	89.62±0.08	83.76±0.08	87.28±0.07
Environmental sample	89.73±0.10	83.71±0.11	87.07±0.18

3.3.3 Reservoir characteristics, microcystin concentration, dynamics of toxigenic and non-toxigenic *Microcystis* spp. and coexistence of *Anabaena* spp.

The ranges of limnological and biological variables during the 19-month sampling period are listed in Table 3.5. Trophic status was assessed using the Carlson's Trophic Status Indices (TSI) (Carlson, 1977). With TSI ranging from 51.5 to 102.9, Kranji Reservoir was categorized as a lake between eutrophic and hypereutrophic status. An independent sample T-test on the variable means demonstrated that there was no limnological difference between Station A and Station B ($P < 0.05$). However, Station C showed significantly higher values of TN, TP and turbidity.

The temporal dynamics of *Microcystis* spp. and *Anabaena* spp. at the sampling sites are illustrated in Figure 3.3. At Stations A and B, the *Microcystis* 16S rRNA gene copy number varied within two orders of magnitude, while the *Microcystis mcyE* and *Anabaena* 16S rRNA gene copy numbers varied within three orders of magnitude. A greater cyanobacterial population fluctuation was observed at Station C. Here, the variability was higher by 3 orders of magnitude compared to those found at Stations A and B. QPCR quantification showed that *Microcystis* was the dominant species in Kranji Reservoir. This result is in agreement with the results obtained from the Public Utilities Board using microscopic identification and counting (unpublished data). *Microcystis* spp. and *Anabaena* spp. were found to coexist at all stations during the sampling period (Spearman's rho, $r_s = 0.498$, $P < 0.001$, $n = 54$).

Table 3.5 Total nitrogen (TN), total phosphorus (TP), total organic carbon (TOC), dissolved oxygen (DO), temperature, conductivity, turbidity, pH, total dissolved solid (TDS), chlorophyll-a, total suspended solid (TSS), Secchi depth, abundances of *Anabaena* spp., *Microcystis* spp. and toxigenic *Microcystis* spp., percentage of toxigenic *Microcystis* and microcystin concentration and trophic status measured at the sampling stations. The first number indicates average value, followed by the minimum and maximum values. Total number of samples collected at each location is shown in parenthesis ().

Variables	<u>Range</u>		
	Station A	Station B	Station C
TN (mg/L)	1.43; 0.64 - 2.51 (18)	1.41; 0.61 - 3.67 (18)	2.30; 1.01 - 4.36 (18)
TP (mg/L)	0.07; 0.03 - 0.19 (18)	0.07; 0.03 - 0.13 (18)	0.12; 0.04 - 0.21 (18)
TOC (mg/L)	11.79; 3.12 - 24.57 (13)	9.30; 3.98 - 19.86 (14)	8.92; 3.50 - 12.82 (14)
DO(mg/L)	6.82; 4.48 - 8.76 (18)	6.52; 4.10 - 9.97 (18)	6.96; 2.97 - 14.40 (18)
Temperature (°C)	29.31; 26.70 - 30.70 (18)	29.48; 26.50 - 30.85 (18)	28.64; 26.20 - 30.25 (18)
Conductivity (µs/cm)	214.9; 168.1 - 289.7 (18)	214.7; 168.9 – 285.0 (18)	236.1; 106.5 - 302.9 (18)
Turbidity (NTU)	44.09; 25.05 - 76.40 (18)	45.74; 13.25 - 119.00 (18)	70.57; 5.76 - 224.50 (18)
pH	8.59; 7.59 - 9.51 (17)	8.42; 7.34 - 9.50 (17)	8.50; 7.10 - 10.35 (17)
TDS (g/L)	0.12; 0.10 - 0.16 (17)	0.12; 0.10 - 0.16 (18)	0.13; 0.06 - 0.18 (18)
Chlorophyll-a (µg/L)	100.2; 41.9 - 272.4 (18)	96.3; 46.2 - 173.5 (18)	268.3; 8.4 - 1584.8 (18)

Table 3.5 Continue.

Variables	<u>Range</u>		
	Station A	Station B	Station C
TSS (mg/L)	24.6; 15.2 - 47.0 (18)	24.4; 10.8 - 37.5 (18)	46.5; 4.4 - 210.0 (18)
Secchi depth (cm)	49.6; 39.0 – 78.0 (17)	53.9; 39.5 - 75.0 (17)	47.2; 19.0 - 100.0 (17)
<i>Anabaena</i>	3.47×10 ⁴ ;	4.77×10 ⁴ ;	5.18×10 ⁴ ;
(16S rRNA gene copies/mL)	1.18×10 ³ – 1.25×10 ⁵ (18)	9.99×10 ² – 3.54×10 ⁵ (18)	2.82 – 5.66×10 ⁵ (18)
<i>Microcystis</i>	1.68×10 ⁶ ;	2.98×10 ⁶ ;	7.83×10 ⁶ ;
(16S rRNA gene copies/mL)	7.62×10 ³ – 4.44×10 ⁶ (18)	2.59×10 ⁴ – 1.34×10 ⁷ (18)	3.94×10 ² – 1.08×10 ⁸ (18)
Toxigenic <i>Microcystis</i> (<i>mcyE</i>)	4.26×10 ⁵ ;	7.06×10 ⁵ ;	1.63×10 ⁶ ;
copies/mL)	4.51×10 ³ – 8.48×10 ⁵ (18)	1.07×10 ⁴ – 2.29×10 ⁶ (18)	4.61×10 ¹ – 2.07×10 ⁷ (18)
Percentage of toxigenic <i>Microcystis</i>	62.7; 24.1 – 118.4 (18)	59.4; 22.9 – 102.2 (18)	45.7; 21.9 – 74.1 (18)
Microcystin (µg/L)	2.22; 0.62 - 8.00 (15)	2.28; 0.50 - 7.50 (15)	3.50; 0.00 - 14.40 (15)
Carlson's trophic status index (chlorophyll-a)	74.8; 67.3 – 85.6 (18)	74.6; 68.2 – 81.2 (18)	76.3; 51.5 – 102.9 (18)
Carlson's trophic status index (TP)	65.0; 53.7 – 79.8 (18)	64.5; 51.8– 73.8 (18)	70.8; 58.7 – 81.3 (18)
Trophic status^a	Eutrophic - hypereutrophic	Eutrophic - hypereutrophic	Eutrophic - hypereutrophic

^a based on TP concentration and chlorophyll concentration.

Figure 3.3 Variations of *Microcystis* and *Anabaena* cell numbers equivalent to 16S rRNA and *Microcystis mcyE* measured from February, 2008 to August, 2009. Two copies of *Microcystis* or *Anabaena* 16S rRNA genes are equivalent to one *Microcystis* or *Anabaena* cell while one copy of *Microcystis mcyE* gene is equivalent to one toxigenic *Microcystis* cell. All data points were calculated from duplicate samples.

The gene copy numbers were converted to equivalent cell numbers assuming: (1) *Microcystis* and *Anabaena* have two rRNA operons per genome (Rinta-Kanto et al., 2005; Rinta-Kanto et al., 2009); (2) toxigenic *Microcystis* contains one *mcyE* gene per genome (Kaebernick et al., 2002) and (3) each cell has only one genome. The equivalent cell numbers of *Microcystis*, toxigenic *Microcystis* and *Anabaena* are illustrated in Figure 3.4.

Figure 3.4 Boxplot for estimated cyanobacterial cell numbers at different stations. The length of the box shows interquartile range and 50% of cases of the variable. The line in the box indicates the median value, while extended lines from the box are the smallest and largest values. Cases extended more than 1.5 box-lengths and 3 box-lengths from the edge of the box are represented by circle (○) and asterisk (★) respectively.

Microcystis cell abundance in Kranji Reservoir was relatively high and stable throughout the year compared with *Microcystis* blooms reported in subtropical and temperate regions (Figure 3.5) (Baxa et al., 2010; Bozarth et al., 2010; Dai et al., 2008; Davis et al., 2009; Fortin et al., 2010; Gobler et al., 2007; Ha et al., 2009; Hotto et al., 2008; Kardinaal et al., 2007; Kim et al., 2010; Kurmayer and Kutzenberger, 2003; Robarts and Zohary, 1984; Sabart et al., 2010; Vaitomaa et al., 2003; Ye et al., 2009; Yoshida et al., 2007). Only three data points reported from Lake Taihu (China) and Hirosawa-no-ike Pond (Japan) showed cell numbers higher than that of this study (Figure 3.5, Cases 6 and 11) where very high TN (0.48 – 7.2 mg/L; 2.8 – 23.8 mg/L) and TP (0.075 – 0.29 mg/L; 0.4 – 2.0 mg/L) concentrations were found in these waters. In comparison, TN and TP values for Kranji Reservoir ranged from 0.61 – 4.36 mg/L and 0.03 – 0.21 mg/L, respectively.

Anabaena cell abundance fluctuated between 0 and 29.6% with an average of 0.78% of the total *Microcystis* cell count. Although *Anabaena* spp. had lower cell numbers, they showed greater fluctuations in abundance at all three locations. The toxigenic *Microcystis* cells contributed 62.67 % (Station A), 59.39 % (Station B) and 45.69 % (Station C) of the total *Microcystis* population, with an overall average of 55.92 %. Percentages of toxigenic cells throughout the study period are shown in Figure 3.6. It is interesting to note that the larger fluctuation of both toxigenic and non-toxigenic *Microcystis* spp. at Station C did not show greater changes in the proportion of toxigenic cells and toxin concentration (Table 3.5). Three samples with toxigenic cell percentage more than 100 were found.

Figure 3.5 Comparison of maximum and minimum *Microcystis* cell numbers in this study with *Microcystis* blooms in subtropical and temperate regions within a year. Water bodies in temperate regions are in solid line (Cases 1 to 15) (Baxa et al., 2010; Bozarth et al., 2010; Dai et al., 2008; Fortin et al., 2010; Gobler et al., 2007; Ha et al., 2009; Hotto et al., 2008; Kardinaal et al., 2007; Kim et al., 2010; Kurmayer and Kutzenberger, 2003; Robarts and Zohary, 1984; Sabart et al., 2010; Vaitomaa et al., 2003; Ye et al., 2009; Yoshida et al., 2007), case 15 (Copco Reservoir) presents only samples during bloom season. Blooms in subtropical regions are in dashed line (Cases 16 and 17) (Baxa et al., 2010; Dai et al., 2008; Fortin et al., 2010; Gobler et al., 2007; Ha et al., 2009; Hotto et al., 2008; Kim et al., 2010; Kurmayer and Kutzenberger, 2003; Sabart et al., 2010; Vaitomaa et al., 2003; Ye et al., 2009; Yoshida et al., 2007). Cell numbers in this study are shown in bold line for Case 18 (Station A), Case 19 (Station B) and Case 20 (Station C). Lake Taihu (Case 16) and Hirosawa-no-ike Pond (Case 6) are two exceptional cases with average *Microcystis* levels exceeding 2×10^7 cells/mL.

Figure 3.6 Proportions of *Microcystis* genotypes in Kranji Reservoir and microcystin concentrations at the sampling stations. Letters A, B and C indicates the names of sampling stations.

3.3.4 Principal component analysis (PCA)

The PCA was performed by using 14 variables based on 45 complete data sets. Only variables with communalities > 0.5 were used for the analysis. Temperature was omitted in the PCA as the communality was < 0.5 , meaning that less than 50% of its variance was accounted for by the analysis. The value of KMO was 0.714, exceeding the recommended value of 0.6 and above (Pallant, 2007). Bartlett's Test of Sphericity was significant ($P < 0.0005$), showing that the analysis was appropriate (Pallant, 2007). The first component (PC1) and second component (PC2) explained 66.49% of the data variation (PC1 = 40.99%; PC2 = 25.50%). Both scree test and parallel analysis suggested only 2 components to be retained in the analysis. Projecting the variables onto the ordination plot (Figure 3.7) shows that PC1 is mainly defined by phytoplankton biomass-related variables including the abundance of the *Microcystis* 16S rRNA and *Microcystis mcyE* genes, chlorophyll-a, TSS and turbidity. PC2 is mainly influenced by physical water quality variables such as pH, conductivity, TDS and DO. Microcystin concentration is negatively correlated with Secchi depth in the PCA.

The score plot of samples (Figure 3.8) shows no significant change in pattern for the samples taken from Stations A and B. However, samples collected from Station C can be categorized into two groups. Group 1 consists of samples taken from June to September, 2008, where a distinct clearance of bloom was observed with low *Microcystis* 16S rRNA gene copy numbers. Group 2 includes samples collected in October, 2008, and from December, 2008 to August, 2009. During this period, a cyanobacterial bloom had started and reached its peak in November, 2008. After a sharp decline in the next month, cyanobacterial cell numbers then remained stable for 8 months. An extreme case was found for the sample collected at Station C in November, 2008, when the cyanobacterial bloom reached a maximum (1.08×10^8 copies 16S rRNA/mL) and formed a thick layer of scum at the water surface.

Figure 3.7 PCA ordination plot of variables for *Microcystis* 16S rRNA (*Mic*-16S rRNA), *Anabaena* 16S rRNA (*Anab*-16S rRNA) and *Microcystis* specific *mcyE* (*Mic-mcyE*) gene copy numbers, chlorophyll-a (Chl-a), total suspended solid (TSS), turbidity, Secchi depth, total nitrogen (TN), total phosphorus (TP), total dissolved solid (TDS), conductivity, dissolved oxygen (DO), pH and microcystin concentration (Toxin). First component (PC1) is mainly defined by *Mic*-16S rRNA and *Mic-mcyE* genes, Chl-a, TSS and turbidity. Second component (PC2) is mainly influenced by pH, conductivity, TDS and DO.

Figure 3.8 Samples' scores for 45 samples on the PCA plot. Samples from Stations A (■) and B (▲) are grouped in a solid circle while samples from Station C (○) are grouped in two dashed circles. Group 1 consists of samples with low cyanobacterial gene copy numbers corresponding to a clearance of bloom observed between June and September 2008. Group 2 are samples with high cyanobacterial concentrations where a bloom had started (October 2008) and was maintained for 8 months (from December 2008 to August 2009).

3.3.5 Microcystin concentrations

Total microcystin concentration was measured for 45 samples taken from June, 2008 to August, 2009. Microcystin concentration ranged from below the detection limit to 14.4 µg/L while 77.8% of the samples contained microcystin > 1 µg/L. The mean concentrations were 2.22 ± 1.82 µg/L at Station A, 2.28 ± 1.85 µg/L at Station B and 3.50 ± 4.11 µg/L at Station C. Variations of microcystin concentrations at three sampling stations are shown in Figure 3.6. Both the highest and lowest toxin concentrations were recorded at Station C, corresponding to the greater fluctuation of *Microcystis* spp. at this location.

Since the Kolmogorov-Smirnov Test showed that most of the environmental variables were not normally distributed ($P < 0.05$), the Spearman rank correlation was selected to correlate microcystin concentration with environmental variables and cyanobacteria abundance. Microcystin concentration was positively correlated with *Anabaena* 16S rRNA gene copy number, *Microcystis* 16S rRNA gene copy number, *Microcystis mcyE* gene copy number, TN, DO, pH, turbidity, chlorophyll-a and TSS but negatively correlated with Secchi depth ($P < 0.005$), as shown in Table 3.6. Temperature was also correlated with microcystin, *Microcystis* 16S rRNA and *Microcystis mcyE* gene concentration but no significant correlation was found ($P > 0.05$).

Table 3.6 Spearman' rho coefficient (r_s) matrices for variables correlated with *Microcystis* 16S rRNA gene and *Microcystis mcyE* gene and microcystin concentration (only significant values are shown).

Variable	<i>Microcystis</i> 16S rRNA (gene copies/mL)	<i>Microcystis mcyE</i> (gene copies/mL)	Microcystin concentration (µg/L)
<i>Anabaena</i> 16S rRNA (gene copies/mL)	0.498**	0.512**	0.311*
<i>Microcystis</i> 16S rRNA (gene copies/mL)		0.950**	0.484**
<i>Microcystis mcyE</i> (gene copies/mL)	0.950**		0.500**
Microcystin concentration (µg/L)	0.484**	0.500**	
Total nitrogen, TN (mg/L)	0.327*	0.282*	0.339*
Total phosphorus, TP (mg/L)	0.308*	0.331*	
Dissolved oxygen, DO (mg/L)			0.314*
Conductivity (µs/cm)			
Turbidity (NTU)	0.566**	0.544**	0.529**
pH		0.296*	0.531**
Total dissolved solid, TDS (g/L)			
Chlorophyll-a (µg/L)	0.575**	0.536**	0.579**
Secchi depth (cm)	-0.587**	-0.512**	-0.563**
Total suspended solid, TSS (mg/L)	0.509**	0.482**	0.561**

* indicates correlation is significant at the 0.05 level (2-tailed)

** indicates correlation is significant at the 0.01 level (2-tailed)

3.3.6 Multiple linear regression (MLR)

MLR models of *Microcystis* 16S rRNA gene copy number, *Microcystis mcyE* gene copy number and microcystin concentration are summarized in Table 3.7. Prior to MLR, Pearson's correlations between 10 predictor variables were checked to identify possible multi-collinearities between variables. Since strong correlations were found between TSS and turbidity (coefficient = 0.802, $P < 0.0005$); chlorophyll-a and turbidity (coefficient = 0.747, $P < 0.0005$) and

conductivity and TDS (coefficient = 0.946, $P < 0.0005$), three highly correlated predictor variables (TSS, chlorophyll-a and conductivity) were omitted in the regression analysis. The beta value of the MLR indicates how well an independent variable is associated with a dependent variable.

Table 3.7 Beta (β) values for variables of multiple linear regression (MLR) models explaining the abundances of *Microcystis* 16S rRNA gene, *Microcystis mcyE* gene and microcystin concentration in Kranji Reservoir.

	<u>Beta value</u>		
	<i>Microcystis</i> 16S rRNA (gene copies/mL)	<i>Microcystis</i> <i>mcyE</i> (gene copies/mL)	Microcystin concentration ($\mu\text{g/L}$)
<u>Predictor Variable</u>			
Total nitrogen (mg/L)	-9.06E6**	-1.48E6**	1.526*
Turbidity (NTU)	5.15E5**	8.51E4**	
pH		-1.26E6*	
Secchi Depth (cm)	3.17E5**		
Constant	-2.36E7**	9.68E6*	0.08
R^2	0.720	0.765	0.238
Adjusted R^2^a	0.703	0.750	0.220
Model Sig.	<0.0005	<0.0005	0.001
F-test	$F_{3,47} = 40.371$	$F_{3,47} = 51.029$	$F_{1,43} = 13.394$

a Adjusted R^2 is a modified statistic term which incorporates a correction for the positive bias of R^2 .

