ANALYSIS OF TOXIC CYANOBACTERIAL ABUNDANCE IN SELECTED AQUACULTURE SYSTEMS AND ITS EFFECT ON *Oreochromis* spp.

ANN ANAK SINDEN

UNIVERSITI PENDIDIKAN SULTAN IDRIS

2016

ANALYSIS OF TOXIC CYANOBACTERIAL ABUNDANCE IN SELECTED AQUACULTURE SYSTEMS AND ITS EFFECT ON *Oreochromis* spp.

ANN ANAK SINDEN

05-4506832 pustaka.upsi.edu.my Perpustakaan Tuanku Bainun Kampus Sultan Abdul Jalil Shah PustakaTBainun ptbupsi

THIS THESIS IS SUBMITTED IN FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF SCIENCE (RESEARCH MODE)

FACULTY OF SCIENCE AND MATHEMATICS
UNIVERSITI PENDIDIKAN SULTAN IDRIS

05-4506832 pustaka.upsi.edu.my Perpustakaan Tuanku Bainun Kampus Sultan Abdul Jalil Shah PustakaTBainun ptbupsi

2016

ABSTRACT

This study aims to analyse the abundance of toxic cyanobacteria in selected aquaculture systems and its effect on *Oreochromis* spp. In this study, a total of forty freshwater fish aquaculture ponds were sampled from ten different locations in Perak, Malaysia. To analyse the effects of naturally-occurring microcystins concentration in Perak aquaculture environments on fish, *Oreochromis* spp. fingerlings were cultured in water treated with cyanobacterial extracts in the laboratory. Study results revealed that the most commonly found cyanobacterial taxa in Perak aquaculture systems was *Microcystis* spp. During the sampling periods, the majority of the sampled ponds water were under cyanobacterial bloom and contained unsafe concentration of microcystins exceeding 20 µg/L. A combination of temperature and pH was correlated to the proliferation of cyanobacteria and its toxicity in the selected aquaculture ponds. Microcystins accumulated in fish tissues were dependent on the concentration of microcystins in the surrounding water. Despite high microcystins bioaccumulation, this study discovered that microcystin concentrations did not give impacts to the survival and growth of *Oreochromis* spp. These findings illustrated the potential health risk of toxic cyanobacteria through fish consumption in Malaysia which can be two to three orders of magnitude higher than the tolerable daily intake guideline (0.04 µg MC-LR / kg body weight per day) recommended by World Health Organization. In conclusion, the abundance of toxic cyanobacteria in Malaysia aquaculture systems may cause accumulation by fish at a harmful level. As an implication, this study can serve as a guide on the occurrence of toxic cyanobacteria in our freshwater systems particularly in aquaculture ponds, as well as its potential bioaccumulation in aquatic organisms which may lead to significant health threat to human through food web.

ANALISIS KELIMPAHAN ALGA BIRU-HIJAU TOKSIK DALAM SISTEM AKUAKULTUR TERPILIH DAN KESANNYA TERHADAP *Oreochromis* spp.

ABSTRAK

Kajian ini bertujuan menganalisis kelimpahan alga biru-hijau toksik dalam sistem akuakultur terpilih dan kesannya terhadap *Oreochromis* spp. Dalam kajian ini, sebanyak empat puluh buah kolam akuakultur ikan air tawar telah disampel daripada sepuluh lokasi yang berbeza di Perak, Malaysia. Bagi menganalisis kesan kepekatan semulajadi mikrosistin dalam persekitaran akuakultur di Perak terhadap ikan, benih *Oreochromis* spp. dikulturkan dalam air yang dirawat dengan ekstrak alga biru-hijau di makmal. Dapatan kajian menunjukkan bahawa taksa alga biru-hijau yang paling kerap ditemui dalam sistem akuakultur di Perak adalah *Microcystis* spp. Semasa tempoh persampelan, majoriti daripada air kolam tersebut berada di bawah paras *bloom* serta mengandungi kepekatan mikrosistin yang tidak selamat melebihi 20 μg/L. Gabungan suhu dan pH didapati bertindak sebagai pemboleh ubah alam sekitar utama yang mencetuskan percambahan alga biru-hijau, serta ketoksikan di dalam kolam akuakultur terpilih. Mikrosistin yang terkumpul dalam tisu ikan adalah bergantung kepada kepekatan mikrosistin yang berada dalam air di sekitarnya. Meskipun pengumpulan biologi mikrosistin yang tinggi, kajian ini mendapati bahawa kepekatan mikrosistin tidak memberi kesan kepada kelangsungan hidup serta pertumbuhan *Oreochromis* spp. Penemuan kajian ini menggambarkan potensi risiko kesihatan yang berkaitan dengan alga biru-hijau toksik melalui pengambilan ikan akuakultur di Malaysia yang berkemungkinan dua hingga tiga kuasa magnitud lebih tinggi daripada garis panduan pengambilan harian boleh diterima (0.04 μg MC-LR / kg berat badan per hari) yang disyorkan oleh Pertubuhan Kesihatan Sedunia. Kesimpulannya, kelimpahan alga biru-hijau dalam sistem akuakultur di Malaysia berkemungkinan menyebabkan pengumpulan oleh ikan pada tahap yang memudaratkan. Implikasinya, kajian ini boleh dijadikan panduan berkaitan kejadian alga biru-hijau toksik dalam sistem air tawar terutamanya dalam kolam akuakultur, serta potensi pengumpulan biologinya dalam organisma akuatik yang boleh membawa kepada ancaman kesihatan yang serius kepada manusia melalui rantai makanan.