* indicates correlation is significant at the 0.05 level (2-tailed)

** indicates correlation is significant at the 0.01 level (2-tailed)

Using the enter method, significant models were built at a 95% significant level. Only statistically significant variables were retained in the final models. *Microcystis* 16S rRNA and *Microcystis mcyE* gene copy numbers were functions of turbidity, TN, pH and Secchi depth. Therefore, an increase in one of the variables (turbidity) was compensated by the decrease in other variables (Seip, 1994). Strong regression models were obtained to predict the abundances of the *Microcystis* 16S rRNA gene and *Microcystis mcyE* gene (adjusted $R^2 > 0.700$). However, the regression model for microcystin concentration was less significant with an adjusted $R^2 = 0.220$.

3.4 Discussion

3.4.1 PCR and qPCR method efficiencies

Three qPCR assays were successfully optimized to monitor the indigenous cyanobacterial populations. The qPCR assays were more sensitive than the conventional PCR assays. This was shown by the positive qPCR amplifications on PCR-negative samples. The triplex reaction was the most sensitive PCR assay in this study where all samples were positively amplified. The significantly lower detection of *mcyC* gene was an unusual observation as toxigenic *Microcystis* spp. generally need a complete *mcy* gene cluster (*mcyA* – *J*) to generate microcystins (Kaebernick et al., 2002). This result was unlikely to be caused by low sensitivity of the assay as several *mcyC*-negative samples have high *mcyE* gene copy numbers and vice versa. The primer set for *mcyC* was designed for a product from the 3' end of *mcyC* to an open reading frame (ORF) downstream of the *mcyC* gene with unknown function named *umaI* (Tillett et al., 2001). Tillett et al. (2001) showed consistent presence of this PCR product amplified from *Microcystis* spp. and suggested that the constant distance between *mcyC* and *umaI* was evidence that all *Microcystis* originally obtained the *mcy* gene cluster but some species gradually lost the function through mutation. However, this is not the case for our present data set. Unsuccessful *mcyC* gene amplification could be due to unique DNA sequences of local species or a different *mcy* gene cluster arrangement. Therefore, isolation of

the local toxigenic *Microcystis* with and without a constant distance of *mcyC* and *umal*, together with DNA sequencing, are necessary to reveal the reason for negative amplifications.

3.4.2 Dynamics of *Microcystis* spp. and *Anabaena* spp. and correlations with environmental factors

Based on the genus-specific PCR results and *mcyA* gene sequences (results not shown), *Microcystis* was the main microcystin producer in this reservoir. The proportion of toxic *Microcystis* genotype was steadily maintained during the sampling period. Different fluctuation patterns were observed at different sampling sites. The relatively stable cyanobacterial dynamics at Station A and Station B (Figure 3.4) are in accordance with the ordination plot of samples for PCA. Their similar characteristics could be attributed to similar environmental conditions found at these two stations as supported by the T-test.

Microcystis largely dominated the aquatic ecosystem with an average of 100-fold higher cell concentrations compared to *Anabaena*. The *Microcystis* cell concentrations in this study (average 4.16×10^6 ; $7.21 \times 10^3 - 1.08 \times 10^8$ copies 16S rRNA/mL) were higher and relatively constant compared to studies reported in temperate regions such as Lake Erie (Canada/United States) (average $\sim 6 \times 10^3$; $22 - 3.4 \times 10^7$ copies 16S rDNA/mL), Lake Mikata (Japan) (average $\sim 2 \times 10^5$; $7 \times 10^2 - 1 \times 10^6$ copies phycocyanin intergenic spacer/mL), Lake Wannsee (Germany) (average $\sim 9 \times 10^4$; $50 - 1 \times 10^6$ copies phycocyanin gene/mL), Lake 't Joppe (Netherlands) ($3 \times 10^3 - 3 \times 10^5$ cell/mL), Copco Reservoir (Northern California) ($5 \times 10^4 - 3 \times 10^6$ cell/mL) and Hartbeespoort Dam (South Africa) ($5 \times 10^3 - 2 \times 10^6$ cell/mL) (Bozarth et al., 2010; Kardinaal et al., 2007; Kurmayer and Kutzenberger, 2003; Mitsuhiro et al., 2007; Rinta-Kanto et al., 2009; Robarts and Zohary, 1984). However, results from this study were comparable to the study of Hirosawa-no-ike Pond (Japan) (average $\sim 3 \times 10^7$; $4 \times 10^3 - 3 \times 10^8$ copies 16S rRNA/mL) which was reported as hypereutrophic (Ha et al., 2009).

Pronounced cyanobacterial fluctuations observed in other countries are typically associated with seasonal changes (Catherine et al., 2008; El Herry et al., 2008; Kurmayer and Kutzenberger, 2003; Wicks and Thiel, 1990; Ye et al., 2009), where blooms in eutrophic waters tend to be dominated by cyanobacteria during the summer and autumn months (Elliott, 2010). Figure 3.5 illustrates that *Microcystis* blooms reported in subtropical or temperate countries generally range from undetectable to 1×10^7 cells/mL while the toxigenic *Microcystis* based on *mcy* genes vary from undetectable to 3.8×10^5 cells/mL. The two exceptional cases are Lake Taihu and Hirosawa-no-ike Pond, which are hypereutrophic systems and have average *Microcystis* levels exceeding 2×10^7 cells/mL.

In contrast to subtropical and temperate climates, the tropical rainforest climate in Singapore is warm and humid for the entire year. The high, uniform temperature in the tropics, corresponding to the brief summer period in temperate regions, offers optimum climatic conditions for sustained cyanobacterial growth. The results from this study show that the high temperatures, typical of an equatorial climate, are able to maintain continuous high levels of cyanobacteria (chlorophyll-a commonly $> 50 \mu\text{g/L}$ with dominance of cyanobacteria), with a relatively higher and more stable proportion of toxigenic *Microcystis* cells (*Microcystis mcyE* equivalent) (range 21.9-100 %; average 55.9 SD 21.1 %), compared to subtropical and temperate regions (e.g. 0.01 to 27% (Baxa et al., 2010), 8.5 to 17.7 % (Rinta-Kanto et al., 2009), 0.5 to 35 % (Mitsuhiro et al., 2007), 0.9 to 38.3 % (Kurmayer and Kutzenberger, 2003), 4 to 80 % and 0 to 100 % (Kardinaal et al., 2007) and 1 to 86 % (Bozarth et al., 2010)). This has important implications especially with the effects of global warming impacting the average temperature of inland lakes. Several studies have suggested a link between global warming and more intense and toxic *Microcystis* blooms (Davis et al., 2009; Jöhnk et al., 2008; Paerl and Huisman, 2008). This study provides evidence that this is indeed possible.

Although high, equatorial temperatures favoured the growth of *Microcystis* and a high percentage of toxigenic species, no significant correlation was observed between temperature and *Microcystis* using both the PCA and MLA models

($p < 0.05$). This is probably due to the ‘small’ temperature variation relative to the wide variability in other parameters. Under such uniform temperature conditions, it was found that nutrient supply was more important in affecting cyanobacterial dynamics in the reservoir, i.e. both TN and TP were positively correlated with total and toxigenic *Microcystis* abundances, consistent with many other studies (Vaitomaa, 2006). This, in turn, is linked to the landuse pattern which includes 5.4% agricultural usage in the form of vegetable farms, aquaculture and poultry farms and 18% urban runoff (residential and industrial activities). As non-point sources dominate this catchment, it seems reasonable that seasonal rainfall (e.g. during the monsoon periods) could have an impact on the nutrient loading to the reservoir. In addition, earlier studies also showed that internal cycling of phosphorus in the reservoir is important (Gin and Gopalakrishnan, 2010).

Early controls on freshwater eutrophication emphasized phosphorus reduction based on the argument that nitrogen is not the limiting nutrient since organic/inorganic nitrogen compounds are more soluble than phosphorus compounds and N_2 fixation replenishes nitrogen depletion (Paerl, 2009). However, this may not be applicable in controlling the proliferation of toxigenic cyanobacteria. It has been suggested in a more recent study that both phosphorus and nitrogen rich conditions could enhance the yield of toxigenic *Microcystis* and vice versa (Vézic et al., 2002). Davis et al. (2009) presented similar findings where toxigenic *Microcystis* is superior to non-toxigenic strains at higher temperature and phosphorus concentration. Furthermore, several studies demonstrated that nitrogen also promoted toxigenic *Microcystis* as it is associated with the optimum growth rate and the syntheses of microcystin peptide and enzymes involved in microcystin production (Davis et al., 2010; Gobler et al., 2007). Thus, the higher proportion of toxigenic cells found in Kranji Reservoir could be explained by the integrated effects of high temperature and nutrient concentration.

In general, Stations A and B showed more stable populations of *Microcystis* and *Anabaena* compared to Station C. In the latter case, a drastic decrease in cyanobacteria abundance was observed between July and September 2008. The

reason for this is uncertain and the trend does not repeat in the following year. One possibility for the difference in spatial dynamics is that Stations A and B represent 'open' waters whereas Station C is in a narrow tributary arm which experiences fluctuating water levels in the stormwater canals draining a high-density residential area.

Cases of toxigenic *Microcystis* cell number being higher than the total population have been reported (Baxa et al., 2010; Ha et al., 2009; Rinta-Kanto et al., 2005) and it was also evident in this study. Although qPCR is a powerful tool to reveal the composition of cyanobacterial communities, it may however, produce a large error when estimating actual cell numbers in water (Rinta-Kanto et al., 2009). Converting gene copy number to equivalent cell number based on available information on 16S rRNA can sometimes produce unrealistic data. To date, there is only one complete *Microcystis* genomic sequence available (*M. aeruginosa* NIES-843). Furthermore, the assumption of two 16S rRNA genes per genome may not be applicable to all species or strains. This is because bacteria may have irregular rRNA gene copy number in response to the resource availability of their natural surroundings (Klappenbach et al., 2000). DNA sequence heterogeneity is another possible explanation for this (Rinta-Kanto et al., 2009), leading to unsuccessful rRNA gene amplification and underestimation of the rRNA gene copy number. A study has shown that *Microcystis* strains exhibited 1 to 9 % dissimilarity in their 16S rRNA sequences (Neilan et al., 1997). In addition, the sequencing result of the 16S-23S rRNA gene internal transcribed spacer (ITS) region of the local *Microcystis* spp. showed 0.2 – 7.2 % dissimilarity (result not shown).

The PCA and MLR analyses also show that *Microcystis* 16S rRNA and *Microcystis* *mcyE* gene copy numbers were both positively correlated with chlorophyll-a, turbidity and TSS, and negatively correlated with the Secchi depth ($P < 0.0005$), indicating that these parameters can be used as biomass indicators for this reservoir.

3.4.3 Toxin concentrations and correlation with environmental factors

The average microcystin concentration in Kranji Reservoir was 2.66 ± 2.80 $\mu\text{g/L}$. This is not surprising considering microcystin concentrations reported in other countries such as the United States, Finland and the Netherlands fell within the range of $1.7 - 4.6$ $\mu\text{g/L}$ (Gobler et al., 2007; Graham et al., 2004; Hotto et al., 2008; Kardinaal et al., 2007; Rantala et al., 2006).

Microcystin concentration showed significant positive correlations ($P < 0.01$) with the abundance of the *Microcystis* 16S rRNA and *Microcystis mcyE* genes, chlorophyll-a, TSS, turbidity (positive correlations) and Secchi depth (negative correlation). Similar results were also obtained at Erie Lake (Rinta-Kanto et al., 2009) and Hirosawa-no-ike Pond (Ha et al., 2009). Spearman's rho coefficient indicated a positive correlation between the abundance of *Microcystis mcyE* gene and the microcystin concentration. This is consistent with results from other studies which used *mcyA* (Pearson $R^2 = 0.66$) and *mcyD* genes (Pearson $R^2 = 0.65$) to quantify toxigenic cell number (Ha et al., 2009; Hotto et al., 2008), suggesting that all the *mcy* genes are equally well correlated with the occurrence of microcystin.

Although a positive correlation was found between *Anabaena* 16S rRNA gene copies and microcystin concentrations, it was improbable that *Anabaena* contributed to the toxin production as PCR showed no *Anabaena mcyE* detected. In fact, this correlation is likely due to the strong positive correlation between the abundances of *Anabaena* and *Microcystis*. Interestingly, the percentage of toxigenic *Microcystis* did not correlate with toxin concentration. Similar findings were also reported by Kardinaal (2007) and Rinta-Kanto (2009) where microcystin production seemed to be better correlated with *Microcystis* abundance rather than the succession of toxic genotypes. Compared to the study at Hirosawa-no-ike Pond (Ha et al., 2009) which has similar *Microcystis* cell numbers but a lower percentage of toxigenic cells, the microcystin concentrations (average 969 $\mu\text{g/L}$) found in the pond was much greater than toxin concentrations in the Kranji Reservoir. This may be explained by the toxicity variation found in natural blooms due to the waxing

and waning of several cyanobacterial species/strains with different toxin quotas (Rantala, 2007; Sivonen and Jones, 1999).

Non-nitrogen fixing species such as *Microcystis* are known to produce more toxin under nitrogen-rich condition (Sivonen and Jones, 1999). In this study, the significant correlation found between TN and toxin concentration was supported by the MLR model. This is consistent with the chemostat study by Van de Waal et al. (2009) showing that excessive nitrogen supply will increase microcystin production. From their findings, the authors suggested a carbon-nutrient balance hypothesis which predicted the surplus nitrogen in cell was allocated to secondary metabolite, thus increasing the toxin production (Van de Waal et al., 2009).

Apart from TN, pH also strongly correlated with microcystin concentration. A study on a subtropical hypereutrophic lake in South Africa also showed similar pH and microcystin correlations with Pearson's $r = 0.52 - 0.67$ (Wicks and Thiel, 1990). It is interesting to note that pH was also positively correlated with *Microcystis mcyE* copies, and the percentage of toxigenic cells ($r_s = 0.309$, $P < 0.05$). The pH values are usually higher during a bloom event (Rantala et al., 2006; Ye et al., 2009) due to the photosynthesis of algae and cyanobacteria (Albertano et al., 2000; Bowmer and Muirhead, 1987). In return, a high pH condition is advantageous to the growth of cyanobacteria with their carbon-concentrating mechanisms (Kardinaal and Visser, 2005). This eventually increases the toxin production as the cells grow under favourable condition (Kaebernick and Neilan, 2001; Orr and Jones, 1998). A controlled laboratory study on the effect of pH ranging from 6.5 – 10.5 in *Microcystis aeruginosa* culture demonstrated that maximum toxicity was found at both ends of the studied range (Wicks and Thiel, 1990). Positive correlation of toxin production with pH (ranging from 7.10 to 10.35) found in this study is consistent with the laboratory result by Wicks and Thiel (1990). An alternative explanation for the positive correlation is that the higher proportion of toxic *Microcystis* found in the reservoir has caused lower CO₂ level and led to an increase of pH. It is supported by a recent study showing that

toxic *Microcystis* was able to reduce the concentration of dissolved CO₂ in water to a lower level as compared to the non-toxic strain (Van de Waal et al., 2011).

3.5 Conclusion

Microcystis spp. and *Anabaena* spp. were detected in Kranji Reservoir throughout the study period where *Microcystis* was the main toxin producer in the reservoir. Toxigenic cells contributed 55.92 % of the total *Microcystis* population and the average microcystin concentration was 2.66 ± 2.80 µg/L. *Microcystis* 16S rRNA gene and *mcyE* gene numbers were positively correlated with TN and TP while microcystin concentrations were positively correlated with TN, pH and DO. Previous studies showed that the *Microcystis* population and microcystin concentration were significantly affected by the availability of both phosphorus and nitrogen (Gobler et al., 2007; Kardinaal and Visser, 2005; Sivonen and Jones, 1999). However the relative importance of these nutrients may vary strongly for different aquatic environments. Our data suggests that increasing concentration of N or P can increase the *Microcystis* and toxigenic *Microcystis* abundance, contrary to several studies showing that the cell abundances correlated positively only to a single nutrient (either N or P), where even negative correlations were sometimes found (Kotak et al., 2000; Rinta-Kanto et al., 2009; Yoshida et al., 2007). Due to the low variability of temperature in the tropics, it was found that temperature was not significantly correlated to the cyanobacterial dynamics in this particular reservoir, although its overall high value maintained high growth rates of cyanobacteria. Consistent with laboratory results showing that toxigenic *Microcystis* spp. are more superior than non-toxigenic strains under high temperature and nutrients condition, findings from this field study suggest that the combined effects of stable and high temperature, light and nutrient conditions in the Kranji Reservoir led to relatively high and constant abundances of total *Microcystis* as well as higher proportions of toxigenic *Microcystis* throughout the year compared to studies conducted in subtropical and temperate regions. This implies that with global warming and

increasing urbanization on the rise, potentially more lakes could experience more intense and toxic cyanobacterial blooms in the future.

CHAPTER 4

Isolation of Indigenous Cyanobacteria and Molecular Characterization of the Cyanobacterial Community and Diversity of *Microcystis*

This chapter consists of two parts: (1) isolation of indigenous cyanobacterial species from Kranji Reservoir and (2) molecular identification of the cyanobacterial community in the reservoir, focusing on the genetic diversity of *Microcystis* species.

4.1 Isolation of Indigenous Cyanobacteria

Isolation and cultivation works are the basis for many cyanobacteria related research efforts including ecological, physiological, genetic and taxonomic studies (Shirai et al., 1989; Shirai et al., 1991). The establishment of laboratory cultures is critical for controlled studies aimed to understand the environmental or genetic factors which affect cyanotoxins production. Usually, clonal or uni-algal culture is sufficient for taxonomic studies. However, sensitive molecular techniques such as PCR and RT-PCR require axenic (bacterial free) cultures (Bolch and Blackburn, 1996). Moreover, it is important to remove contaminants trapped in mucilaginous colonies before conducting a controlled study to ensure heterotrophic bacteria do not affect the experimental result.

Several cyanobacterial species from genera *Microcystis* and *Anabaena* were isolated from Kranji Reservoir. There were characterized genetically using 16S rRNA sequences. In the second part of this chapter, two toxic *Microcystis* isolates obtained in this study were also characterized using ribosomal internal transcribed spacer (ITS) and microcystin producing genes. These isolates were used as the controls for molecular detection. Lastly, a purified *Microcystis* isolate was used in

the controlled study to evaluate their toxin production under controlled conditions (Chapter 5). Isolation works were carried out using a combination of three standard isolation methods: (1) enrichment of culture medium (2) direct isolation by micropipette and (3) isolation using agar.

4.1.1 Isolation of *Anabaena* species

For the isolation of *Anabaena* species, culture medium enrichments were used as the preliminary method before the direct isolation method. The deficiency or enrichment of one or more nutrients altered the competitive strength of one species relative to the others. This method imposed a positive selection on members of cyanobacteria which were most suitable to thrive and reproduce in the medium, under laboratory culture conditions.

After arrival of water samples at the lab, 1 mL of water sample was added into culture flask containing Z45/4 medium, a recipe similar to Z-medium (Zehnder and Gorham, 1960) but without nitrogen sources. After 2-3 cycles of culture transfer, the surviving stock was dominated by nitrogen-fixing species such as *Anabaena*. Using the direct isolation method, *Anabaena* trichomes or colonies were picked from diluted stock culture under a microscope with micropipettes, washed several times by repeated transferring and picking up in sterile medium. The washed isolates were then deposited in culturing medium. The purpose of this technique was to eliminate contaminants or other attached plankton / bacteria as much as possible (Anderson and Kawachi, 2005; El Herry et al., 2008). Two *Anabaena* species were obtained by this method (Figure 4.1).

Figure 4.1 Cyanobacterial isolates obtained in this study. A: *Anabaena* sp. (Isolate A), B: *Anabaena* sp. (Isolate B), C: *Synechococcus* sp., D: *Limnothrix* sp., E: *Microcystis* sp. (Isolate C) F: *Microcystis* sp. (Isolate E).

4.1.2 Isolations of *Synechococcus* and *Limnothrix* species

Although isolating target cells on agar plate is a common method, it cannot be applied to all cyanobacterial species. This method was found to be more useful for coccoid algae (Anderson and Kawachi, 2005). In this study, apart from

Synechococcus, filamentous cyanobacteria species was also successfully isolated by agar isolation method. Agar plates were prepared by mixing BG-11 medium (Allen and Stanier, 1968) with 0.8 to 1.5% of Bacto agar (Dafco). Agar washing steps were added prior to isolation to remove the impurities which may be toxic to the growth of cyanobacteria. Concentrated agar was prepared by dissolving agar powder in nanopure water, autoclaving and cooling. Solidified agar was cut into small cubes and soaked in nanopure water. The nanopure water was changed daily for 6 to 8 days. After that, washed agar blocks were dried and autoclaved again, while double-strength medium was also prepared and autoclaved separately. To avoid the formation of toxic substances, agar and medium was mixed together slightly above the agar melting temperature to form 1× concentration agar medium (Watanabe, 2005).

The field sample was serially diluted and plated onto agar plates. The agar plates were later sealed with parafilm and incubated at 25°C under a continuous light source of 2500 lx. Green colonies or filaments appeared after 2-3 weeks of incubation. Cyanobacterial colonies were picked up by sterile inoculation loops and then streaked onto new agar plates. The streaked agar plate was incubated again until colony/filament growths were observed. Colonial or filamentous isolates were selected from the plates and grown in liquid culture medium. Morphology of the *Synechococcus sp.* and *Limnothrix sp.* isolates from agar the method are illustrated in Figure 4.1.

4.1.3 Isolation of *Microcystis* species

Isolation of *Microcystis* colonies was initially conducted by using the direct isolation method. However, the isolated colonies were unable to be maintained after several cycles of culturing. It was observed that other bacteria species instead of *Microcystis*, dominated the culture flask. The presence of bacteria could be due to the tight association of contaminating bacteria with the gelatinous material encompassing colony (Rippka, 1988). Therefore, a modified agar isolation method

with a two-step centrifugation (Shirai et al., 1991) was used in order to reduce contaminants.

Fresh water samples from Kranji Reservoir were 100× diluted with ultrapure water and vortexed vigorously for 30 min to disperse the *Microcystis* colonies as much as possible. 1 mL of diluted sample was then transferred into a 2-mL microcentrifuge tube and centrifuged at a low speed of $150 \times g$ for 30 min at 20 °C. This was followed by another round of centrifugation at higher speed of $3000 \times g$ for 5 min at 20 °C. Centrifugation at different speeds allowed the gas-vesiculate cells (*Microcystis*) to float to the surface and be separated from contaminants at the bottom (Shirai et al., 1991). After centrifugation, 1 µL of sample was taken from the surface layer with a micropipette. The sample was serially diluted and plated onto washed agar (0.8%, Bacto agar) containing 1× MLA medium (Bolch and Blackburn, 1996). Agar plates were incubated for 1 month for cyanobacterial colonies to grow. Randomly selected green colonies were identified using PCR assay targeting *Microcystis* 16S rRNA gene. Successful *Microcystis* isolates were transferred to liquid MLA medium for continuous growth.

Two *Microcystis* isolates (Isolates C and E) obtained by this two-step centrifugation and agar method were able to survive in the liquid culture. These isolates are toxic species confirmed by microcystin ELISA kit and PCR assays on *mcyA-E*. In liquid medium, Isolate C formed compact dark green colonies while the Isolate E formed loose green colonies which were easily broken by shaking (Figure 4.1). However, isolates captured from this method were not axenic.

4.1.4 Purification of *Microcystis* species

Microcystis Isolates C and E were further purified using lysozyme treatment (Kim et al., 1999), antibiotic treatments (Bolch and Blackburn, 1996) and the sulphide selection gradient method (Parker, 1982). Interestingly, only one isolate (Isolate E) was successfully purified using the sulphide selection gradient method. *Microcystis* isolates could neither tolerate the treatments nor be purified using other

purification methods. To purify *Microcystis* using the sulphide selection gradient method, 1 ml of culture was first dispersed by repeated drawing and releasing through a needle (0.8 × 38mm, 21G × 1.5) with the aid of a syringe. The culture was then diluted to obtain sample of 1/20, 1/200 and 1/2000 dilutions. 100 µL of each diluted sample was plated onto 0.8 % Bacto agar with MLA medium and left to dry. 3 drops of 1.3 M sodium sulphide (Na₂S) were then dropped onto one side of the plate with a pre-marked spot on the underside of the plate. Agar plates were covered and left in the biohazard cabinet for approximately 2 hours to allow Na₂S to absorb into the agar. They were then double sealed with parafilm, inverted and incubated at 30 °C under a light intensity of 2000 lux.

Green colonies started to appear after 3 weeks of incubation. The Na₂S solution created a clear zone as shown in Figure 4.2. Colonies appearing in the clear zone were picked and the purity was verified using phase contrast microscopy and fluorescence microscopy with acridine orange stain (Darzynkiewicz, 1990).

Figure 4.2 Purification of *Microcystis* Isolate E. A: Black circle indicates location where Na₂S was added, creating a clear zone free of bacterial contaminants. B: Cell purity check with epi-fluorescence microscopy with acridine orange stain before (left) and after (right) treatment.

4.1.5 Morphological and genetic characteristics of cyanobacterial isolates

The morphological features of cyanobacterial isolates observed under inverted light microscope are summarized in Table 4.1. Under laboratory culturing conditions, vegetative cells and heterocysts were present in two *Anabaena* isolates (Isolates A and B) but no akinete was detected. The *Microcystis* Isolate C formed big and compacted colonies compared to *Microcystis* Isolate E. Purification treatment changed the morphological characteristics of the culture, as shown in *Microcystis* Isolate E. It was observed that after sulphide selection gradient treatment, the culture (Isolate E) lost its mucilage sheath and formed loose colonies which were easily disrupted by vigorous shaking.

Genetic identification was also carried out for the isolates by comparing their partial 16S rRNA sequences to the GenBank database. The PCR products of these isolates were obtained using primers CYA106F and CYA781R (Nubel et al., 1997) following methods discussed in Chapter 3 (sections 3.2.3 and 3.2.5). The amplicons were directly sequenced with forward primer, edited and aligned with sequences deposited in Genbank using the BLAST comparative program (Altschul et al., 1990). The three closest matched sequences from cultured strains are listed in Table 4.2. Genetic identifications based on the 16S rRNA sequences were in agreement with morphological results for *Microcystis*, *Synechococcus* and *Limnothrix* isolates. However, the sequences for two *Anabaena* isolates could not be determined due to the presence of multiple PCR products contributed by the heterotrophic bacteria. Hence, to study the genetic information of these *Anabaena* isolates, further culture purification to eliminate non-target bacteria is necessary.

Table 4.1 Morphological characteristics of isolates from Kranji Reservoir.

Isolates	Cell organization	Gas vesicles	Mucilage sheath	Cell size	Shape	Colour
<i>Anabaena</i> sp. Isolate A	Solitary, straight, free-floating	✓	Very thin and colourless	Vegetative cells: 4-6 µm Heterocysts: 5-6 µm Akinetes: Not observed	Spherical to barrel-shape Spherical	Blue green
<i>Anabaena</i> sp. Isolate B	Solitary or bundled, colied, free-floating	✓	Very thin and colourless	Vegetative cells: 5-7 µm Heterocysts: 5-6 µm Akinetes: Not observed	Spherical to barrel-shape Spherical	Dark green to brownish
<i>Synechococcus</i> sp.	Solitary	Not observed	Not observed	4-7 µm		Green
<i>Limnothrix</i> sp.	Loosely entangled or solitary, free-floating	Not observed	Not observed	Width ~ 2 µm	Straight or flexuous, long trichome	Green
<i>Microcystis</i> sp. Isolate C	Densely clustered, free-floating	✓	Surrounding the colony, colourless	4-7 µm	Spherical or nearly spherical	Blue green
<i>Microcystis</i> sp. Isolate E	Loosely clustered or solitary, free-floating	✓	Not observed	2-3 µm	Spherical or nearly spherical	Green

Table 4.2 Comparison of partial 16S rRNA sequence identity between cyanobacterial isolates from this study with other strains based on GenBank database.

Matched culture in Genbank	Compared 16S rRNA fragment	Coverage	Identity	Accession No.
<u>Synechococcus isolate</u>				
<i>Synechococcus</i> sp. CENA108	582 bp	100%	98%	EF088334
<i>Cyanobium</i> sp. JJ2-3		100%	98%	AM710363
<i>Synechococcus</i> sp. PCC 7920		100%	98%	AM709627
<u>Limnothrix isolate</u>				
<i>Limnothrix redekei</i> 2LT25S01	593 bp	100%	99%	FM177493
<i>Limnothrix</i> sp. CENA110		100%	99%	EF088338
<i>Planktothrix</i> sp. FP1		100%	99%	AF212922
<u>Microcystis Isolate C</u>				
<i>Microcystis aeruginosa</i> strain UWOC C1	591 bp	100%	99%	AF139315
<i>Microcystis smithii</i> CHAB139		100%	99%	GQ496077
<i>Microcystis wesenbergii</i> NIES 104		100%	99%	AJ133174
<u>Microcystis Isolate E</u>				
<i>Microcystis aeruginosa</i> UADFM7	580 bp	99%	99%	HM854736
<i>Microcystis smithii</i> CHAB110		99%	99%	GQ496078
<i>Microcystis aeruginosa</i> LMECYA 142		99%	99%	EU078501

4.2 Molecular Characterization of Cyanobacteria and Diversity of *Microcystis* species in Kranji Reservoir

Cyanobacteria that cause harmful algal blooms in freshwater systems are diverse, including members of different genera. In the past, classification of these bacteria has concentrated on their features of morphology and development (Hayes et al., 2007), but the current knowledge on taxonomy is still insufficient to distinguish many cyanobacterial species. Two major obstacles of using morphological identification are (1) distinctive characteristics are limited and (2) plastic morphology maybe present when environmental condition changes (Neilan et al., 1995). To study bloom dynamics and species diversity, a more detailed and

accurate taxonomic system is required that utilizes genetic information including gene sequences and RNA expressions (Neilan et al., 1995). Furthermore, studies of the genetic properties of cyanobacteria, for example, common genetic features present in the bloom-forming species, may assist in understanding their response to environmental challenges (Hayes et al., 2007).

The bloom-forming cyanobacteria, *Microcystis* spp., are the most well studied freshwater phytoplankton species because of their wide-spread occurrence around the world. These organisms frequently dominate the lakes and ponds in tropical and temperate regions (Humbert et al., 2005). Recent advances in molecular techniques make it possible to differentiate toxic and non-toxic *Microcystis* species using specific primers targeting non-ribosomal peptide synthetases (NRPSs) or polyketide synthases (PKSs) that produce over 90 microcystin variants (Hotto et al., 2008). Molecular probes designed for this purpose were mainly applied for detecting *mcyA*, *B*, *C*, *D*, *E* and *J* genes (Bittencourt-Oliveira, 2003; Furukawa et al., 2006; Kim et al., 2010; Kurmayer and Kutzenberger, 2003; Rinta-Kanto et al., 2005; Tillett et al., 2001; Vaitomaa et al., 2003). The *mcy* genes have also been used to study the diversity of toxic cyanobacteria. Several studies have used the partial *mcyA*, *mcyB* and *mcyJ* fragments to reveal the genetic richness of microcystin producing cyanobacteria from genera *Microcystis*, *Anabaena* and *Planktothrix* (Bozarth et al., 2010; Hisbergues et al., 2003; Hotto et al., 2007; Kim et al., 2010; Mikalsen et al., 2003; Rinta-Kanto and Wilhelm, 2006; Ye et al., 2009). However, knowledge and potential use of other *mcy* genes for phylogenetic analysis are still relatively unexplored.

The traditional identification of *Microcystis* based on morphological characteristics is generally insufficient to distinguish *Microcystis* species especially for identification below genus level (Janse et al., 2004). Moreover, single species may present multi morphotypes which complicate the attempt. Currently, the construction and comparison of phylogenetic trees are the best approaches to understand the evolutionary process of toxigenic genes that exist in some cyanobacteria species (Rantala et al., 2004). Molecular approaches have been used

in differentiating *Microcystis* genotypes by comparing the genetic diversity of 16S rRNA, phycocyanin intergenic spacer (PC-IGS), c-phycocyanin BA (*cpcBA*), RNA polymerase (*rpoC1*) and rRNA internal transcribed spacer (ITS) sequences (Bozarth et al., 2010; Briand et al., 2009; Iteman et al., 2000; Neilan et al., 1995; Nishizawa et al., 2007; Otsuka et al., 1999; Sabart et al., 2009; Tillett et al., 2001). In general, many studies showed that *Microcystis* genotypes did not correlate to their phenotypic characteristics (Haande et al., 2007). Molecular techniques have been shown to be more reliable for the recognition of *Microcystis* species and their toxin synthesis property.

Despite great interest in understanding tropical *Microcystis* ecology and population dynamics, little is known about the genetic diversity of this phytoplankton. In contrast to temperate regions, cyanobacterial blooms in the tropics lack obvious seasonal fluctuation due to uniform temperatures throughout the year. However, tropical plankton species may still be influenced by other seasonal changes such as local climatic conditions (i.e. precipitation) and modifications in the chemical and physical characteristics of water (Figueredo and Giani, 2001). Hence, in this chapter, the temporal changes in *Microcystis* cell number and genetic diversity of the cyanobacterial community in Kranji Reservoir were investigated. Cyanobacterial 16S-23S rRNA ITS was selected to study population genetics as it has been shown to be useful for phylogenetic analysis (Briand et al., 2009; Haande et al., 2007; Janse et al., 2004; Otsuka et al., 1999). Moreover, an extensive database for this sequence region, which is essential for genetic comparison, is available on GenBank. A primer set detecting the putative ABC transporter gene, *mcyH*, was designed to evaluate the variability of the microcystin producing gene. The results were then compared with the diversity of partial *mcyA* gene.

4.2.1 Materials and methods

4.2.1.1 Sampling method

Sampling events were carried out monthly at three sampling stations as discussed in Chapter 3. Samples were collected approximately at mid-depth (2 m at Station A; 4 m at Station B and 0.5 m for Station C) using a Niskin water sampler and stored in sterile amber-glass bottles. A total of 54 samples (18 from each station) were collected and transported to the laboratory on ice for processing. Several physical, chemical and biological variables were measured including water temperature, TN, TP, chl-a and microcystin concentrations, abundance of total *Microcystis* equivalent to 16S rRNA gene and toxigenic *Microcystis* equivalent to *mcyE* gene. All related methodology is presented in Chapter 3.

4.2.1.2 PCR, cloning and DNA sequencing

The genetic diversity of the cyanobacteria and *Microcystis* spp. was examined quarterly (i.e. in Feb, May, Aug and Nov 2008 and Feb, May and Aug 2009). PCR assays were performed to amplify a cyanobacterial ITS fragment and two microcystin producing gene fragments from *mcyA* and *mcyH*. The respective oligonucleotide primers are listed in Table 4.3.

Table 4.3 PCR primers used for phylogenetic study.

Target	Primer	Sequence (5' – 3')	Reference
<i>mcyA</i>	<i>mcyA</i> -Cd 1F	AAAATTAAAAGCCGTATCAAA	(Hisbergues <i>et al.</i> , 2003)
	<i>mcyA</i> -Cd 1R	AAAAGTGTTTTATTAGCGGCTCAT	
<i>mcyH</i>	<i>mcyHF</i> 137	GGGGAATGCTGTTTCTCCTYS	This study
	<i>mcyHR</i> 140	CAAGTTCTCCATCGGTCATTTAC	
ITS	322	TGTACACACCGCCCGTC	(Iteman <i>et al.</i> , 2000)
	340	CTCTGTGTGCCTAGGTATCC	

For ITS and *mcyH* PCR reactions, 5 µL of nucleic acid template was mixed in a total reaction volume of 50 µL which contained 10 µL of 5 × PCR buffer, 1.5 mM of MgCl₂, 0.2 µM of dNTPS, 0.5 µM for each forward and reverse primers, 1.25 U of Taq polymerase (GoTaq® Flexi DNA Polymerase, Promega) and 300 ng/µL of bovine serum albumin for amplification enhancement. The reagent mix and amplification for *mcyA* is described in Chapter 3, section 3.2.5. The amplification profile for the *mcyH* gene consisted of an initial denaturation at 95 °C for 3 min, followed by 40 amplification cycles of 95 °C for 40 s; 56 °C for 50 s and 72 °C for 1 min. A final elongation step was carried out at 72 °C for 5 min. The ITS fragments were amplified as described by Iteaman *et al.* (2000). To ensure reaction quality, two positive controls and one negative control (nuclease-free water) were included in each reaction set. For the positive controls, nucleic acids from *Microcystis aeruginosa* PCC 7806 (CSIRO Collection of Living Microalgae, Australia) and a local toxic *Microcystis* isolate (Isolate E) were used. PCR amplicons were analyzed on 1.5% agarose gel stained with ethidium bromide and visualized under UV illumination.

PCR amplicons of the *mcyA* gene were purified using the Wizard SV Gel and PCR Clean-up System (Promega) before direct sequencing from both directions with forward and reverse primers. However, ITS and *mcyH* amplification products were unable to be sequenced directly due to the presence of multiple genotypes. Therefore, the products of these two PCR assays were purified and subsequently cloned into the pGEM-T easy Vector System (Promega) according to the manufacturer's instructions. Three to eight white colonies were randomly picked and the correct target fragments were confirmed by using PCR. Successful transformants were grown overnight in LB broth with ampicillin for plasmid extraction. Plasmid DNA sequencing was performed in the ABI big Dye Terminator Cycle Sequencing Kit and Prism 3100 Genetic Analyzer using commercial promoter primers T7 (5'-TAA TAC GAC TCA CTA TAG GG-3') and SP6 (5'-ATT TAG GTG ACA CTA TAG-3').