CONTENTS

CHAPTER 3 METHODOLOGY

05-4506832 pustaka.upsi.edu.my Perpustakaan Tuanku Bainun Kampus Sultan Abdul Jalil Shah PustakaTBainun ptbupsi

05-4506832 pustaka.upsi.edu.my Perpustakaan Tuanku Bainun Kampus Sultan Abdul Jalil Shah PustakaTBainun ptbupsi

LIST OF TABLES

05-4506832 pustaka.upsi.edu.my Perpustakaan Tuanku Bainun Kampus Sultan Abdul Jalil Shah PustakaTBainun ptbupsi

05-4506832 pustaka.upsi.edu.my Perpustakaan Tuanku Bainun Kampus Sultan Abdul Jalil Shah PustakaTBainun ptbupsi

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
APHA	American Public Health Association
CB	Cyanobacterial biomass
chl-*a*	Chlorophyll-*a*
DO	Dissolved oxygen
DoF	Department of Fisheries
EDI	Estimated daily intake
EPA	United States Environmental Protection Agency
eq.	Equivalent
FAO	Food and Agriculture Organisation
F_T	Instantaneous chlorophyll fluorescence
GPS	Global Positioning System
HAB	Harmful Algal Bloom
HDPE	High-density polyethylene
HPLC	High performance liquid chromatography
IFRPC	Indigenous Fisheries Research and Production Centre
Ks	Half-saturation constants
MC	Microcystin
MC-LR	Microcystin-LR
MIB	2-Methylisoborneal
NAHRIM	National Hydraulic Research Institute of Malaysia

PDA	Photodiode array
RM	Ringgit Malaysia
rpm	Rotation per minute
r.u	Relative unit
SPE	Solid phase extraction
SRP	Soluble reactive phosphate
TDI	Tolerable Daily Intake
TFA	Trifluoroacetic acid
TN	Total nitrogen
TP	Total phosphorus
UNEP	United Nation Environmental Protection
USD	U.S. Dollar
UV	Ultra-violet
v/v	Volume per volume
WHO	World Health Organisation

05-4506832 pustaka.upsi.edu.my Perpustakaan Tuanku Bainun Kampus Sultan Abdul Jalil Shah PustakaTBainun ptbupsi
05-4506832 pustaka.upsi.edu.my Perpustakaan Tuanku Bainun Kampus Sultan Abdul Jalil Shah PustakaTBainun ptbupsi

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Cyanobacteria, also known as blue-green algae are prokaryotic organisms possessing photosynthetic pigments and can proliferate in water bodies such as ponds, lakes, reservoirs and slow moving streams (Butler, Carlisle, Linville, & Washburn, 2009; Chorus & Bartram, 1999). Just like other phytoplanktons, cyanobacteria is part of the microbial community and acts as the primary producer for aquatic organisms (Palmeri, Barausse, & Erik, 2013). Other than being important in the aquatic food chain, cyanobacteria also assimilate ammonia as its nitrogen source for growth, hence minimising the accumulation of this toxic compound in water systems (Paerl & Tucker, 1995).

In aquaculture ponds, large amount of nitrogen is introduced into the water systems as most of the manufactured fish pellets contain about 32 to 45% of protein (Pandey, 2013). Nearby human activities such as rapid urbanisation, industrialisation and intensifying agriculture also contributing to nutrient inputs into the aquaculture water bodies and result in eutrophication (Yang, Wu, Hao, & He, 2008).

Eutrophication is an excessive nutrient enrichment in water bodies (Kaufman & Franz, 2000). Phosphorus and nitrogen that are available in human sewage and livestock excrement, as well as synthetic fertilisers are believed to be the main contributors to eutrophication (Schindler, 2012). Eutrophication has been considered as a rapidly growing environmental crisis in freshwater and marine systems worldwide (Selman & Greenhalgh, 2009). According to United Nation Environmental Protection (UNEP), about 30 to 40% of lakes and water reservoirs all over the world have been affected by eutrophication (Yang et al., 2008). Eutrophication is also a critical issue in Malaysia. The preliminary desktop study on the status of lake eutrophication in Malaysia indicated that more than 60% of the lakes reviewed out of 90 lakes in Malaysia were eutrophicated (National Hydraulic Research Institute of Malaysia [NAHRIM], 2005; Zati & Salmah, 2008).

Eutrophic water body causes excessive growth of phytoplanktons which usually leading to the dominance of cyanobacteria (Havens, 2008). The dominance of cyanobacteria over other phytoplanktons in water bodies are mainly due to its buoyancy characteristic that enable this species to compete for nutrients (Bellinger & Sigee, 2010). The overgrowth of cyanobacteria disturbs the natural balance of the

aquatic ecosystem and ultimately result in cyanobacterial bloom (Selman & Greenhalgh, 2009).

Cyanobacterial bloom is a common issue in aquaculture industry (Rodgers, 2008). This phenomenon causes depletion of oxygen in water column of aquaculture ponds leading to mortality of aquatic species (Snyder, Goodwin, & Freeman, 2002), a condition known as hypoxia ("Health and Ecological Effects," 2015). Cyanobacterial bloom can cause severe economic losses (Landsberg, 2002; Rodgers, 2008). Preliminary study conducted in United States revealed that the country lost more than USD 40 millions per year and at least USD 1 billion per decade due to harmful algal blooms (HABs) in aquaculture sector (Landsberg, 2002; Rodgers, 2008). In Malaysia, lossess of not less than RM 20 millions were reported in relation to massive fish kills at finfish farms in Penang due to prolonged HAB event from 2005 to 2006 (Sin Chew Daily, 2005 as cited in Lim, Gires, & Leaw, 2012).

Some species of cyanobacteria such as *Oscillatoria* spp., *Anabaena* spp., and *Microcystis* spp are capable of synthesising two highly odorous compounds called geosmin and 2-methylisoborneal (MIB) that can cause earthy-musty taste on fish (Paerl & Tucker, 1995; Tucker, 2000; Schrader & Dennis, 2005; Zhong et al., 2011). Despite being non-toxic to human, these compounds are nuisance to the public ("Health and Ecological Effects," 2015) as they can alter the natural taste of aquatic products. The production of off-flavour compounds are more common in freshwater aquaculture systems as compared to marine and brackish water due to acceptable salinity range as well as nutrients abundance (Paerl & Tucker, 1995). This problem usually adds about 10 to 20% to the production cost of aquaculture practise (Keenum

& Waldrop., 1988; Paerl & Tucker, 1995) which can sum up to USD 60 millions annually (Tucker, 2000).

In addition, several species of cyanobacteria are also capable to produce secondary metabolites known as cyanotoxin. Cyanotoxin can be classified into three categories based on the mode of action: hepatotoxin, neurotoxin and dermatotoxin (Rodgers, 2008). Among all, microcystin is the most commonly found cyanobacterial toxin in freshwater system (Poste, Hecky, & Guildford, 2011; Schmidt et al., 2013).

Microcystin falls under the group of hepatotoxin and its contamination in aquaculture industry has long been reported in many past literatures (Barros, de Souza, Tavares, & Amaral, 2010; Peng et al., 2010). This toxin enters fish body via the gills, diet and food chain (Poste et al., 2011; Schmidt et al., 2013), destroys the liver tissues and leads to fish death (Hudnell, 2008; Schmidt et al., 2013). Besides, microcystin can also accumulate in fish tissues and pose health risk to human through fish consumption (Peng et al., 2010; Poste et al., 2011).

Malaysia is located in tropical region with an average temperature of 26 to 28 °C throughout the year (Malaysian Meteorological Department, 2015). Hot climate in this country is expected to induce the growth of cyanobacteria and promotes the persistence of toxic blooms (Ferrão-Filho & Kozlowsky-Suzuki, 2011). Hence, there is a possibility for the toxic cyanobacterial biomass to be present in majority of water bodies in Malaysia (Sinang, 2012b) including the aquaculture systems.

1.2 Problem Statements

Malaysia produces large amount of fish through aquaculture practices (Department of Fisheries Malaysia [DoF Malaysia], 2013a) in order to cope with the demand of increasing human population (The World Bank, 2015). As mentioned earlier, excessive growth of cyanobacteria disturbs the water quality of aquaculture ponds and leads to fish death (Zimba, Khoo, Gaunt, Brittain, & Carmichael, 2001; Jewel, Affan, & Khan, 2003). The survived fish, however, may have accumulated cyanotoxin, particularly microcystin which can be dangerous enough to pose health threat to human (Peng et al., 2010; Poste et al., 2011 ; Schmidt et al., 2013).

Due to the potential health risk of microcystin contamination, World Health Organisation (WHO) has established the provisional guidelines for Microcystin-LR which are 1.0 µg/L for drinking water and 0.04 µg/kg body weight per day for tolerable daily intake (TDI) (Chorus & Bartram, 1999). However, the information on the risk associated with the consumption of aquatic products from eutrophicated water system is still lacking (Peng et al., 2010). Most of the previous studies conducted on toxin accumulation in aquatic species focused on toxicological concern with the key objectives of determining the target organ of cyanotoxin (Peng et al., 2010; Zhang, Xie, Liu, & Qiu, 2009).

In Malaysia, a number of research has been conducted on cyanobacteria, however, the assessment of this noxious species in our freshwater aquaculture system is still limited. The evaluation of cyanobacteria compositions in Sarawak aquaculture systems reported the presence of *Anabaena* spp., *Chamaesiphon* spp., *Lynbya* spp.,

Microcystis spp., *Oscillatoria* spp., and *Spirulina* spp. (Mohd. Nasarudin & Ruhana, 2011b; Ramlah, 2005 as cited in Mohd. Nasarudin & Ruhana, 2011b). Among all of the detected genera, *Microcystis* spp., the primary producer of hepatotoxic microcystin (Mioni et al., 2011), was found at the most abundant in earth aquaculture ponds (Mohd. Nasarudin & Ruhana, 2011b). Since cyanobacteria community varies on spatial scales (Sinang, 2012a), more research is needed to assess the types of cyanobacteria present in water column of aquaculture especially the occurrence of potentially toxic strains.