All sequences were edited with BioEdit Sequencing Alignment Editor version 7.0.5 (Hall, 1999) and aligned with ClusterW in Mega4 (Tamura *et al.*, 2007). Phylogenetic trees were constructed by the neighbor-joining method with *p*-distance. The bootstrap analyses were set to 1000 replicates. Cyanobacterial genera presented in Kranji Reservoir were identified using the 16S-23S rRNA ITS sequences. Based on the same ITS sequences, phylogenetic relationships of local *Microcystis* spp. were compared with species/samples isolated from Asia (Otsuka *et al.*, 1999), East Africa (Haande *et al.*, 2007) and France (Briand *et al.*, 2009; Humbert *et al.*, 2005). The sequence similarities of *mcyA* and *mcyH* genes were compared and aligned with available sequences deposited in the GenBank database (www.ncbi.nlm.nih.gov/blast/). The GenBank accession numbers for sequences in this study are JN171700 to JN171844.

4.2.2 Results

4.2.2.1 Site conditions and abundances of *Microcystis* populations

A detailed study on the correlations between environmental variables and occurrence of *Microcystis* and toxins using surface water samples is discussed in Chapter 3. In this chapter, samples from middle depths (Stations A and B) were used. The microcystin concentration varied from undetectable to 14.4 µg/L with an average value of 2.40 ± 2.78 µg/L. Table 4.4 summarizes the nutrient levels, temperatures, rainfalls, total and toxigenic *Microcystis* cell numbers and microcystin concentrations obtained during the sampling period from February 2008 to August 2009. The dynamics of *Microcystis* populations at mid-depths of Stations A and B were similar to those found at the surface (Figure 4.3). Average *Microcystis* abundance at Stations A, B and C were 1.29×10^6 , 1.36×10^6 and 7.77×10^6 copies *Microcystis* 16S rRNA gene/mL, respectively. Overall, the average of total *Microcystis* was 1.74×10^6 cell/mL and 7.97×10^5 cell/mL for toxigenic *Microcystis*. Based on *mcyE* and *Microcystis* 16S rRNA gene concentrations, the proportions of toxigenic genotype in the entire *Microcystis* population are shown in Figure 4.4. Using Spearman's rho coefficients, the abundance of total *Microcystis*

cells, toxigenic *Microcystis* cells and microcystin concentration were correlated with monthly rainfall and concentrations of TN, TP and chl-a (Table 4.5).

Table 4.4 Trophic conditions, temperatures, monthly rainfall, abundances of *Microcystis* and microcystin concentrations recorded in Kranji Reservoir.

Sampling site and month	Monthly Rainfall (mm)	TN (mg/L)	TP (mg/L)	Total <i>Microcystis</i> (16S rRNA genes/mL)	Toxigenic <i>Microcystis</i> (<i>mcyE</i> genes/mL)	Water Temperature (°C)	Microcystin concentration (µg/L)
Station A							
Feb 08	84.6	1.110	0.1214	4.31E+04	1.76E+04	28.3	-
May 08	206.4	1.970	0.1071	2.67E+06	1.08E+06	30.0	-
Aug 08	201.4	0.870	0.0505	1.82E+06	3.39E+05	29.2	1.40
Nov 08	231.2	0.910	0.0872	1.26E+06	2.76E+05	28.5	0.99
Feb 09	211.2	1.210	0.1140	9.78E+05	3.76E+05	28.9	1.58
May 09	196.0	1.600	0.0471	1.92E+06	2.72E+05	30.2	2.05
Aug 09	97.6	1.368	0.0290	1.19E+06	3.64E+05	29.4	2.22
Station B							
Feb 08		0.730	0.1159	7.51E+04	5.34E+04	28.6	-
May 08		1.380	0.0820	1.84E+06	7.46E+05	30.2	-
Aug 08		0.910	0.0618	2.08E+06	4.90E+05	29.6	1.62
Nov 08		0.950	0.0627	1.18E+06	3.61E+05	28.4	0.55
Feb 09		1.080	0.0784	8.59E+05	2.74E+05	28.5	1.36
May 09		1.260	0.0438	7.31E+05	1.55E+05	30.7	6.30
Aug 09		1.453	0.0920	1.27E+06	3.62E+05	29.6	0.81
Station C							
Feb 08		1.740	0.2090	3.10E+06	8.17E+05	28.6	-
May 08		2.440	0.0979	5.46E+05	1.24E+05	30.3	-
Aug 08		1.010	0.0618	1.51E+03	1.71E+02	28.5	0.33
Nov 08		2.340	0.1730	1.07E+08	2.12E+07	26.2	2.94
Feb 09		2.430	0.2111	5.76E+06	1.40E+06	28.7	4.28
May 09		4.050	0.1560	5.82E+06	1.48E+06	29.7	14.40
Aug 09		1.880	0.0440	8.89E+05	2.14E+05	29.1	2.21

Figure 4.3 Comparison of *Microcystis* abundance at the water surface and at mid-depths at Stations A and B.

Table 4.5 Correlations of *Microcystis* 16S rRNA gene copy, *Microcystis mcyE* gene copy and microcystin concentration with environmental variables using Spearman's rho coefficients (r_s).

Variable	r_s	P	n
<i>Microcystis</i> 16S rRNA (copies/mL)			
TN (mg/L)	0.460	<0.0005	54
TP (mg/L)	0.436	0.001	54
Chl-a (µg/L)	0.716	<0.0005	54
<i>Microcystis mcyE</i> (copies/mL)			
TN (mg/L)	0.384	0.004	54
TP (mg/L)	0.454	0.001	54
Chl-a (µg/L)	0.674	<0.0005	54
Microcystin (µg/L)			
TN (mg/L)	0.395	0.009	43
Chl-a (µg/L)	0.609	<0.0005	43
Monthly rainfall (mm)	-0.428	0.004	43
<i>Microcystis mcyE</i> (copies/mL)	0.516	<0.0005	43

Figure 4.4 Quarterly observations of the proportion of *Microcystis* genotypes in Kranji Reservoir. Letters A, B and C denote the sampling stations. The cell numbers equivalent to 16S rRNA gene copies are shown in solid lines.

4.2.2.2 Cyanobacterial 16S-23S rRNA ITS in Kranji Reservoir

The PCR product generated from primer set 322 and 340 (Table 4.3) consisted of partial 16S rRNA gene (~155 bp), complete ITS (224-899 bp) and partial 23S rRNA gene (~46 bp). The following discussion focuses on the ITS sequences between 16S and 23S rRNA genes.

A total of 153 clones were randomly picked and their ITS lengths were examined by gel electrophoresis. 91 sequences of different lengths were selected for sequencing. The majority of ITS sequences were contributed by *Microcystis* species, which is discussed in the next section. Other cyanobacterial ITS found in this study were identified using a phylogenetic tree analysis as shown in Figure 4.5.

Figure 4.5 Cyanobacterial genotypes detected in the Kranji Reservoir (excluding *Microcystis*) based on ribosomal ITS sequencing. Sequences in the rectangular boxes share 100% identity. Sequences in this study are in bold type and named according to the format X/Y/Z; where X is the sequence type ('I' for ITS sequence), Y indicates sampling station and date (letters for Stations A, B or C; figures for month and year) and Z is the different genotype detected in the same sample. Bootstrap value $\geq 60\%$ are shown at the node (based on 1000 bootstrap iteration).

Clustering of the sequences shows that besides *Microcystis*, *Anabaena*/*Aphanizomenon* and *Synechococcus* were the prevalent genera identified in the reservoir. Two sequences were unable to have their genera identified by comparing the sequences with Genbank database. However, they were closely related to the genus *Oscillatoria* spp. from phylogenetic analysis. For the clones clustered with *Anabaena*/*Aphanizomenon*, both shorter and longer spacers of the ribosomal operon were detected, of which 11 clones were the shorter spacers (ITS-S) and only 3 were the longer spacers (ITS-L). The exact taxonomy of clones in this cluster could not be determined as *Anabaena* and *Aphanizomenon* are phylogenetically undistinguishable (Gugger et al., 2002). Despite frequent detection, the ITS-S sequence was low in diversity with 8 clones having exactly identical sequence (Figure 4.5). Sequence identity for ITS-S was high (> 0.992) but slightly lower for ITS-L (0.987-0.997). In contrast to *Anabaena*/*Aphanizomenon* ITS, all *Synechococcus* clones detected (17 clones) possessed different genotypes with identity between 0.185-0.998.

Anabaena/*Aphanizomenon* ITS-S differed from ITS-L by the absence of tRNA^{Ile}, tRNA^{Ala} and conserved region of D3 domains as shown in Figure 4.6. Conserved domains D1, D1', D2, D4 and D5, and boxes A and B were found in both ITS-S and ITS-L, except for two ITS-S sequences containing the incomplete D5 domain. Sequence divergence and variation in length were shown in regions between the conserved domains and box B. The conserved domains D1-D5 are important for creating the secondary structure of rRNA operon transcripts, while the antiterminator sites are responsible for ensuring the rRNA transcription in correct stoichiometric ratio (Condon et al., 1995; Iteman et al., 2000). The D1 domain, located at the immediate start of ITS, was paired with the D1' domain to form a stem-loop. D2 and D3 domains are responsible for bending of the rRNA operon structure before the downstream region that contains two tRNA. The antiterminator region with box A and box B follows after the tRNA region. The highly conserved D4 domain forms another bending in the operon and the D5 domain situated at the 3' end of the ITS before the start of 23S rRNA (Iteman et al., 2000).

Figure 4.6 Alignment of *Anabaena/Aphanizomenon* ITS-S and ITS-L sequences found in this study. Identical nucleotides (dots) and gaps (dashes) are shown. Reference sequences for ITS-S (*Aphanizomenon issatschenkoi* 16-1, *Anabaena planktonica* 1-3 and *Anabaena circinalis* 13-3) and ITS-L (*Anabaena* sp. OKARE-25) are included for comparison. The positions of conserved domains (D1 – D5), tRNA genes and antiterminators box B and box A are indicated. Highly conserved domains are shaded in grey, less conserved domains are in boxes and domains which are present in ITS-L only are marked with double lines.

It is interesting to note that different genera produced ITS sequences of variable size but in certain lengths as summarized in Table 4.6. Findings in this study were similar to other reports on cyanobacterial ITS studies, thus suggesting that ITS sequence length is useful for the identification at genus level. Using information of the ITS lengths, the percentages of various cyanobacterial clones during the study period are illustrated in Figure 4.7. Overall, *Anabaena* contributed 16.3% to the total ITS clones, 19.0% was from *Synechococcus*, 1.8% were clones close to *Oscillatoria* and the rest were *Microcystis*. Based on genera found, the diversity at Station C was low compared to Stations A and B, as four samples from Station C gave only clones of a single genus. *Microcystis* was detected in all samples except for the sample from Station C on August 2008 when a clearance of bloom was observed. *Synechococcus* was the only genus detected in this sample. Overall, *Synechococcus* and *Anabaena* presented in 11/21 and 8/21 samples respectively. The percentages shown in Figure 4.7, however, may not reveal the actual cyanobacterial dynamics since only a portion of clones were randomly examined.

Table 4.6 Comparison of ITS sequence lengths of different cyanobacterial genera.

Genera	ITS length in study	ITS length reported in other study	
<i>Anabaena</i> / <i>Aphanizomenon</i>			
ITS-S	224 – 280 bp	249 – 300 bp	(Gugger et al., 2002; Itean et al., 2002)
ITS-L	497 bp	450 – 550 bp	
<i>Synechococcus</i>	644 – 899 bp	660 – 913 bp	(Chen et al., 2006; Choi and Noh, 2009)
<i>Microcystis</i>	355 – 365 bp	354 – 370 bp	(Haande et al., 2007; Humbert et al., 2005; Janse et al., 2004; Otsuka et al., 1999; Sabart et al., 2009)
Sequences closely related to <i>Oscillatoria</i> sp.	425 – 441 bp	540 – 632 bp	(Cadel-Six et al., 2007)

Figure 4.7 Percentage of 16S-23S rRNA ITS clones detected in Kranji Reservoir based on the sequence lengths.

4.2.2.3 Genetic diversity of *Microcystis* 16S-23S rRNA ITS

Based on the ITS sequence length, 97 out of 153 colonies (63.4%) were contributed by *Microcystis* spp. (Table 4.7). 58 of these colonies were selected for sequencing. Considering strains with identical sequences (100% similarity) are of the same genotype, 35 different *Microcystis* genotypes were found based on the ITS sequencing results. Only a single band was obtained for *Microcystis* ITS. The sizes of the whole PCR product (ITS + partial 16S and 23S rRNA) ranged from 554 to 562 bp with sequence identities varying between 0.930 and 0.998 (average of 0.968 ± 0.021). For ITS alone, sequence identities varied between 0.898 and 0.997 (average of 0.968 ± 0.028). The diversity of *Microcystis* spp. from different sampling dates and stations were also compared (Table 4.7). Overall, the aligned ITS sequences showed that there were nucleotide variations at 70 positions (19.1%) including gaps or at 50 positions (14.5%) excluding gaps. The conserved domains of D1, D1', D2, D3, D4, D5 and antiterminators, boxes A and B (Iteman *et al.*, 2000), were found in all sequences as illustrated in Figure 4.8. All domains were highly conserved with only one point mutation found in the D3 and D5 domains respectively. In contrast to other genera such as *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis* and *Oscillatoria* which show the presence of two tRNA genes (tRNA^{Ile} and tRNA^{Ala}) (Cadel-Six *et al.*, 2007; Gugger *et al.*, 2002; Gugger *et al.*, 2005), *Microcystis* ITS sequences contained only tRNA^{Ile} with very few variations (2 point mutations in 74 nucleotides). Four main variable regions were found in the alignment: antiterminator box B, regions between D1 and D1', between D1' and D2, and between tRNA^{Ile} and box B. These variable regions were characterized by both nucleotide variations and gaps.

Table 4.7 Diversity of the *Microcystis* 16S-23S rRNA ITS clones based on sampling dates and sites.

	No. of clones	No. of <i>Microcystis</i> clones	No. of sequenced <i>Microcystis</i> clones	No. of genotypes	Nucleotide similarity $\pm$ standard deviation	Cluster
Sampling date						
Feb 08	23	13	11	9	0.971 ± 0.019	II, I, IV, III
May 08	20	17	11	9	0.971 ± 0.022	II, I, IV
Aug 08	20	12	7	6	0.963 ± 0.021	II, I, IV
Nov 08	21	15	7	7	0.965 ± 0.025	II, I
Feb 09	32	13	7	6	0.969 ± 0.016	II, I, IV
May 09	19	18	9	5	0.969 ± 0.024	II, I
Aug 09	18	10	6	5	0.972 ± 0.016	II, III
Sampling site						
Station A	48	32	18	13	0.967 ± 0.020	
Station B	59	31	23	15	0.966 ± 0.022	
Station C	46	35	17	15	0.969 ± 0.018

Figure 4.8 Alignment of the *Microcystis* ITS sequences found in this study. Sequences from three reference strains are also included. Identical nucleotides (dots) and gaps (dashes) are shown. Highly conserved domains are shaded in grey and the less conserved domain is indicated with a double-line.

Neighbor-joining analysis based on the ITS sequences created a phylogenetic tree that divided into four major clusters from Cluster I to Cluster IV (Figure 4.9). Although with low bootstrap support, a similar tree topology as described by Otsuka *et al.* (1999) was obtained in this study. 90 % of the sequences were grouped into Clusters I, II or III, corresponding to the three major clusters commonly used for *Microcystis* ITS gene studies (Humbert *et al.*, 2005; Otsuka *et al.*, 1999). A new cluster (Cluster IV) containing six ITS sequences was observed. This cluster does not group into any of the other major clusters reported previously but it is phylogenetically close to Clusters II and III. The phylogenetic tree of ITS revealed no significant spatial genotype variations for samples from different stations. However, temporal changes in *Microcystis* genotypes were observed (Table 4.7).

The dominant *Microcystis* genotype (24% of *Microcystis* clone) was from Cluster II and is closely related to the toxic *Microcystis* species from Japan and China (Otsuka *et al.*, 1999) and an isolate from France (Briand *et al.*, 2009). This genotype was detected in at least one sampling station for all sampling months. In addition to this dominant genotype, another 4 genotypes were also detected in more than one sample, each of them grouped into different clusters (rectangles in Figure 4.9). The occurrence of *Microcystis* from Cluster II was the highest (7 months), followed by Cluster I (6 months) and subsequently Cluster IV (4 months). Genotypes from Cluster III were only detected in February 2008 and August 2009. In addition, a distinct genotype (5 identical sequences) from Cluster I was only detected in May 2009.

Figure 4.9 Neighbor-joining tree based on the cyanobacterial 16S – 23S rRNA ITS sequences detected from Kranji Reservoir. The main clusters obtained are shown to the right. Two local toxic *Microcystis* species (Isolates C and E) are included in the analysis. Sequences in the rectangular boxes share 100% identity. Bootstrap values $\geq 60\%$ are shown at the nodes. Sequences isolated in this study are in bold type and designated according to the format X/Y/Z; where X is the sequence type (I for ITS sequence), Y indicates sampling station and date (letters for Stations A, B or C; figures for month and year) and Z is the different genotype detected in the same sample. Reference sequences were obtained from GenBank with their countries of origin presented. The accession numbers are in parentheses. Symbols ‘+’ and ‘–’ indicate microcystin producer and non producer respectively, no symbol for unknown production.

4.2.2.4 *mcyA* and *mcyH* sequences analyses

All *mcyA* sequences detected in Kranji Reservoir samples have 291 bp and clustered with the *Microcystis mcyA* fragments. The novel clade of *Microcystis*-like sequences with 6-bp additional insertion (Hotto et al., 2007; Rinta-Kanto and Wilhelm, 2006) was not found in the samples. Only three different sequences were found which were differentiated by two nucleotide variations at positions 168 and 170, but these were silent mutations which resulted in no amino acid variation. The dominant *mcyA* partial sequence was identical with sequences isolated from Japan (*M. aeruginosa* and *M. viridis*) (Hisbergues et al., 2003) and China (*Microcystis* clone) (Ye et al., 2009), while a unique sequence was detected from Station C in August 08 and May 09, as shown in Figure 4.10.

Figure 4.10 Neighbor-joining tree of partial *mcvA* genes showing the relationship between sequences collected from Kranji Reservoir and those reported from other countries. Sequence name indicates the type of gene ('A' for *mcvA*), sampling station and date.