Additionaly, the toxicity of cyanobacteria in Malaysia freshwater system is also rarely studied. Sinang et al. (2015) reported the presence of microcystins in all of the water samples collected from freshwater lakes in Selangor. Connecting that fact, it is possible that microcystin is present in Malaysia aquaculture system. Jasmina, Samsur and Ruhana (2010) assessed the toxicity of cyanobacteria, however, the study was only carried out on the laboratory-cultured sample. Due to limited study on cyanobacterial toxicity particularly the assessment of environmental sample, more research focusing on cyanobacterial toxin in actual aquaculture system is urgently needed.

Since there is lack of scientific studies on cyanobacterial bloom and cyanobacterial toxicity carried out in Malaysia (Lim, Leaw, & Usup, 2003; Sinang et al., 2015), it is not exaggerating to say that our present knowledge on the potential health risk of cyanobacterial toxin especially on aquatic products is still inadequate. To address the issue, this study was undertaken to establish a profile for cyanobacterial diversity, abundance, and toxicity in selected fish aquaculture systems

in Perak, Malaysia. Apart from that, this study also aimed to investigate the bioaccumulation of microcystin on Red Tilapia (*Oreochromis sp.*) tissues and its impact on the survival and growth of fish.

1.3 Research Questions

This study was carried out based on research questions as below:

1. Does potentially toxic cyanobacterial genera present in the selected Perak aquaculture systems?
2. How abundant is the occurrence of toxic cyanobacteria in water column of aquaculture ponds in terms of biomass and microcystin produced?
3. What triggers the occurrence of toxic cyanobacterial bloom in aquaculture ponds?
4. Does microcystin bioaccumulation in fish tissues affects the survival and growth of fish?

1.4 Research Objectives

The research aimed to investigate the presence and abundance of toxic cyanobacteria in aquaculture systems, as well as its toxic accumulation in fish tissues. In more specific, this study aimed to:

1. Determine the presence of potentially toxic cyanobacterial genera in the selected Perak aquaculture systems.

2. Quantify the cyanobacterial biomass and microcystin concentration available in water body of aquaculture ponds.
3. Identify the main trigger of toxic cyanobacterial bloom in aquaculture ponds in Perak.
4. Analyse the bioaccumulation of microcystin in *Oreochromis* spp. tissues and its effect on fish survival and growth.

1.5 Significance of Study

Since there is a lack of cyanobacteria research in Malaysia, this study is important to enhance our present knowledge on the occurrence of toxic cyanobacteria, particularly in Malaysia aquaculture systems. This research is also essential for public health risk protection. Through microcystin bioaccumulation experiment, this study revealed how dangerous is the naturally-occurring microcystin concentration available in our aquaculture water, especially to the Red tilapia (*Oreochromis* spp.) consumers. Understanding of the relationships between cyanobacterial biomass, microcystin production, and environmental parameters, as well as the information on the potential microcystin accumulation in aquatic products, will assist fish farmers in aquaculture monitoring. The application of these knowledges certainly will give positive impacts to both fish yield and the farmer's income.

1.6 Scopes and Limitations of Study

This study was carried out within the scopes and limitations as below:

1. Water samples were collected from commercialised freshwater fish aquaculture ponds in Perak, Malaysia.
2. Potentially toxic cyanobacteria identified were those that capable of producing microcystin according to Sivonen & Jones (1999) namely *Microcystis* spp., *Anabaena* spp., *Anabaenopsis* spp., *Oscillatoria* spp., *Planktothrix* spp., and *Nostoc* spp.
3. Total chlorophyll-*a* was used as a proxy to estimate the cyanobacterial biomass.
4. Selected environmental parameters were temperature, pH, dissolved oxygen, nitrate, phosphate, nitrite, magnesium, calcium, and ammonium.
5. Microcystin is the only cyanotoxin quantified in this study and the concentration was expressed in microcystin-LR (MC-LR) equivalents.
6. Microcystin concentrations quantified from the aquaculture water samples were only extracted from the intracellular cells.
7. The cultured fishes were exposed to the microcystin through immersion technique and the accumulation was studied only on *Oreochromis* spp. tissues. The source of microcystin was from crude cyanobacterial extracts dominant with *Microcystis* spp.