PCR for the partial *mcyH* gene gave a product of 1260 bp which encodes for an open reading frame for 420 amino acid residues. Two or three *mcyH* clones from each sample were randomly chosen for the sequencing and the resulting phylogenetic tree is illustrated in Figure 4.11. 49 of the 60 selected clones were shown to be genetically distinct. All clones were clustered together with three known *Microcystis mcyH* sequences, which is in agreement with the phylogenetic result from *mcyA*. The sequence identity among all *mcyH* gene fragments from Kranji Reservoir was high (nucleotide: 0.988 ± 0.006 ; amino acid: 0.985 ± 0.008). Significantly high identity was also found when comparing our sequences with *Microcystis* species from other countries (nucleotide: 0.983 ± 0.004 ; amino acid: 0.976 ± 0.006), but less similarity was observed when compared with *Planktothrix* spp. (nucleotide: 0.815 ± 0.002 ; amino acid: 0.824 ± 0.003), *Anabaena* sp. (nucleotide: 0.763 ± 0.001 ; amino acid: 0.739 ± 0.001), and the ABC transporters of *Nodularia spumigena* (nucleotide: 0.754 ± 0.002 ; amino acid: 0.750 ± 0.002) and *Nostoc* sp. (nucleotide: 0.705 ± 0.002 ; amino acid: 0.659 ± 0.002).

Several amino acid residue sequences from this study were aligned with the *mcyH* gene from other cyanobacterial genera (*Planktothrix* and *Anabaena*). Two representative members of ABC transporters from *Nodularia* (putative nodularin ABC transporter, *ndaI*) and *Nostoc* (nostopeptolide syntheses, NosG) (Moffitt and Neilan, 2004) that are structurally and functionally similar (Figure 4.12) were also included. The *mcyH* amino acid sequences presented in this study consist of two parts: 1) a hydrophobic N-terminal module from residues 1 to 356 that forms the transmembrane domains (TMDs), and 2) a partial sequence of the hydrophilic C-terminal module from residues 357 to 420, which forms the nucleotide binding domains (NBDs) including the Walker-A motif and partial Q-loop (Davidson et al., 2008; Jones and George, 2002). Walker-A motif (GPSGRGKS) together with the Walker-B motif provide the nucleotide binding fold (Holland and Blight, 1999). The Q-loop not only links several motifs in the NBDs but also aids the interaction between TMDs and NBDs in the ABC transporter (Davidson et al., 2008).

Figure 4.11 Neighbor-joining tree based on the partial *mcyH* gene (1260 bp) isolated from Kranji Reservoir. The putative ABC transporters from *Nodularia spumigena* NSOR10 (*ndaI*) and *Nostoc* sp. GSV224 (NosG) were aligned with the *mcyH* sequences from *Microcystis* spp., *Planktothrix* spp. and *Anabaena* sp. Sequence names starting with 'H' indicated *mcyH* sequences.

Figure 4.12 Amino acid sequence alignment of the *mcyH* genes obtained in this study. Conserved region of Walker A motif marked in the box and the partial Q-loop is shown by arrows.

4.2.3 Discussion

4.2.3.1 Diversity of cyanobacteria in Kranji Reservoir based on ITS sequences

Using the ITS sequence variations, the temporal cyanobacterial community of Kranji Reservoir was revealed. However, a discrepancy was found between the genetic results from this study and microscope analysis from PUB, Singapore. For example, ITS result showed that *Microcystis*, *Anabaena/Aphanizomenon*, *Synechococcus*, and possibly *Oscillatoria* species were present in the reservoir (Figures 4.5 and 4.9). In contrast, microscope observations showed the presence of *Microcystis*, *Oscillatoria*, *Anabaena*, *Anabaenopsis*, *Lyngbya*, *Merismopedia* and *Raphidiopsis* but not *Aphanizomenon* and *Synechococcus* (PUB, unpublished data).

Considering that not all colonies were chosen for sequencing in this study, it is therefore possible that some cyanobacterial genera were undetected due to random selection. Nevertheless, incongruent results from cyanobacterial phenotypic and genotypic characteristics have been widely reported. For instance, studies have shown that *Anabaena* and *Aphanizomenon* could not be differentiated according to their 16S rRNA, ITS or *rbcLX* nucleotide sequences (Iteman et al., 2002; Lyra et al., 2001). Although these two genera exhibit distinctive morphological features, their 16S rRNA sequence similarity is found to be > 97.5% (Gugger et al., 2002), exceeding the lower boundary of 95% which defines a genus (Stackebrandt and Goebel, 1994). A study on the RNA polymerase gene of *Anabaena* and *Aphanizomenon* also provided similar results which showed these two genera were clustered together (Fergusson and Saint, 2000b). In another study by Palinska et al. (Palinska et al., 1996), isolates from *Merismopedia* and *Synechococcus* genera have revealed sequence similarity more than 96%. These findings may help explaining our observation why *Aphanizomenon* and *Synechococcus* were not detected using the microscopic method. To resolve problems associated with the taxonomic validity of cyanobacterial genera, a combined approach of both morphological and molecular methods is recommended.

Comparing the cyanobacterial ITS obtained in this study, it was shown that five conserved domains of D1, D1', D2, D4 and D5 essential for the correct folding of the rRNA operon were found in all the sequences regardless of their lengths and genera. D2 and D4 are of particular importance as they direct the transcription to form the 16S rRNA and 23S rRNA processing stems (Iteman et al., 2000). The antiterminator box A region (GAACCTTGAAAA) was highly conserved in sequences from all genera. However, antiterminator box B was a diverse region with many nucleotide deletions and variations. This result supports previous studies that suggested only the box A region is needed to keep the rRNA transcription at an appropriate ratio (Gourse et al., 1986; Iteman et al., 2000). Findings on the ITS size and primary structure of various cyanobacterial genera reported in this study are valuable for molecular characterizations. When a sufficiently complete genetic database is available, the species identification of cyanobacteria at a higher resolution and accuracy can be achieved.

4.2.3.2 Genetic variation and dynamics of *Microcystis* based on ITS sequences

In Kranji Reservoir, the abundance of the total *Microcystis* population and toxigenic population showed strong positive correlations with TN and TP concentrations which confirmed the effect of nutrients on bloom formation. Microcystin concentration was positively correlated with TN and *mcyE* copies but negatively correlated with monthly rainfall (Table 4.5). It is interesting to note that although higher rainfall seemed to reduce the microcystin concentration, no significant correlation was found between the rainfall and the abundance of *Microcystis* population. A possible explanation for this is that the rainfall pattern changes the *Microcystis* genotypic composition in the reservoir, instead of diluting the toxin concentration.

Microcystin synthesis genes are useful for the detection of toxin producers. However, to investigate the genetic variability of the whole *Microcystis* population, the same diagnostic platform for both toxic and non-toxic members is required, such as the use of ITS sequence. In order to illustrate the *Microcystis* population

genotypes in this study, ITS sequences from other regions including sequences from Asia, Uganda, Kenya and France were used (Briand et al., 2009; Haande et al., 2007; Otsuka et al., 1999). Two toxic *Microcystis* species isolated from Kranji Reservoir (Isolates C and E) whose microcystin producing abilities have been confirmed by ELISA and PCR based on *mcyA*, *B*, *C*, *D*, *E* and *H*, were also included in the analysis. From the tree topography, Isolate C is grouped in Cluster II whereas Isolate E belongs to Cluster I (Figure 4.9). The *Microcystis* ITS phylogenetic tree produced in this study is similar to the tree structures demonstrated in other previous studies (Humbert et al., 2005; Otsuka et al., 1999). Several efforts have been made to characterize toxic and non-toxic species by utilizing the ITS sequences. For example, it was shown that all *Microcystis* from Cluster II were toxic species while Cluster III *Microcystis* were non-toxic species (Otsuka *et al.*, 1999). Janse *et al.* (2004) also supported this idea by showing that toxic and non-toxic *Microcystis* were distinguishable based on their rRNA ITS. These findings might be applicable while analyzing the *Microcystis* bloom in Kranji Reservoir.

With a large number of ITS clones grouped as Cluster II *Microcystis*, current results suggest that the *Microcystis* bloom in this reservoir was dominated by toxic species. This could be supported by another two observations: (1) a toxic local *Microcystis* isolate, Isolate C, was also grouped in Cluster II; (2) one sample that contained 100% toxigenic *Microcystis* (Station B, Feb 2008) had 80% ITS clones appearing in Cluster II. In addition to these, the high percentage of clones of possible toxigenic species from Cluster II (50% of the clones) were in agreement with our previous result which indicated that 55.92% of the *Microcystis* population was toxigenic (Te and Gin, 2011). However, for the exact identification of *Microcystis* genotypes and their toxin quota, it is necessary to isolate as many *Microcystis* genotypes as possible that exist in the reservoir.

Higher sequence heterogeneity of Cluster I shown in earlier studies was also evident in the present results. Cluster I *Microcystis* contains sequences from many regions (Asia, Europe, Africa and New Zealand), suggesting a worldwide distribution of these *Microcystis* genotypes. In the present study, 27.6 % of the

sequenced clones fell within this cluster, but their toxin production abilities were undetermined since both toxic and non-toxic species coexist in Cluster I. It is interesting to note that the appearance of a distinct Cluster I genotype from all stations in May 2009 corresponded to the highest microcystin concentrations at Stations B and C. Succession of toxic *Microcystis* genotypes with different toxin quotas could be the explanation for this observation as demonstrated in many field and laboratory studies (Carrillo et al., 2003; Haande et al., 2007; Kardinaal and Visser, 2005). However, the increase in microcystin concentration could also be due to the lysis of bloom species or stimulation from other environmental factors.

This study demonstrates the high genetic diversity of the *Microcystis* population in Kranji Reservoir. This variability remained high even for an extreme bloom event recorded at Station C in Nov 2008 (all isolated colonies are of different genotypes). Located in the tropics with high light and temperature throughout the year, this reservoir provided favorable growing conditions for *Microcystis*. Moreover, the relatively stable hydrodynamic conditions and uniform temperatures might help to reduce the loss of genetic diversity due to natural selection, thus allowing the survival of different genotypes. However, changes in *Microcystis* genotype composition in response to environmental factors still remain unknown unless the complete *Microcystis* ITS clone library, together with comprehensive monitoring of environmental variables are available for a substantial period of time. Potential microcystin producers from the genera *Anabaena* and *Planktothrix*, were also detected in the reservoir using a microscope during the sampling period, but PCR assays showed none of them contained the microcystin synthesis genes (Te and Gin, 2011). Sequencing results of the partial *mcyA* and *mcyH* genes in this study complemented each other and confirmed that *Microcystis* was the only microcystin producer.

4.2.3.3 *mcyA* and *mcyH* genes diversity

To our knowledge, there has been no previous study using the *mcyH* gene to evaluate the diversity of toxic *Microcystis* species. In this study, greater genetic

variation was shown in the *mcyH* sequence than the *mcyA* sequence when the nucleotide variations of both sequences were compared. Earlier research using *mcyA* gene fragments to study the divergence of microcystin producers gave sufficiently good results to differentiate producers from different genera (Hisbergues *et al.*, 2003) or to differentiate producers from the same genus but differing in geographical and hydrological conditions (Hotto *et al.*, 2008). Note that current result on *mcyA* diversity is consistent with the result from a temperate lake in China, i.e. Lake Taihu (Ye *et al.*, 2009), where it was found that *mcyA* sequences were low in diversity.

The ABC transporters (exporters) in prokaryotes are responsible for the excretion of lipids, proteins, polysaccharides, drugs and toxins (Davidson *et al.*, 2008). Regardless of the variety of substrates (allocrites) being moved across cellular membranes via these transporters, all ABC transporters consist of two TMDs and two NBDs. The conserved NBDs fuel the movement of allocrites through the membrane, while the less conserved TMDs recognize specific allocrites and provide the translocation pathways (Holland and A. Blight, 1999). It is believed that the substrate-binding sites that exist in TMDs contribute to the sequence diversity (Jones and George, 2002). This may have implication on the current study as *Microcystis* spp. are able to produce a wide range of allocrites (microcystin variants). Since individual *Microcystis* sp. may have different toxin quotas, the TMDs may be compromised structurally in order to mediate the transport of a specific microcystin congener or a pool of variants produced by the cell. Therefore, the TMDs sequences in *mcyH* genes might have evolved to optimize microcystin binding and export. A similar phenomenon has been described for the P-glycoprotein (pgp) plasma membrane transporter that gives multi-drug resistance in eukaryotic cells (Devine *et al.*, 1992). It was found that amino acid mutations in a pgp transmembrane domain influenced substrate specificity and changed the drug efflux (Devine *et al.*, 1992).

The genetic diversity of the ABC transporters within bacterial species has important implications. For example, the development of an effective

pneumococcal vaccine was greatly complicated by the nucleotide divergence of the lipoprotein sequences of the *Streptococcus pneumonia* ABC transporter (Whalan *et al.*, 2006). A more relevant finding on the mutated plant root ABC transporter showed that a single gene mutation could alter the photochemical exudates from plants and subsequently change the microbial communities in soils (Badri *et al.*, 2009). Thus, it is possible that genetic changes of the *Microcystis* ABC transporter could affect the synthesis of the toxin spectrum (different microcystin variants) and the export of toxins to the environment.

While the mutation of plant ABC transporter modified the secretion of root exudates, it was found that a mutation on the putative ABC transporter gene (*mcyH*) in *Microcystis* PCC7806 resulted in loss of microcystin production (Pearson *et al.*, 2004). From their result, Pearson and colleagues suggested that the *mcyH* gene not only encodes for the ABC transporter but is also involved in stabilizing microcystin compounds during enzyme-based biosynthesis. In nature, wild-type *Microcystis* species are subject to natural variation and random mutation which may cause alteration or inactivation of the *mcyH* function. Following this, the production of microcystin could be disturbed or destroyed. As such, genetic information of the *mcyH* sequence may provide important insights into controlling the level of microcystin and types of variants produced.

4.2.4 Conclusion

Phylogenetic trees based on the ITS sequences have revealed the presence of cyanobacteria from genera *Microcystis*, *Anabaena/Aphanizomenon*, *Synechococcus* and possible *Oscillatoria* in Kranji Reservoir. A novel cluster of *Microcystis* ITS sequences was also identified from local samples, suggesting unique indigenous cyanobacterial strains inhabit our reservoirs. By sequencing the *mcyH* gene fragments obtained from environmental samples that included a TMD and partial NBD, it was shown that the *mcyH* gene is highly varied. Therefore, this gene fragment can be used as an alternative tool to study the genetic diversity of toxic *Microcystis* populations in a natural bloom.

CHAPTER 5

Effect of Light Wavelength on *Microcystis* Growth and Microcystin Content

5.1 Introduction

Microcystis is one of the most intensively studied freshwater cyanobacterial species as it is known to form blooms with varied toxicity (Fastner et al., 1999; Visser et al., 2005). Worldwide, the hepatotoxin, microcystin, is the most commonly detected toxin found in freshwater blooms, where cyanobacteria of genera *Microcystis*, *Anabaena*, *Planktothrix* and *Nostoc* are the major producers (Sivonen and Jones, 1999). To date, the exact role of microcystins in the lake ecosystem remains unknown despite extensive studies carried out to obtain the answer. Nevertheless, these studies reveal that the dynamics of microcystins in a bloom is modulated by two factors: (1) changes in cyanobacterial genotypic composition that lead to populations with different toxin potentials; and (2) changes in environmental parameters that affect bloom formation or toxin biosynthesis (Fastner et al., 1999; Wiedner et al., 2003). Although the abundance and genotype of toxin producers in waters seem to control the toxin variation on a larger scale, there are cases which indicate that environmental variables can affect the toxicity of a *Microcystis* bloom (Kardinaal and Visser, 2005). Environmental factors that have been shown to affect toxin production include light, pH, temperature, nitrogen, phosphorus, iron and inorganic carbon (Deblois and Juneau, 2010; Jahnichen et al., 2007; Long et al., 2001; Lyck, 2004; Orr and Jones, 1998; Rapala et al., 1997; Sivonen, 1990; Utkilen and Gjølme, 1992; Utkilen and Gjølme, 1995; Westhuizen and Eloff, 1985; Wiedner et al., 2003). Although several studies suggested that the highest microcystin production corresponded to optimal cell growth (Long et al., 2001; Orr and Jones, 1998), more recent evidence show that the highest microcystin/cyanotoxin concentration did not correspond to the maximum cell

growth or an inverse correlation was observed between growth rate and microcystin production (Deblois and Juneau, 2010; Tonk et al., 2009; Wiedner et al., 2003).

Recently, an interesting hypothesis for the ecological function of microcystin postulated that it might have an internal function related to the cell growth and photosynthesis process (Jahnichen et al., 2007). Indeed, many findings derived from previous experiments support this putative function of microcystin. These experiments involved a comparison of the photosynthetic pigments and intracellular carbon deficiency between toxic *Microcystis* PCC7806 and its non-toxic mutant cell (Hesse et al., 2001; Jahnichen et al., 2007); identifying the allocation of microcystin in cells (Gerbersdorf, 2006; Shi et al., 1995; Young et al., 2005); examining transcription levels of microcystin producing genes; and investigating how light quantity/quality affected toxin amount and variants generated by cyanobacteria (Deblois and Juneau, 2010; Lyck, 2004; Tonk et al., 2005; Utkilen and Gjølme, 1992; Watanabe et al., 1989; Westhuizen and Eloff, 1985).

Further support has been given in a recent study suggesting that microcystin protects cells from oxidative stress. Comparing protein profile differences between the wild type *Microcystis* (microcystin producer) and its mutant cell (microcystin deficient), Zilliges et al. found that the protein profile change in mutant cells was related to the enzymes of the Calvin cycle and phycobiliproteins. In addition, their study also showed that microcystin was bound to photosynthesis-related proteins (i.e. RubisCo, phycocyanin and allophycocyanin subunits) in the cell. The binding was enhanced under high light condition and reduced the proteases activity against the microcystin-bound-protein (Zilliges et al., 2011).

Changes in light not only affect the microcystin quota in cells, but also influence the growth and pigment content of cyanobacteria (MacIntyre et al., 2002; Wyman and Fay, 1986). The ability to manipulate pigment combinations, also known as chromatic adaptation, is important for many cyanobacteria because it serves a general function for light absorption in the cells (Wyman and Fay, 1986).