1.7 Research Framework

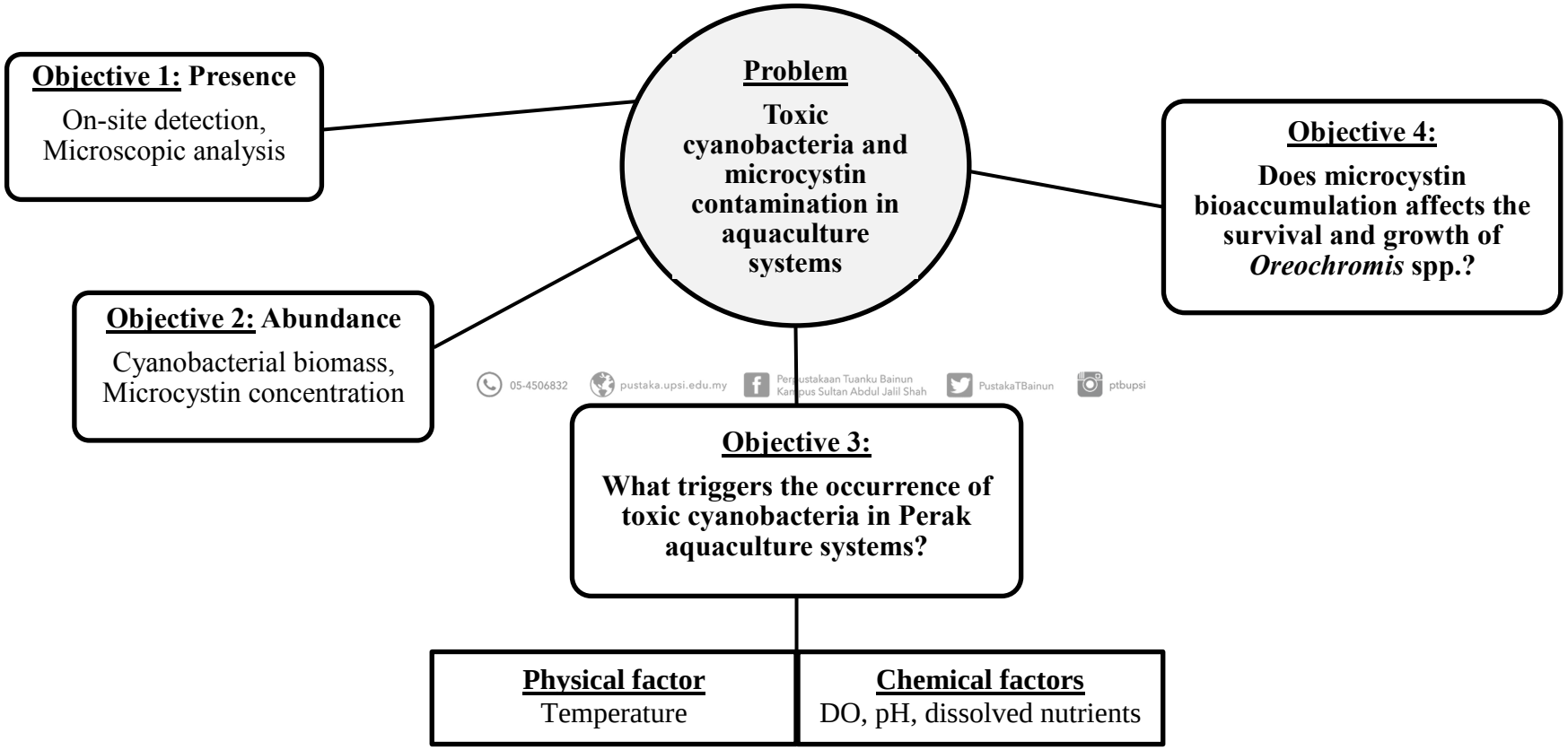

Figure 1.1. Research framework

1.8 Research Design and Hypothesis

This research was divided into two parts: (i) assessment of environmental samples, and (ii) laboratory experiment. Brief descriptions of the research design (see Table 1.1) and hypothesis are as described below.

(i) Part I: Assessment of environmental samples

A total of 10 commercialised aquaculture farms focusing on the production of freshwater fish were sampled from December 2013 to March 2014 to investigate the presence and abundance of toxic cyanobacteria in the aquaculture systems, as well as to identify the main environmental factor that triggered its occurrence. This part of research was conducted based on the following hypothesis:

a. Toxic cyanobacteria present in abundance in Perak aquaculture systems.
b. Microcystin concentration in aquaculture ponds is positively correlated with the biomass of cyanobacteria.
c. The occurrence of toxic cyanobacteria in aquaculture systems is significantly correlated with the physical and chemical environmental factors.

(ii) Part II: Laboratory experiment

Oreochromis spp. fingerlings were cultured in water immersed with different concentrations of microcystin for a duration of seven days to investigate the

bioaccumulation of microcystin in fish tissues, as well as its effect to the survival and growth of the fish. This part of research was conducted based on the following hypothesis:

a. Microcystin accumulated in fish tissues is positively related to the concentration of microcystin in the surrounding water.
b. Survival and growth of fish are inversely related to the concentration of microcystin in the surrounding water.
c. Survival and growth of fish are inversely correlated to the concentration of microcystin accumulated in fish tissues.

Table 1.1

Simplified research design

Part	Objective	Parameter	Methodology	Data Presentation / Analysis
I	1	- Instantaneous chlorophyll fluorescence (F_T) of cyanobacteria	- On-site measurement with AquaPen-C at 620 nm	- Bar graph with error bars
		- Cyanobacteria identification (microcystin-producing)	- Microscopic examination of preserved water samples with inverted microscope	- Photos
	2,3	- Total chlorophyll-*a*	- Analysed according to Standard Methods (APHA, 1998) and quantified with revised Lorenzen (1967) equation	- Bar graph with error bars, Scatter plot with regression line, Bivariate correlation with Pearson's test, One-way ANOVA with Tukey's HSD test, Multiple linear regression with forward selection
		- Microcystin concentration (extracted from cyanobacteria intracellular cells)	- Analysed according to Lawton et al. (1994). Extracted microcystin samples were purified with SPE cartridge and quantified with HPLC-PDA detector at 238 nm	
		- Temperature, pH, and DO	- On-site measurement with YSI 550A probe	
		- NO_3^-, PO_4^{3-}, NO_2^-, Mg^{2+}, Ca^+, and NH_4^+	- Analysed with IC	
II	4	- Microcystin bioaccumulation (extracted from the entire body of fish)	- Red tilapia were cultured in water immersed with crude cyanobacterial extracts (17.96, 40.66, 78.32 µg/L MC-LR equivalent) for 7 days.	- Table, One-way ANOVA with Tukey's HSD test, Kaplan-Meier survival curve with Log-Rank test
			- Analysed according to Lawton et al. (1994). Extracted microcystin samples were purified with SPE cartridge and quantified with HPLC-PDA detector at 238 nm	
		- Fish survival	- Visual observation	
		- Fish growth	- Final weight – initial weight	