Light intensive research has been carried out to show how chromatic adaption increases competition advantages to phytoplankton, promoting biodiversity in water columns and raising the global primary productivity (Kehoe and Gutu, 2006; Kehoe, 2010; Stomp et al., 2004; Stomp et al., 2007b). However, few studies have investigated the effects of light spectra (i.e. wavelength) on microcystin production in comparison to those focused on light intensity. Among those limited studies of light spectra on microcystin production, some showed there was no significant toxin variation when *Microcystis* cells were treated at different wavelengths of light (Gerbersdorf, 2006; Kaebernick et al., 2000), while other studies showed that light spectra could disturb the toxin circadian rhythm, toxic gene expression and toxin amount per cell (Carneiro et al., 2009; Kaebernick et al., 2000; Utkilen and Gjølme, 1992). Due to contradicting experimental outcomes, no conclusive observation has been drawn on the response of microcystin production to different light wavelengths.

This study aimed to extend the knowledge of the effects of light spectra on *Microcystis* growth rate as well as their toxin (microcystin) production. Batch culturing system was chosen for this experiment to mimic the behaviour of a natural bloom from its start to collapse. *Microcystis* cultures were grown under controlled conditions and exposed to different light spectra/wavelengths. Changes in cell quotas of microcystin, pigments and microcystin-producing gene were monitored during cell growth, from exponential phase to stationary phase.

5.2 Materials and Methods

5.2.1 Cell culturing and experimental setup

An axenic local toxic *Microcystis* species, Isolate E, was obtained from a tropical reservoir in Singapore (1°25'N; 103°44'E). The isolate was obtained by a two-step centrifugation and agar method, and its purity was verified using phase contrast microscope and acridine orange stain as mentioned in sections 4.1.3 and 4.1.4. Prior to the experiment, *Microcystis* cells were grown until mid-exponential

phase in liquid medium. The time required for the *Microcystis* Isolate E to reach the mid-exponential growth phase was pre-determined. To avoid changes in the culture conditions due to sampling (i.e. reduction in volume), 24-27 culturing flasks were prepared for each culture batch. For each sampling, several flasks were randomly selected and sacrificed for measurements without disturbing other growing cells. Since the *Microcystis* culture was a colony-forming species, the culture was vortexed vigorously before spiking into the tissue culture flasks (Techno Plastic Products AG, 25 cm²) containing 45 mL of sterile MLA medium (Bolch and Blackburn, 1996). These flasks were equipped with 0.2 µm filters at the caps that allowed sterile air exchange. Flasks spiked with culture were put in a temperature room at a constant temperature of 29 °C.

Cells were grown under a series of white florescent lamps (Philips TL-D 18W/54 Daylight) at a photon irradiance of 20-28 µmol/m²·s by adjusting the distance between flasks and lamps. A photometer equipped with quantum sensor (SKP 200, Skpe Instruments Ltd) was used to measure the photosynthetically active radiation (PAR). To examine the effect of different light colours on the *Microcystis* species, four culturing systems were set up: white (control), red, green and blue light dominant conditions. Irradiances with different light spectra were accomplished by covering the culture flasks with non-monochromatic plastic foils. The absorbance wavelengths for the colour foils were measured by wrapping a cuvette with each colour foil and scanning with a spectrophotometer. The wavelength scans for the colour foils are shown in Figure 5.1 and Table 5.1. Figure 5.2 showed the spectral profile for white florescent lamp. The experimental set up is illustrated in Figure 5.3.

Figure 5.1 The spectral distribution of colour foils used in this study (colour lines) comparing with the visible light spectra shown in colour bar.

Figure 5.2 The light spectral fluorescent lamp (Philips TL-D 18W/54) used in this study.

Table 5.1 The absorbance of wavelengths provided by colour foils.

<i>Filter colour</i>	<i>Range of wavelength</i>	<i>Peak</i>
Red	572 - 750	700 - 750
Green	493 - 565	528
Blue	419 - 534	481

Figure 5.3 Experimental set up showing local *Microcystis* species, Isolate E, grown under different light spectra condition: (A) white light (B) red light dominant (C) green light dominant and (D) blue light dominant conditions.

To ensure homogenous cell growth in all flasks, flasks were randomly shuffled daily to eliminate the variations in light intensity due to different positions on the shelves. Also, the flasks were shaken twice per day to disaggregate colonies formed during the culturing process. *Microcystis* cultures were allowed to grow for 13–15 days and samples were drawn for analysis in triplicate. Sampling was carried

out two days after the culture spiking and repeated every 32–40 hours. During sampling, randomly selected flasks were kept in the dark immediately to avoid further cell growth. Samples were processed within 4 hours and then stored at either 4 or -20 °C, according to the requirements of measured parameters. Six parameters were measured from exponential phase to stationary phase including *Microcystis* cell number, *Microcystis mcyE* gene copy numbers, and the concentrations of chlorophyll-a (chl-a), phycobiliproteins and microcystin.

5.2.2 Cell counting

Samples for cell count were stained and preserved with Lugol's solution (Utkilen et al., 1999). Depending on the cell concentration, 1 to 5 mL of sample was added with Lugol's solution to a final concentration of 1 % and kept in the dark at 4 °C. Before counting, the sample was vortexed for 5 min to break up the colonies completely. Then the sample was diluted or concentrated to a concentration of 5×10^3 – 2×10^5 cell/mL. 1-mL diluted/concentrated sample was filled in a Sedgwick-Rafter Counting Cell (Camlab, UK) and left for 15 min to allow settling of cells. The number of cells was counted in duplicate using an inverted microscope. Cell concentration in the original sample was determined using the equation: $[Cell] = \frac{N \times F_{total}}{F} \times \frac{D}{S}$, where $[Cell]$ is the cell concentration, N is the number of cells counted, F_{total} is the total number of fields in the Sedgwick-Rafter cell, F is the number of fields counted, D is the diluted/concentrated volume and S is the sample volume.

5.2.3 Determine *Microcystis mcyE* gene copy numbers, chl-a and microcystin concentrations

For DNA extraction, 5 mL of sample was first filtered onto a Whatman-AH filter and transferred into a 2-mL sterile tube (screw cap) containing ¼ filled sterile 2 µm glass beads (Sigma-Aldrich) and kept frozen at -20 °C. The detailed DNA extraction method is discussed in Chapter 3 (3.2.3). The abundance of *Microcystis* toxin synthesis *mcyE* gene were determined using two qPCR assays described

previously (3.2.6 and 3.2.7). For chl-a concentration, 10-15 mL of sample was harvested on a 0.45 µm pore size cellulose nitrate filter and stored at -20 °C in the dark. Procedures to extract and determine the chl-a content is listed discussed in Chapter 3 (3.2.2). Samples for microcystin concentration measurement were kept in glass bottles, wrapped with aluminium foil and stored at -20 °C. Total microcystin concentration was analyzed using a microcystin/nodularin ELISA kit (Abraxis LLC). Toxin samples were sonicated for 30 min before the measurement. Since the ELISA kit has a quantification limit between 0.15 and 5 µg/L, samples exceeding the upper quantification limit were diluted accordingly and remeasured. Only total microcystin concentration was measured in this study.

5.2.4 Phycobiliprotein extraction and measurement

5 to 10 mL of cell culture was harvested onto a Whatman glass microfibre membrane and transferred to a micro-centrifuge tube with ¼ filled acid-washed glass beads and stored at -20 °C. Phycobiliprotein was extracted with the modified method by Kursur et al. (Kursar et al., 1983b) using a sodium phosphate buffer (NaPi) at pH 5.5. The preparation of NaPi buffer was done by adding 150 mL of dibasic phosphate solution (Na₂HPO₄, 24 g/L) and 235 mL of monobasic phosphate solution (NaH₂PO₄·H₂O, 27.6 g/L) to 615 mL of ultrapure water. The pH of the final solution was tested and then adjusted accordingly with hydrochloric acid (1 M). For cell disruption, 1.5 mL of NaPi buffer was added to the microfibre membrane, frozen at -80 °C and then thawed in a 20 °C water bath twice in the dark. This was followed by mechanical beating at a speed of 40 for 10 s three times. Lastly, the mixture was centrifuged at 8000 rpm for 30 min and the supernatant was collected for phycobiliprotein measurement. During processing, samples were kept in the dark at 4 °C at all times unless stated, to avoid pigment degradation.

To determine phycobiliprotein concentrations, the absorbances of the supernatant were determined using a spectrophotometer at 498.5 nm, 614 nm, 651 nm and 750 nm. The readings for 750 nm were subtracted from the readings of the

other wavelengths as a correction factor. The contents of phycocyanin (PC) and phycoerythrin (PE) were then calculated using the following equations (Kursar et al., 1983b), where A is the absorbance at different wavelengths.

$$\text{PC } (\mu\text{g/mL}) = 151.1 A_{614} - 99.1 A_{651}$$

$$\text{PE } (\mu\text{g/mL}) = 155.8 A_{498.5} - 40.0 A_{614} - 10.5 A_{651}$$

5.2.5 Statistical analysis

The cell quotas for toxin (microcystin) (Q_{MCYST}), chl-a (Q_{chl}), PC (Q_{PC}), and *mcyE* gene copy number (Q_{mcyE}) were calculated for different growth phases. This was done by dividing the variable concentrations with their respective cell concentrations (e.g. chl/mL was divided by cell/mL). The cell specific cell growth rate (μ_{cell}) and production rates for chl-a (μ_{chl}), PC (μ_{PC}) and microcystin (μ_{MCYST}) were determined by calculating the difference between successive sample points using the equation below, assuming that the growth or production rates followed a simple first-order reaction.

$$\mu = \frac{\ln(N_2 - N_1)}{t_2 - t_1}$$

In this equation, μ is the specific growth rate or production rate, N_1 and N_2 are the numbers of cells or the concentrations of cell component at time t_1 and t_2 . All units were expressed in hour^{-1} .

Data analyses were carried out using the statistical software package PASW Statistics 18 (SPSS Inc.). Data sets of each measured variable were checked for normality using the Kolmogorov-Smirnov and Shapiro-Wilk tests. For data sets which were not normally distributed, non-parametric analyses were used for data comparison. For the multiple sample comparison, analysis of variance was performed using the Kruskal-Wallis (KW) test which is equivalent to the parametric one-way ANOVA. It was followed by the Bonferroni t test conducted manually to discover those means that were different (Glover and Mitchell, 2004). This was

carried out by a two-tailed comparison of the means for two conditions (e.g. i and j) calculated using the test statistic Z with the following equation:

$$z_{ij} = \frac{\left| \frac{R_i}{n_i} - \frac{R_j}{n_j} \right|}{\sqrt{\frac{N(N+1)}{12} \left(\frac{1}{n_i} + \frac{1}{n_j} \right)}}$$

where z_{ij} is the test statistic for the mean difference between conditions i and j , R_i is the sum of ranks from condition i , n_i is the sample number of condition i and N is the total sample number. Comparison for the differences between two growth phases was determined with the Mann-Whitney U test for non-normally distributed populations (Glover and Mitchell, 2004).

Finally, the growth/production rates of different compounds (μ) as well as their quotas in cell (Q) were tested with Spearman's correlation coefficient (r_s) based on the samples' ranks to reveal significant correlations existing between the variables.

5.3 Results

5.3.1 Cellular growth rate and growth phase

Under growing conditions provided in this study, the growth curves of *Microcystis* Isolate E can be divided into three phases – exponential, late exponential and stationary, as shown in Figure 5.4. The growth phases were determined by comparing the change in the specific cell growth rate (μ_{cell}) between two successive samples using the Mann-Whitney U test ($P < 0.05$). The resulting comparison is illustrated in Figure 5.5. All culture batches had exponential growth for 9-11 days (222–262 hours) before reaching the stationary phase. At the beginning of the experiment, culture batches experienced a 5-day (118 hours) fast-growing exponential phase. After that, culture batches entered the late exponential phase which lasted for 4 (red and green) or 6 days (white and blue) before reaching the stationary phase. During the first 5 days of exponential growth, differences in growth rate were found among culture batches growing under different spectral

conditions (KW test: $\chi^2 = 9.86$, $df = 3$, $P < 0.05$). Cultures grown under blue light showed significantly lower specific growth rate (Bonferroni t : $P < 0.05$). However, this difference was not observed in the late exponential and stationary phases where all culture batches showed similar specific growth rates (Table 5.2). The slower growth in blue light during the initial exponential phase, caused a significantly lower cell count at the stationary phase (average 7.3×10^5 cell/mL) compared to the batches grown under white, red and green light (average 1.1 - 1.7×10^6 cell/mL) (KW test: $\chi^2 = 17.5$, $df = 3$, $P < 0.005$). Overall, *Microcystis* cells in the exponential phase proliferated 3 times faster than in the late exponential phase with average doubling times of 18.4 hours and 53.3 hours, respectively (Table 5.2).

Figure 5.4 Growth curves of *Microcystis* culture batches incubated under different dominant spectra of light. Error bars indicate standard deviations for triplicate samples. Separation of growth phases are indicated by vertical lines. All culture batches have 5 days of exponential phase (solid vertical line). Batches grown under red and green spectra entered the stationary phase at 222 hour, while those grown under white and blue spectra entered at 262 hours.

Figure 5.5 Specific cell growth rates of *Microcystis* Isolate E under various spectra of light. The vertical length of the box indicates 50% of cases of the variable (between the 25th and 75th percentiles), the horizontal line in box shows median value, while extended lines from the box are the smallest and largest values that are not outliers. For outliers, cases extended more than 1.5 box-lengths and 3 box-lengths from the edge of the box are represented by circles (○) and asterisks (★) respectively.

Table 5.2. Cell specific growth rate (μ_{cell}) and doubling time (T_d) at different growth phases.

Spectrum	Exponential	Late exponential	Stationary
	<u>Average cell specific growth rate, μ_{cell} (day⁻¹)</u>		
White	1.08±0.26	0.33±0.18	-0.05±0.04
Red	1.14±0.09	0.37±0.11	0.06±0.06
Green	0.97±0.23	0.29±0.14	0.13±0.12
Blue	0.73±0.16 *	0.34±0.10	0.08±0.14
	<u>Doubling time, T_d (hour)</u>		
White	16.3±4.8	53.4±24.7	NA
Red	14.6±1.2	49.7±17.3	NA
Green	18.5±6.5	55.0±21.6	NA
Blue	24.2±7.4*	55.2±21.1	NA

* Significant difference was found using the KW test ($P < 0.05$).

NA: not applicable

5.3.2 Microcystin quotas in cells

The change in microcystin content was normalized to cells (Q_{MCYST}) in pg microcystin/cell for comparison. The general trend of Q_{MCYST} in the course of the experiment can be seen in Figure 5.6. Under all conditions of different spectra of light, Q_{MCYST} content increased from day 2 to day 8/9 until the maximum was reached. Thereafter, a rapid decrease in Q_{MCYST} content was observed when cultures entered the stationary phase. Further comparison of Q_{MCYST} was performed by averaging values based on the exponential, late exponential and stationary phases (Figure 5.6). During the exponential phase, the batch grown under red light showed significantly higher Q_{MCYST} (average 0.52 pg/cell) compared to batches under other colours of light (KW test: $P < 0.05$, $df = 3$). The batch under blue light exhibited the lowest Q_{MCYST} with an average of 0.26 pg/cell. In the late exponential phase, however, Q_{MCYST} of the white and green light microcosms (average 0.84 and 0.81

pg/cell, respectively) were higher compared to those grown under red and blue light (average 0.60 pg/cell). An approximately twofold (2.03–2.35) increase in Q_{MCYST} was observed in all culture batches when cultures progressed from the exponential phase to the late exponential phase. An exceptional case was observed for the red light microcosm in which no significant Q_{MCYST} difference was detected during the exponential and late exponential phases (Mann-Whitney U test: $P > 0.05$). In the stationary phase, Q_{MCYST} of all batches were reduced to similar levels of 0.38-0.46 pg/cell (KW test: $P > 0.05$).

Figure 5.6 Change of *Microcystis* cell quotas for microcystin (Q_{MCYST}), *mcyE* gene (Q_{mcyE}), chlorophyll-a (Q_{chl}) and phycocyanin (Q_{PC}) during the experiment.

Figure 5.7 *Microcystis* cell quotas of microcystin (Q_{MCYST}), *mcyE* gene (Q_{mcyE}), chlorophyll-a (Q_{chl}) and phycocyanin (Q_{PC}) based on different growth phases. Cases extended more than 1.5 box-lengths and 3 box-lengths from the edge of the box are represented by circle (○) and asterisk (★) respectively.

5.3.3 Chlorophyll-a and phycobiliprotein quotas in cell

As illustrated in Figure 5.6, for all culture batches, the chl-a content per cell (Q_{chl}) increased from day 5 (118 hr) and reached a peak on day 8 (190 hr) where the highest quota ($Q_{chl} = 1.51$ pg/cell) was found in the batch grown under white light. This was followed by those grown under blue and green light (1.08 and 1.06 pg/cell), and lastly under red light (0.71 pg/cell). The maximum variation in Q_{chl}

was observed in the white light microcosm with an approximately 5-fold variation; while for the batch grown under red light, only a 1.6-fold change in Q_{chl} content was observed. Overall, the batch under red light exhibited the lowest Q_{chl} during the entire experiment (KW test: $\chi^2 = 9.08$, $df = 3$, $P < 0.05$).

Concentrations of two phycolipoproteins, PC and PE, were measured during the cell growth. However, all data for PE were discarded due to unrealistic negative readings recorded from all batches, suggesting the absence or negligible quantities of this pigment in the *Microcystis* strain. In fact, studies have shown that PE is not a common accessory pigment for *Microcystis* spp. (Otsuka et al., 1998a; Otsuka et al., 1998b). In addition, the observation of colour change of the cells during the experiment showed that the *Microcystis* Isolate E varied from light green to dark green when grew under different light wavelengths. This also suggests that Isolate E is a non-PE-containing strain.

The overall pattern described for Q_{chl} during the entire growth period was also observed for Q_{PC} . The Q_{PC} value fluctuated between 0.22 and 2.78 pg/cell. It is interesting to note that the red light microcosm exhibited the lowest Q_{PC} and Q_{chl} compared to other microcosms. Significantly higher Q_{PC} was also observed in the blue light microcosm during stationary phase (KW test: $\chi^2 = 11.65$, $df = 3$, $P < 0.05$) which resulted in a higher PC/chl-a ratio (KW test: $\chi^2 = 10.40$, $df = 3$, $P < 0.05$).

5.3.4 *Microcystis mcyE* gene quotas in cell

Relationships between the *mcyE* gene copy number and counted cell number for different light spectra conditions are listed in Table 5.3. Pooling all the data together, it can be seen that approximately 59 copies of *mcyE* gene equivalent to one *Microcystis* cell (Figure 5.8). The *mcyE* gene copy per cell (Q_{mcyE}) largely ranged from 2.2 to 80.2 gene copies per cell. The general trend for Q_{mcyE} differed from those found for Q_{MCYST} , Q_{chl} and Q_{PC} , as shown in Figure 5.6. The Q_{mcyE} gradually increased from the exponential phase, and was maintained at a stable level when entering into the stationary phase. A Q_{mcyE} peak was only observed in

the white and green batches. At stationary phase, Q_{mcyE} for batches grown under white and red spectra were significantly higher than those under green and blue spectra, with an approximate difference of 20 gene copies per cell.