CHAPTER 2

LITERATURE REVIEW

2.1 Cyanobacteria

Cyanobacteria are prokaryotic bacteria that have both the characteristics of algae and bacteria. It has photosynthetic pigments, hence is capable to perform photosynthesis. Besides containing chlorophylls and carotenoids, this prokaryotic also has water-soluble pigment called phycocyanin which gives the organism blue-green colour in appearance. Cyanobacteria can live in diverse environments in terrestrial, fresh, brackish or marine water and capable to survive in extreme environments such as hot springs, mountain streams, Arctic and Antarctic lakes, snow and ice (Chorus & Bartram, 1999).

Cyanobacteria, which are also known as blue-greens, blue-green algae, myxophyceans, cyanophyceans, cyanophytes and cyanoprokaryotes (Chorus &

Bartram, 1999), is one of the earliest organism living on earth and is called the pioneer of the early earth (Brock, 1973). This is confirmed by the discovery of cyanobacteria fossils in sedimentary rocks formed about 3,500 million years ago (Wilmotte, 1994). At that time, this prokaryote could be the chief primary producer of organic matter, and the first organism to produce oxygen into the atmosphere (Chorus & Bartram, 1999). Chloroplast in plant and eukaryotic algae are also reported to be evolved from an endosymbiotic relation with cyanobacteria (Deusch et al., 2008).

Unlike eukaryotic microalgae, cyanobacteria are simple prokaryotic cell; it does not have nuclei and membrane-bound organelles, while the cell wall is made up of peptidoglycan. Hence, it is classified within the gram-negative eubacteria (Vincent, 2009). Despite being able to perform photosynthesis, this organism actually does not have chloroplast. Cyanobacteria belongs to the domain of prokaryota and the kingdom of monera (Komarek & Hauer, 2013).

Prokaryotic cyanobacteria has been the research interest of global scientists for over a century (Palinska & Surosz, 2014). Despite so, the total number of cyanobacterial species remained uncertain until today (Nabout, da Silva Rocha, Carneiro, & Sant'Anna, 2013) due to confusion existed between Botanical and Bacteriological Codes (Palinska & Surosz, 2014). Guiry (2012) estimated that there are about 8,000 species of cyanobacteria available on earth, while the statistical analysis with discovery curve revealed that the total species could be 6,280, whereby 3582 species are yet to be discovered (Nabout et al., 2013).

Cyanobacteria in general have two types of morphology known as unicellular and multicellular filamentous. Based on the general morphology, mode of reproduction, presence / absence of specialised cells and the nature of branching, cyanobacteria can be classified into five orders: (i) Chroococcales, (ii) Pleurocapsales, (iii) Oscillatoriales, (iv) Nostocales, and (v) Stigonematales (Castenholz, 2012; Vincent, 2009). General characteristics of each taxonomic order as well as the examples of cyanobacteria commonly present in freshwater systems are as described in Table 2.1.

Table 2.1

General characteristics and examples of cyanobacteria in freshwater systems

Type	Taxonomic order	Characteristics	Cyanobacterial genera
Unicellular	(i) Chroococcales	- Coccoid cells, aggregates to form colony - Reproduce by binary fission, some followed with budding - Some form exocytes	*Aphanocapsa* spp. *Chamaesiphon* spp. *Chroococcus* spp. *Gomphosphaeria* spp. *Merismopedia* spp. *Microcystis* spp. *Synechococcus* spp. *Synechocystis* spp.
	(ii) Pleurocapsales	- Coccoid cells, aggregates or pseudo-filaments - Reproduce by multiple fission and the formation of baeocytes	*Pleurocapsa* spp.
Multicellular Filamentous	(iii) Oscillatoriales	- Uniseriate filaments that divide only in one plane - Do not contain heterocysts and akinetes	*Oscillatoria* spp. *Phormidium* spp. *Planktothrix* spp. *Spirulina* spp.
	(iv) Nostocales	- Uniseriate filaments that divide only in one plane - Contain heterocysts and akinetes - Some possess false branching	*Anabaena* spp. *Anabaenopsis* spp. *Aphanizomenon* spp. *Cylindrospermopsis* spp. *Nostoc* spp.
	(v) Stigonematales	- Multiseriate filaments that divide in more than one plane - Contain heterocysts and akinetes - Possess true branching	*Fischerella* spp. *Stauromatonema* spp. *Stigonema* spp.

Note. The characteristics of cyanobacteria are adapted from Vincent (2009) and Chorus & Bartram (1999), whereas the examples of freshwater cyanobacteria are adapted from Bellinger and Sigee (2010).