Table 5.3 Relationships between the cell numbers and the *mcyE* gene copy numbers using the simple linear regression.

Light spectrum	Slope	r^2
White	68.13	0.906
Red	59.47	0.868
Green	48.12	0.867
Blue	45.97	0.924

Figure 5.8 Comparison between microscope-counted cell numbers and *mcyE* gene copy numbers (by qPCR) for all culture batches.

5.3.5 Correlation analysis

In this experiment, the maximum cell quota variations within the culture batches were 4-fold for Q_{MCYST} (white), 5.6-fold for Q_{chl} (white), 7.1-fold for Q_{PC} (white) and 132-fold for Q_{mcyE} (blue). Comparing the differences between culture batches for each sampling time point, cell quota variations were 2.4-fold for Q_{MCYST} , 2.1-fold for Q_{chl} , 6.8-fold for Q_{PC} and 103-fold for Q_{mcyE} . Table 5.4 indicates the Spearman's correlation analysis between individual cell quotas by pooling all the data together. As indicated in the table, Q_{MCYST} was positively correlated with Q_{chl} , Q_{PC} and Q_{mcyE} ($P < 0.005$). Significant positive correlations were also found for Q_{chl} with Q_{PC} and Q_{mcyE} ($P < 0.005$). However, these significances were not valid for all individual culture batches for the different spectral conditions, as shown in Table 5.4. A strong positive correlation was found between Q_{MCYST} and Q_{chl} which accounts for >70% variability for the overall and individual batches. Comparing specific cell growth rate (μ_{cell}) with various cell quotas, significant inverse relationships were observed between the μ_{cell} and Q_{chl} , Q_{MCYST} and Q_{mcyE} ($P < 0.005$).

When comparing μ_{cell} with specific production rates of other cellular compounds using all data, positive correlations ($P < 0.05$) were found for all studied parameters (μ_{chl} , μ_{PC} , μ_{MCYST} and μ_{mcyE}). In addition, the μ_{MCYST} was found to be correlated strongly with μ_{chl} ($r_s = 0.83$) but less so with μ_{PC} and μ_{mcyE} ($r_s > 0.5$). Meanwhile, a strong linear correlation was also found between μ_{chl} and μ_{PC} ($r_s = 0.79$). No significant correlation was observed between production rates of *mcyE* (μ_{mcyE}) and photosynthetic pigments (μ_{chl} and μ_{PC}). Notable positive correlations were found among μ_{cell} , μ_{chl} and μ_{MCYST} . These correlations were not only detected in the overall data, but were also observed for individual microcosms grown under the different light spectra.

Table 5.4 Correlation analyses for the cell quotas of Q_{MCCYST} , Q_{chl} , Q_{PC} and Q_{mcyE} , and the specific cell growth rate (μ_{cell}) and specific production rates of chlorophyll-a (μ_{chl}), phycocyanin (μ_{PC}), microcystin (μ_{MCCYST}) and *mcyE* gene (μ_{mcyE}).

	<u>Spearman's correlation coefficient, r_s</u>				
	White	Red	Green	Blue	Overall
Q_{MCCYST} VS Q_{chl}	**0.794	**0.728	**0.831	**0.848	**0.735
Q_{MCCYST} VS Q_{PC}	**0.857	*0.491	0.486	0.433	**0.594
Q_{MCCYST} VS Q_{mcyE}	**0.694	-0.117	**0.826	0.396	**0.543
Q_{chl} VS Q_{PC}	**0.882	**0.795	*0.572	0.447	**0.778
Q_{chl} VS Q_{mcyE}	**0.679	0.119	**0.751	0.374	**0.389
μ_{cell} VS Q_{chl}	** -0.597	-0.039	-0.401	** -0.623	** -0.419
μ_{cell} VS Q_{PC}	-0.246	-0.351	0.163	* -0.505	-0.135
μ_{cell} VS Q_{MCCYST}	-0.429	0.306	-0.310	** -0.669	** -0.272
μ_{cell} VS Q_{mcyE}	** -0.802	* -0.481	-0.420	** -0.683	** -0.563
μ_{cell} VS μ_{chl}	*0.560	**0.868	**0.672	**0.614	**0.644
μ_{cell} VS μ_{PC}	-0.018	*0.539	*0.699	0.357	*0.334
μ_{cell} VS μ_{MCCYST}	*0.489	**0.732	*0.439	*0.513	**0.572
μ_{cell} VS μ_{mcyE}	0.224	**0.756	0.309	-0.036	*0.278
μ_{chl} VS μ_{PC}	**0.807	**0.928	0.549	**0.754	**0.790
μ_{MCCYST} VS μ_{chl}	**0.717	**0.931	**0.744	**0.883	**0.828
μ_{MCCYST} VS μ_{PC}	*0.631	**0.924	0.302	**0.901	**0.619
μ_{MCCYST} VS μ_{mcyE}	**0.797	*0.579	**0.843	-0.072	**0.532

Significant correlations were shown in bold.

* Correlation is significant at the 0.05 level (2-tailed).

** Correlation is significant at the 0.01 level (2-tailed).

5.4 Discussion

Four growth conditions at different dominant light colours were provided in this study using colour foils as filters. Since the colour foils could not entirely exclude non-target wavelengths, the presence of other wavelengths of light at low intensity was expected. This setup restricted the investigation of individual light colours effect on *Microcystis* species. Nevertheless, it is still reasonable to assume that the *Microcystis* were grown under the influences of different dominant colours of light provided by selected foil materials. Such conditions are relevant and important in investigating the response of *Microcystis* to actual aquatic environments where underwater spectra are dominated by a single colour and transmission of light of certain wavelengths / colours are reduced instead of being entirely absent. For example, in eutrophic waters the dominant wavelengths are red while green wavelengths are dominant in mesotrophic waters.

Not only does light intensity show depth-dependent changes, but the spectral profile of light penetrating through the water also changes according to the water depth (Kehoe, 2010; Van et al., 1977; Wyman and Fay, 1986). Changes in light spectra in the water column are characterized by the spectral absorption coefficients of various wavelengths which are also affected by the water characteristics (Mitchell et al., 2000; Stomp et al., 2007b). In natural waters, turbidity results in attenuation of blue wavelengths and shifting of the light spectra towards the red (Stomp et al., 2007b). For clear blue oceans, blue light dominates in water. For mesotrophic or intermediate-turbid waters, underwater spectra are usually dominated by green wavelengths. In nutrient-rich water bodies, the dissolved and inorganic substances absorb blue wavelengths, diminishing these wavelengths from reaching deeper water layers, and hence, red wavelengths are the dominant light in these waters (Wyman and Fay, 1986). These natural variations in light spectra could be of particular importance for the growth and physiological functions of cyanobacteria.

5.4.1 *Microcystis* growth rate and photosynthetic pigments

Changes in photon irradiance have been shown to affect photosynthetic activities and is known as photoacclimation (Deblois and Juneau, 2010). Cyanobacteria may alter the pigments or photosynthetical units in their cells by changing the amounts or stoichiometric ratios. Photoacclimation is accomplished for the purposes of: (1) increasing the efficiency of light utilization of the photosynthetic process when PAR (photosynthetically active radiation) is low; or (2) protecting the photosynthesis apparatus against damage of photooxidation at light saturation conditions (MacIntyre et al., 2002; Wyman and Fay, 1986).

The pigment chl-a absorbs blue (400-450 nm) and red (650-700 nm) wavelengths of light and it is essential for photosynthesis in all cyanobacteria. However, chl-a in cyanobacteria is not involved in external light harvesting because all chl-a molecules are combined with proteins associated with photosystems I and II (PSI and PSII) to become inner light harvesting pigment-protein complexes (Sun et al., 2009; Zhang et al., 2007). Besides the main photosynthetical unit, chlorophyll-a, the *Microcystis* culture possesses several accessory pigments including carotenoids and phycobilisomes which allow cells to utilize light energy from a broad range of spectra (Mullineaux, 2008; Sun et al., 2009). Phycobilisomes capture light spectra that are poorly absorbed by chl-a and transfer energy to PSI and occasionally to PSII (Sun et al., 2009). The phycobiliproteins, PC and APC (allophycocyanin), are found in all *Microcystis* spp. as the external antennas to harvest and transport light energy to the reaction centre (Raps et al., 1985). PC is a blue/blue-purple coloured pigment responsible for energy capture from light wavelengths 580-630 nm, corresponding to the yellow-orange-red light regime (Morcos et al., 1988; Sun et al., 2009). Although APC is present in all cyanobacterial species, it is of less amount with respect to PC and PE (Sun et al., 2009). While PE-containing *Microcystis* isolates have been reported, they are not common species (Otsuka et al., 1998a). Apart from the phycobilisomes, carotenoids also serve as accessory pigments in cyanobacterial cells for two functions: they absorb light between 450 and 570 nm, i.e. from blue to green wavelengths, and transfer captured energy to chlorophyll thereby increasing photosynthesis

production (Paerl, 1984; Tyystjärvi et al., 2002). Although not measured in this study, carotenoids are strongly associated with enhanced light utilization for *Microcystis aeruginosa* (Paerl et al., 1983; Raps et al., 1983).

Based on previous information obtained for cyanobacterial pigment compositions and the pigment results in this study, it was concluded that the main photosynthetic pigments responsible for the light harvesting in this study were chl-a, phycobiliprotein PC and carotenoids. Since PE was not detected in the phycobiliproteins measurement and the measured APC content was low in concentration (results not shown), analyses on pigment changes were focused on chl-a and the phycobiliprotein PC.

For the same light intensity, white, red and green microcosms showed similar growth rates but a slower growth was found in the blue light microcosm. There are two possible explanations for the results obtained. First, it could be an outcome of photoinhibition that caused a reduced photosynthesis capacity in which the photo flux density was in excess of the quantity needed for saturated photosynthesis (Vonshak and Tomaselli, 2000). A recent study on photoinhibition induced by different wavelengths of lights found that given the same excess light intensity, blue light caused more severe damage than green and red light for capsicum and spinach leaves (Oguchi et al., 2011).

Cyanobacteria are reported to be more light sensitive than other plankton and relatively susceptible to photoinhibition (Paerl et al., 1983; Paerl, 1984). Previous in-situ investigations showed that the *Anabaena* species is photoinhibited by visible light especially the blue spectrum (Codd and Stewart, 1980). Under photoinhibitory stress, the growth rate of cyanobacteria can be suppressed, as shown in a study conducted on Antarctic cyanobacteria (Roos and Vincent, 1998). This finding is similar to our observation for the blue light microcosm during the exponential phase. After this period, a gradual increase in the growth rate with time observed in the cultures may imply an acclimation process of *Microcystis* cells to the stressed condition (Roos and Vincent, 1998). Another study on photoinhibition

showed that *Microcystis* and *Anabaena* species under blue light exhibited inhibitory effects on their ribulose biphosphate carboxylase enzyme activities that decreased the overall growth (Codd and Stewart, 1980).

Alternatively, a slower growth rate in *Microcystis* culture could be attributed to the light harvesting pigments composition in the cell that caused lower photosynthetic rate when the culture was under blue light irradiance in comparison to other light spectra (Whitelam and Codd, 1983). A reduction in photosynthetic photoelectron transport rate has also been found when *Microcystis aeruginosa* was exposed to blue light (Wyman and Fay, 1986). From the photosynthetic pigment composition obtained, our *Microcystis* strain absorbs light principally in the red regime. Incubation dominated by red light provided preferential spectra for the accessory pigment, PC, resulting in faster growth during the exponential phase. Although this PC-rich *Microcystis* was less sensitive to green light, its carotenoids content, nevertheless, was still able to assist in the energy transfer to the photosynthesis reaction center (Paerl et al., 1983; Schagerl and Müller, 2006). Therefore, using the carotenoids, *Microcystis* cultures under green light were able to maintain their photosynthetic efficiency and achieve growth rates similar to that recorded for the white light microcosm.

Blue light was poorly captured by the PC-rich cyanobacteria. This is because the blue light excited chl-a mostly binds with the photosystem proteins and the carotenoids may help to absorb only some wavelengths in the blue regime (Whitelam and Codd, 1983; Wyman and Fay, 1986). Although most of marine phytoplankton are well-adapted to blue light, the dominant wavelengths in clear deep ocean (Stomp et al., 2007a), but a reduced photosynthetic efficiency for cyanobacteria under blue light has been evidenced in studies on freshwater cyanobacteria (*Microcystis*, *Oscillatoria* and *Gloeotrichia*) and a few marine cyanobacteria / algae (Figuerola et al., 1995; Whitelam and Codd, 1983; Wyman and Fay, 1986), possibly as an adaptive divergence to the light wavelengths available in natural environments (Stomp, 2004).

5.4.2 Effects of light spectra and cell growth on microcystin production

Photoirradiance is known to induce microcystin production in the cell. The influence can be quantitative (light intensity) or qualitative (light spectrum/colour) (Deblois and Juneau, 2010; Hesse et al., 2001; Kaebernick et al., 2000; Utkilen and Gjølme, 1992; Westhuizen and Eloff, 1985; Wiedner et al., 2003). For most studies, microcystin was found to positively correlate with PAR intensity under light-limited conditions until the light-saturated state was reached. After that, toxin production would decrease or be maintained at the highest level (Deblois and Juneau, 2010; Utkilen and Gjølme, 1992; Wiedner et al., 2003). The effect of light colour on the toxin variation in cells has been less investigated and the results are inconsistent and only focus on the steady-state condition or short-term exposure (Kaebernick et al., 2000; Utkilen and Gjølme, 1992). Furthermore, many reported studies have found that microcystin production is affected by the cell specific growth rate (Lyck, 2004; Orr and Jones, 1998; Wiedner et al., 2003), suggesting the microcystin content in cells changes at various stages of growth. Thus, understanding the effects of light spectra and the different phases of cell growth are relevant to exploring toxicity changes in a natural bloom which is under the influence of different spectra due to the hydraulic condition and water characteristics (turbidity and trophic level).

One of the reasons that hinders the comparison between results of different studies is the use of units to present microcystin data. Using microcystin content per cell component such as protein, chl-a and biomass may lead to over/under estimation of toxin content as these cell components can vary in the course of the growth cycle (Long et al., 2001; Wiedner et al., 2003). This idea is supported by our results showing irregular amounts of cellular compounds (Q_{chl} and Q_{PC}) throughout the experiment. Also, Lyck (2004) denoted in her study that no relationship was found between Q_{chl} and microcystin/protein ratio, implying different trends of development for the two variables. In addition, data analysis will be complicated if the cell components and microcystin respond similarly to environmental variables. Therefore, to avoid misleading results or bias in data interpretation, the microcystin content was expressed on a per cell basis. Following this, other cellular contents

measured in this study (chl-a, PC and *mcyE*) were also normalized to a unit cell for direct comparison.

This study demonstrates the effects of light spectra on microcystin production at different growth phases. A previous report by Kaebernick et al. (2000) showed that the microcystin synthesis gene transcriptions (*mcyB* and *mcyD*) were elevated when *Microcystis* cultures were transferred from white light to red light and incubated for 2 hours. This result is in agreement with our study in which red light was found to induce Q_{MCYST} significantly during the exponential phase, by at least 30% higher compared with batches of other light spectra. The accessory pigment, PC, of *Microcystis* enhanced the light harvesting system PSII under red light and increased the efficiency of photosynthesis. This eventually led to increased microcystin production, consistent with other studies showing its connection with the photosynthetic process (Deblois and Juneau, 2010; Jahnichen et al., 2007; Shi et al., 1995; Young et al., 2005; Zilliges et al., 2011). As microcystin biosynthesis is an ATP-dependent process (Bickel and Lyck, 2001), enhanced light harvesting ability in red light also helps to fuel the *Microcystis* cell to an energetic state favourable for the toxin production.

It was shown that *Microcystis* under red light at $16 \mu\text{mol}/\text{m}^2\cdot\text{s}$ produced high toxin amounts that were comparable to incubation under white with a high intensity of $68 \mu\text{mol}/\text{m}^2\cdot\text{s}$ (Kaebernick et al., 2000). In comparison, the red light microcosm in this study was provided with an irradiance of $\sim 24 \mu\text{mol}/\text{m}^2\cdot\text{s}$, which is substantially higher. Moreover, prolonged incubation under red light also increases photo-irradiance that is harmful to the PSII units by blocking the electron transport chain (Whitelam and Codd, 1983). To overcome this, *Microcystis* culture may photoacclimate, allowing the cell to adjust its pigment content or dissipate excess energy (Deblois and Juneau, 2010). In fact, an indication of photoacclimation can be seen in our study from the reduced chl-a and PC contents in the cell under red light during the late exponential phase (Figures 5.4 and 5.5), as demonstrated in studies by Raps et al. as well (Raps et al., 1983; Raps et al., 1985). As a result of

this acclimation, Q_{MCYST} for the red light microcosm stopped increasing during the late exponential phase while the other microcosms showed increasing trends.

A positive correlation was found between the specific cell growth rate (μ_{cell}) and microcystin production rate (μ_{MCYST}), which was also reported by previous related studies (Deblois and Juneau, 2010; Long et al., 2001; Lyck, 2004; Oh et al., 2001; Orr and Jones, 1998). However, μ_{cell} and μ_{MCYST} in this study differ from those obtained in another study by Long et al. (2000) where an inverse relationship was found for Q_{MCYST} and μ_{cell} . Our results, together with those observations by Lyck (2004) and Deblois et al. (2010), showed enhanced microcystin production when cells were grown in favourable conditions (Rapala et al., 1997), giving rise to the hypothesis that microcystin is a constitutively synthesized metabolite. The Q_{MCYST} values in our experiment ranged from 0.22 to 0.97 pg/cell, where the variation during the growth cycle differed by 2.3-4 fold; while the variation between different colour microcosms showed 1.2-2.6 fold differences. Similar to findings of 1.5-3 fold in Q_{MCYST} variation (Deblois and Juneau, 2010; Lyck, 2004; Wiedner et al., 2003) regardless of the types of treatment imposed on *Microcystis*, our results support the suggestion of constitutive microcystin production. Before division, the cell will produce cellular compounds in sufficient amounts to equally allocate the compounds to daughter cells (Alberts et al., 2002). Thus, approximately a two fold change in Q_{MCYST} is foreseen for a growing culture. However, the doubling of cellular compounds may not be regulated precisely at a factor of two (Alberts et al., 2002). As observed in this study, Q_{MCYST} was “diluted” when cells were dividing rapidly in the exponential phase but it gradually increased during the late exponential phase when cell growth was slowing down. This explains why a positive correlation between μ_{cell} and μ_{MCYST} and inverse correlation between Q_{MCYST} and μ_{cell} were detected concurrently in our experiment.