Chroococcales and Pleurocapsales have unicellular structures but differed in the mode of reproduction. The cells are divided through binary fission in Chroococcales and may remain solitary, or aggregated together to form colonies afterward. Budding process occurred after binary fission in some cyanobacterial genera for instance *Chamaesiphon* sp. This genus produces exospores during reproduction that will budded off from the upper end of cells. Pleurocapsales on the other hand reproduce by multiple fissions in the parent cells. The cells normally aggregated together and sometimes give the parent cells filament-like structure (pseudofilaments). Besides, Pleurocapsales are also capable to form small cells called baeocytes or also known as endospores in their cells. The baeocytes will then be released from the parent cells and will go through another stage of cell enlargement, multiple fissions and the formation of baeocytes. Both Chroococcales and Pleurocapsales do not possess specialised cells known as heterocysts and akinetes. Heterocysts are cells that capable to fix nitrogen, while akinetes are thick walled dormant cells that responsible in keeping reserve materials to enable cyanobacteria to survive in unfavourable conditions (Bellinger & Sigee, 2010; Castenholz, 2012; Chorus & Bartram, 1999; Komarek & Hauer, 2013; Vincent, 2009).

Filamentous cyanobacteria reproduced through repeated cell divisions in a single plane to the main axis of the filament known as trichome fragmentation. Alike the mode of reproduction, filamentous cyanobacteria consists of chain of cells, called trichomes (Chorus & Bartram, 1999). Oscillatoriales, Nostocales and Stigonematales are cyanobacterial orders with filamentous structures. Similar to Chroococcales and Pleurocapsales, Oscillatoriales do not have heterocyst and akinete cells. Nostocales contain heterocysts and akinetes, but without true branching (false branching).

Stigonematales on the other hand possess heterocysts and akinetes, as well as true branching (Bellinger & Sigee, 2010; Castenholz, 2012; Chorus & Bartram, 1999; Komarek & Hauer, 2013). The schematic diagrams of cyanobacteria for each taxonomic order are as shown in Figure 2.1 to Figure 2.5.