During the experiment, Q_{MCSYT} was found to be significantly correlated with all cell quotas (Q_{chl} , Q_{PC} and Q_{mcyE}). Comparing the overall correlation coefficients (r_s), Q_{chl} turned out to be the most suitable parameter in estimating Q_{MCYST} . Examining this relationship for each batch culture, it was found that the light

spectrum did not affect the strong correlation between Q_{MCYST} and Q_{chl} . Furthermore, our results also show that the production of microcystin (μ_{MCYST}) and chl-a (μ_{chl}) were well-correlated, where 83% of the μ_{chl} variation could be attributed to the variation in μ_{MCYST} . The connection between microcystin and chl-a presented is in agreement with several studies on microcystin production using varied approaches. The study by Long et al. on N-limited chemostat *Microcystis* cultures demonstrated a constant microcystin/chl-a ratio at different growth rates (Long et al., 2001). Experiments conducted in the laboratory and field also indicate that photoirradiance affected microcystin cell quota as well as the chl-a quota in a similar way (Deblois and Juneau, 2010; Lyck and Christoffersen, 2003; Lyck, 2004).

In addition to the above, *Microcystis* mutant cells deficient in microcystin production have been shown to possess less chl-a compared to wild-type cells capable of toxin synthesis (Hesse et al., 2001). By using immune-gold labeling techniques to identify the microcystin locations in cells, it was revealed that the toxin mainly accumulated at the thylakoid membranes (Shi et al., 1995; Young et al., 2005). Since the thylakoid membranes change with photoirradiance and affect the chl-a content (Deblois and Juneau, 2010; Kana and Glibert, 1987), it is possible that the association between microcystin and chl-a is linked to the changes in thylakoid caused by photosynthesis. Indeed, a recent study has proven that the electron transport rate involved in photosynthetic activity can be a good indicator of microcystin content in cells (Deblois and Juneau, 2010). Collectively, our data together with the above findings, suggest that photosynthesis not only regulates chl-a content but also microcystin synthesis in cells.

5.4.3 *Microcystis mcyE* quota (Q_{mcyE})

A surprisingly huge variation in the *mcyE* gene copy number cell quota (Q_{mcyE}) was detected. Correlating gene copy numbers with measured cell numbers showed that one *Microcystis* cell was equivalent to 45-68 copies *mcyE* gene with R^2 between 0.87 and 0.92 (Table 5.3). From genomic sequencing, it is known that *Microcystis* sp. has one *mcy* gene cluster per genome (Christiansen et al., 2003).

Therefore, ideally, molecular quantification using qPCR that measures gene copy number should produce the same result as the microscopic count, but this is not so for our case. Despite the contradiction, our observation coincides with other studies which show that molecular counts were higher than cell counts by orders of magnitude (Ha et al., 2009; Rinta-Kanto et al., 2005). A possible explanation for this is a changing genome copy number at different growth phases, as indicated in a previous report that showed *Synechococcus* sp. has more copies of genome during the early culturing stage (Becker et al., 2002). However, data discrepancies caused by under-estimation of cell number or over-estimation of gene copy number could not be excluded in this study. Possible errors include: (1) by using a Sedgwick-Rafter Counting Cell, cells sank in the deeper layer or overlapped and were hard to detect; (2) cells broke down during sample processing or storage; or (3) presence of free-DNA that increased the amount of *mcyE* gene when cells lysed naturally in the culture flask. A study to investigate change of toxin gene copy during cell growth is necessary to reveal the internal genomic dynamics of cells. While molecular methods become more popular for cyanobacterial studies, it is important to understand the potential shortcomings when using *mcy* gene number to quantify the toxic *Microcystis* population.

5.5 Conclusion

From the results presented, it is concluded that light quality/spectra has positive impacts on the growth and toxin synthesis of *Microcystis* species. Cell quotas of microcystin, chl-a, PC and *mcyE* changed during the course of the experiment. Red light increased the microcystin content significantly in the first 5 days of the exponential phase. An obvious photoacclimation process was also detected when *Microcystis* was grown under red light, in which the chl-a and PC quotas in cells were significantly reduced. Current data also suggest that microcystin is a possible constitutive metabolite, and its cell quota is closely associated with cell growth rate and chl-a quota. Q_{chl} was the best parameter to predict microcystin content in cells. The results of this study support the hypothesis

that the photosynthesis process may be involved in the modulation of microcystin production.

CHAPTER 6

Conclusions and Recommendations

6.1 Conclusions

Massive proliferation of cyanobacteria in freshwater aquatic systems such as rivers, lakes and reservoirs is a concern for water consumers, water related industries and public health and environmental agencies due to the production of a diverse range of toxins associated with cyanobacterial blooms. With the commonly reported concentration levels persisting in water bodies used for drinking or recreational purposes, cyanotoxins pose potential risks of illness and fatality to humans and live-stock. Currently, studies on tropical cyanobacterial blooms are limited compared to those conducted in temperate and cold regions. This thesis describes the first *Microcystis* bloom study in Singapore using advanced molecular techniques. The dynamics of *Microcystis*, together with its toxic compound, microcystin, were monitored. Local cyanobacterial strains were also isolated for fundamental studies on toxin production, specifically the effects of light spectra on toxin biosynthesis.

The findings of this thesis regarding cyanobacterial blooms in Kranji Reservoir suggested that both nutrients (N & P) influence the abundance of cyanobacteria (*Microcystis*) populations, different from several field studies showing *Microcystis* abundance correlated only to a single nutrient. Increases in N or P concentration had positive impacts on the growth of total *Microcystis* and toxigenic *Microcystis* species, as reflected in the PCA analysis and the significant correlations between these variables as shown by the Spearman's rho coefficients. In comparison, the total microcystin concentration showed less response to changes in nutrient concentrations. This could be due to switching of cyanobacterial genotypes, possibly with different toxin potentials, as shown in Chapter 4. Although the significant relationship between *in-situ* microcystin and TN concentrations

reported here is in agreement with several laboratorial outcomes from other studies, continuous culture experiments using local toxin-producing strains are necessary to confirm the results and to provide additional information on the strategies for reservoir management and restoration.

PCR and qPCR methods optimized in this study have been successfully applied for the detection and numeration of local cyanobacterial species. For the detection of toxic cyanobacteria (*Microcystis*) species, results generated from different PCR assays were similar to each other, except for the *mcyC* gene reaction. The design of *mcyC* PCR is based on the consistent presence of *umal* located downstream of the 3' end of *mcyC*, showing that the whole *mcy* cluster is unlikely to be transferred horizontally. Therefore, further investigation on the low detection rate for *mcyC* in this study is important from a phylogenetic perspective. Instead of using tedious methods of strain isolation and cultivation with toxin analysis, it is possible to monitor cyanobacterial blooms more easily by coupling molecular methods and biochemical toxin kit (ELISA).

Results from PCR and toxigenic gene (*mcyA*) sequencing showed that *Microcystis* was the major microcystin producer in Kranji Reservoir. *Anabaena* spp. coexisted with the *Microcystis* spp. at all times, with Spearman's rho coefficient, $r_s = 0.498$ ($P < 0.001$). The average concentrations of *Microcystis* and *Anabaena* (equivalent to 16S rRNA) were 4.16×10^6 gene copies/mL (std 1.46×10^7 gene copies/mL) and 4.47×10^4 gene copies/mL (std 9.16×10^4 gene copies/mL) respectively while the percentage of toxigenic *Microcystis* spp. was 55.92 % (std 21.1 %), whereas no *Anabaena*-specific microcystin producing gene was detected. Using the ITS sequences, it was revealed that the major cyanobacterial genera in the reservoir was *Microcystis*, *Anabaena/Aphanizomenon*, *Synechococcus* and *Oscillatoria*, comparable to the morphological identification results by the local water agency, PUB. The molecular data on cyanobacterial species and their abundance provides additional information to the routine cyanobacteria monitoring carried out by PUB. In particular, the quantification data on toxigenic species is an important addition to the current database which relies on microscopic identification

and counting. In this study, the total microcystin concentration measured using an ELISA kit ranged from below the detection limit to 14.4 µg/L with a mean concentration of 2.66 ± 2.80 µg/L. These results are higher than those reported by PUB (from below detection limit to 5.31 µg/L) where LC-MSMS was used to quantify four variants of microcystin (LR, RR, LA and YR) from all reservoirs of Singapore (Wong et al., 2012). This discrepancy could be due to the presence of other microcystin variants that were not detected by the LC-MSMS measurement, thus highlighting the need of a more thorough survey of natural toxin variants in local waters.

As shown in the genus-specific *mcyE* PCR detection (*Microcystis mcyE* and *Anabaena mcyE*), as well as the nucleic acid sequences on partial *mcyA* and *mcyH* genes, *Microcystis* was the sole microcystin producer found in Kranji Reservoir. Although the fluctuation of *Microcystis* biomass in the reservoir was relatively stable throughout the study period, cloning sequences for cyanobacterial 16S-rRNA revealed that divergent genotypes prevailed all the time. From the 16S-rRNA phylogenetic tree constructed using local *Microcystis* gene sequences, a novel cluster was detected implying the presence of unique genotypes in Singapore waters. This emphasizes the need for an in-depth study and characterization of indigenous cyanobacterial strains.

Using a new primer set to amplify the partial *mcyH* gene which encodes for a putative ABC transporter in *mcy* gene cluster, followed by PCR product cloning and sequencing, the sequence variability of this gene was unfolded in this study. Current results indicate high sequence diversity for *mcyH* as compared to the partial *mcyA* proposed by Tillett et al. (2001), commonly used to study the diversity of the microcystin-producing population. Therefore, it is suggested that the partial *mcyH* gene sequences can be an alternative to discover genetic diversity in the toxic *Microcystis* population. The sequence data for microcystin synthesis genes and the cyanobacterial 16S-rRNA gene produced in this study contribute to the understanding of the divergence and distribution of toxin producing strains globally.

Isolation of local cyanobacterial species were carried out to obtain monocultures. However, different techniques have to be applied for different genera as there is no single technique suitable for isolating all types of cyanobacteria. Several cyanobacterial strains were obtained in this study and one of the isolates was subjected to the light microcosm study. Axenic *Microcystis* cultures were unable to be achieved using only direct isolation or agar isolation methods because the heterotrophic bacteria present were easily shielded in mucilage layer encompassing the entire colony. Two *Microcystis* species were isolated from Kranji Reservoir and they are toxic genotypes, corresponding to the qPCR results which showed a high percentage of toxigenic *Microcystis* during the sampling period.

The light experiment conducted on a local *Microcystis* isolate grown from exponential to stationary phases has led to a conclusion which supports the hypothesis that microcystin is produced constitutively and has an intracellular function related to the photosynthesis process. Under the experimental setup in the present study, red light stimulated microcystin content during the exponential phase but reduced the chl-a and PC contents in cells as a result of photoacclimation. Strong positive correlations between microcystin and chl-a concentrations were found not only in the light spectra experiment ($r_s = 0.735$, $P < 0.01$) but also in the field study in Kranji Reservoir ($r_s = 0.579$, $P < 0.01$).

This thesis provides important data for freshwater cyanobacterial blooms in tropical waters. The DNA and antibody based techniques (PCR, qPCR, DNA sequencing and ELISA) used in this study could be applied as routine monitoring tools to identify the presence of toxigenic species and cyanotoxins in other local reservoirs. These high throughput analyses are useful for the prediction, evaluation and control of blooms in local water bodies. The field study which focused on a eutrophic reservoir in Singapore, pointed out the vulnerability of tropical reservoirs to prolonged and intense toxic blooms when the water quality declines. This will contribute to fundamental knowledge on the biosynthesis of cyanotoxins and eventually help in reducing the spread of harmful blooms from anthropogenic activities and their toxicity.

6.2 Recommendations

For more comprehensive monitoring, toxic cyanobacterial species from all genera should be included as targets for DNA-based screenings. Many gene clusters involved in the biosynthesis of hepatotoxins and neurotoxins have been recently identified and sequenced. To date, primers and probes are available for the detection and quantification of toxic cyanobacteria from various genera capable of producing microcystin (*Microcystis*, *Anabaena*, *Planktothrix*), nodularin (*Nodularia spumigena*), cylindrospermopsin (*Cylindrospermopsis*, *Aphanizomenon*), anatoxin-a (*Oscillatoria*) and even saxitoxin (*Anabaena circinalis*) (Al-Tebrineh et al., 2010; Cadel-Six et al., 2009; Christiansen et al., 2003; Koskenniemi et al., 2007; Mihali et al., 2008; Moffitt and Neilan, 2004; Rantala et al., 2006; Rouhiainen et al., 2004; Vaitomaa et al., 2003). This has made possible the detection of all major cyanotoxin producers in aquatic systems. However, these primers and probes are not necessarily ready for immediate use in local environments and assay optimizations are often needed in advance. This is because the designs of these oligonucleotides generally refer only to a subset of target strains, new strains and unique sequences can be excluded from amplification (Sivonen, 2008). Moreover, the presence of PCR inhibitors such as phenolic compounds, humic acids, and heavy metals are highly dependent on the water matrices of samples (Wilson, 1997) and this differs from place to place. Nevertheless, even if the detection of all toxic species is possible, it is a time-consuming task because most of the PCR/qPCR involved have to be done separately for different targets. To overcome this problem, multiplex reactions can help to reduce analytical procedures and allow faster data generation. However, a DNA database containing substantial local cyanobacterial gene sequences is the prerequisite for the development of multiplex PCR/qPCR assays suitable for local usage.

Continuous isolation work is essential for future cyanobacterial studies. DNA-based methods help to predict toxin potential of cyanobacterial species, but their toxin production capabilities can be confirmed only when laboratory cultures

are available. Cyanobacteria culture isolated from local water bodies containing both toxic and non-toxic members from different genera is an invaluable resource (Sivonen, 2008). First, they can be used to investigate the dynamics of cyanobacteria and their responses to environmental factors leading to their widespread occurrence in nature. Second, they provide a reservoir of genetic information which is useful for phylogenetic studies and the development of sensitive/specific molecular tools as mentioned previously. Lastly, isolated cultures can be utilized in controlled experiments to identify the physiological functions and synthesis of cyanotoxins. This can be done by comparing the competitive advantages/disadvantages between toxic and non-toxic strains when encountering environmental stresses. Another approach is to inactivate the toxin synthesis mechanism of a known toxin producer by gene mutation using the knockout technique (Dittmann et al., 1997) and examining the difference before and after genetic alteration.

Many cyanobacterial species are capable of producing multiple microcystin variants simultaneously (Tonk et al., 2008). However, currently available primers/probes were designed for probing general toxic gene sequences, and are not selective for the nucleotide divergence which causes different microcystin variants. While the proportion of microcystin variants produced by a cyanobacterial strain is affected by environmental factors (i.e. light, temperature, nitrogen and amino acid) (Rapala et al., 1997; Tonk et al., 2008), the type of variants synthesized is related to (1) less specific substrate recognition of the adenylation domains and (2) genetic variations in the microcystins synthesis genes (Mikalsen et al., 2003). Studies have shown that polymorphism of adenylation domains in the *mcyA2* and *mcyB1* gene modules of *Microcystis* strains resulted in different microcystin variant profiles (Allender et al., 2009; Mikalsen et al., 2003). Similarly, a study conducted on *Planktothrix* strains demonstrated that varied *mcyA* and *mcyB* genotypes correlated significantly with certain amino acid residues at positions 7 and 2 (Kurmayer et al., 2005). These studies suggest that there could be a molecular basis (genetic tool) for predicting the actual production of microcystin variants. By increasing the gene sequence database responsible for coding specific toxin variants, a library can be

established, from which new primers and probes can be developed to better evaluate and assess the toxicity of a bloom.

Controlling harmful algae blooms through nutrient reduction (i.e. N and P) is one of the most common approaches.. Although there are studies showing that reduction of single nutrient loading is effective for some water bodies (Edmondson and Lehman, 1981; Likens, 1972), however, control of both N and P inputs is usually required for long-term bloom management (Paerl, 2008). In fact, in this study, the positive correlations between nutrients and *Microcystis* abundance demonstrated in Chapter 3 suggests that N and P management is an option for mitigating blooms in the Kranji Reservoir. Controlling blooms via hydrological manipulations such as vertical mixing and flushing have also proven effective for some water bodies (Huisman et al., 2004; Mitrovic et al., 2011; Verspagen et al., 2006; Visser et al., 1996). These hydraulic disruptions either suppress thermal stratification which reduces the surface migration of buoyant cyanobacterial cells or dilute the cells and nutrients availability in affected waters, thus mitigating the growth of blooms (Mitrovic et al., 2011; Paerl, 2008). However, this practice requires water management planning and design to avoid contamination of other water resources by the diverted flow (Qin et al., 2010). Using algicides such as copper sulfate and low concentration hydrogen peroxide to remove blooms is another possible strategy to resolve eutrophication problems (Barrington et al., 2011; Griffiths and Saker, 2003; Matthijs et al., 2012); however, the environmental impacts of chemical addition should also be considered.

An alternative approach to controlling or eliminating harmful cyanobacteria is the use of biological control agents such as cyanophages and lytic bacteria (Sigee et al., 1999). Cyanophages have been thought to manipulate the succession of a bloom and account for the diversity and composition of cyanobacterial population (Yoshida et al., 2008). Viral control could be attractive because cyanophages can replicate rapidly and they have selective and specific host range (Yoshida et al., 2008). However, having a specific host range restricts cyanophages to limited infection that usually effective only to a small portion of target species. In addition,

it is also observed that cyanobacteria can mutate to become phage-resistant, while the phages can also evolve to attack mutant strains (Gumbo et al., 2008; Sigee et al., 1999). The complexity of the phage/cyanobacteria interaction is a great challenge for the use of phages as biocontrol agents. However, cyanophage dynamics and their occurrence together with their hosts in waters deserve further attention in the study of harmful blooms. Lytic bacteria possess characteristics which make them better control agents than phages. For example, they can switch to other food sources during non-bloom periods and problems such as mutations in cyanobacterial cells and non-susceptible strains have no major impacts on them. Lytic bacteria can penetrate into cyanobacteria, multiply and kill the cells, or they produce enzymes and chemicals that lead to cell death (Rashidan and Bird, 2001). In order to protect the original water environment, it is suggested that biocontrol agents should be indigenous to the water body and not undergo any genetic mutation or enhancement (Gumbo et al., 2008). In recent years, a wide range of lytic bacteria such as *Cytophaga*, *Bacillus*, *Saprospira* and *Sphingobium* (Gumbo et al., 2008; Rashidan and Bird, 2001; Takao et al., 2010; Tolba, 2011) have been isolated to attack *Microcystis*, *Anabaena*, *Oscillatoria* and *Planktothrix* species. Thus, isolating lytic bacteria to control local bloom events could be another promising method for the future.

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