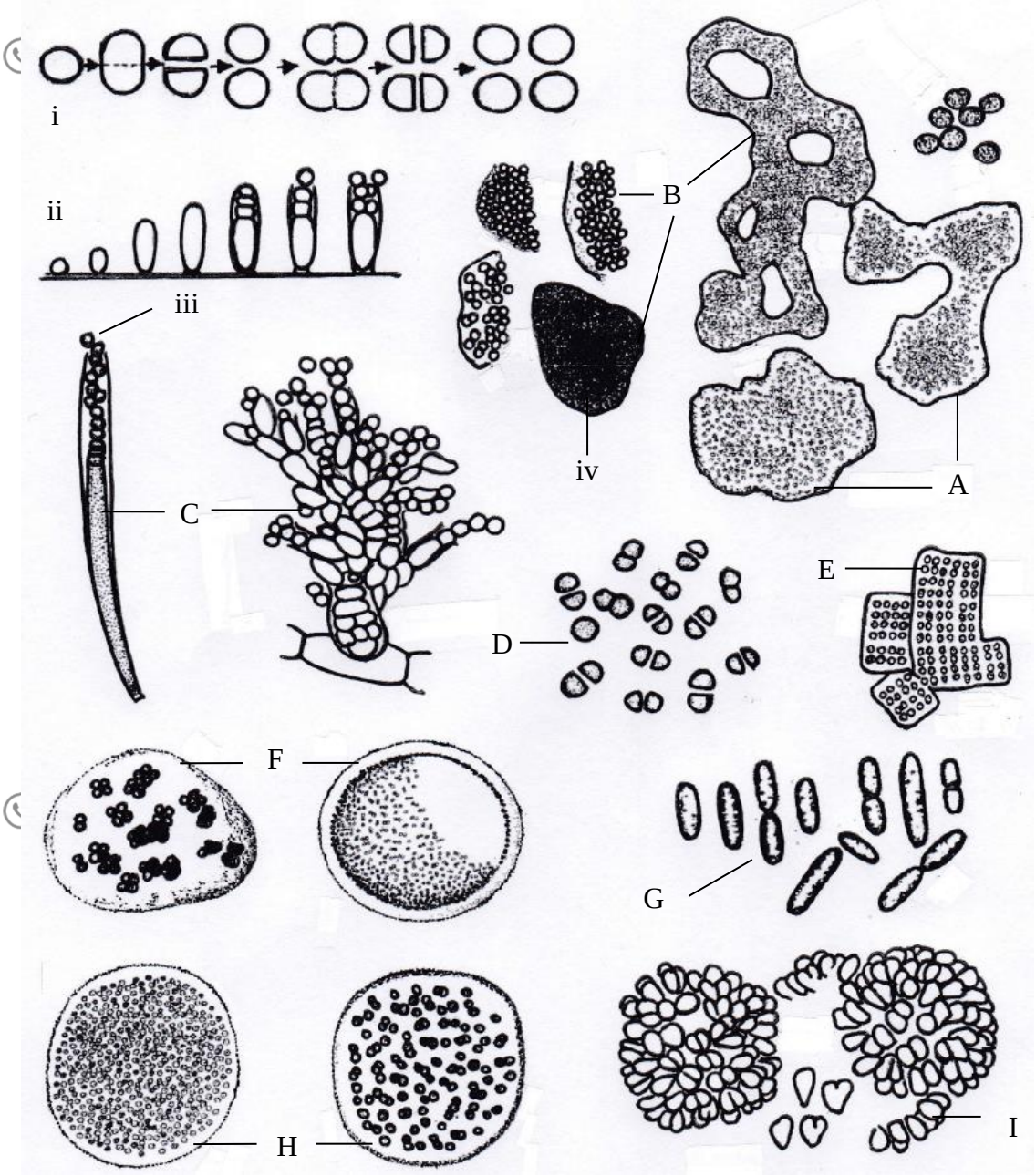

Figure 2.1. Schematic diagrams of Chroococcales. (A-B) *Microcystis* spp., (C) *Chamaesiphon* spp., (D) *Synechocystis* spp., (E) *Merismopedia* spp., (F) *Chroococcus* spp., (G) *Synechoccus* spp., (H) *Aphanocapsa* spp., (I) *Gomphosphaeria* spp., (i) Binary fission mechanism, (ii) Budding mechanism, (iii) Exocytes, and (iv) Densely arranged cells (Chorus & Bartram, 1999; Komarek & Hauer, 2013)

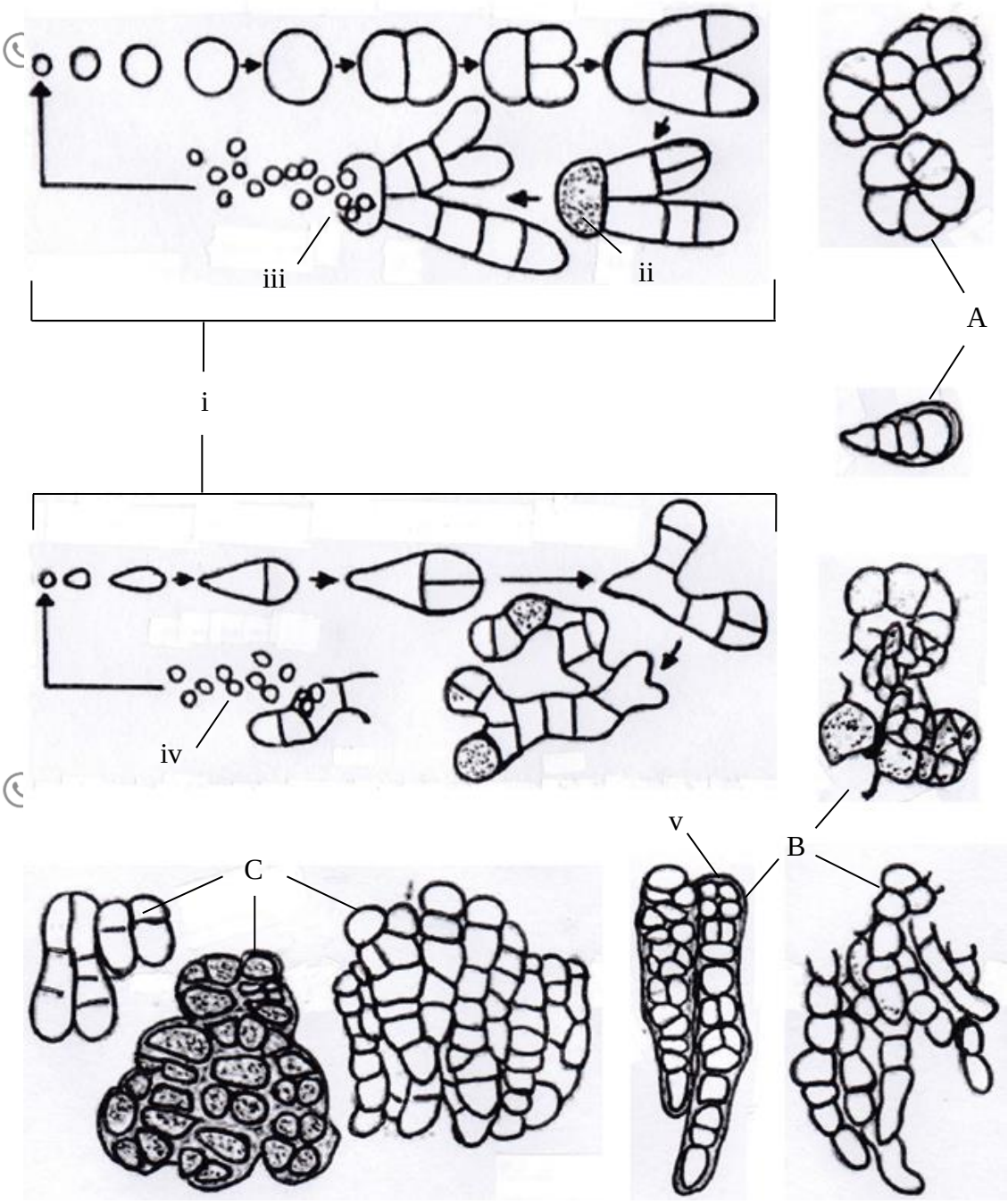

Figure 2.2. Schematic diagrams of Pleurocapsales. (A-C) *Pleurocapsa* spp., (i) Facultative monocyte formation mechanism (started from cell enlargement, multiple fissions and baeocytes formation), (ii) Baeocytes, (iii-iv) Baeocytes released from the parent cells, and (v) Pseudofilaments (Komarek & Hauer, 2013)

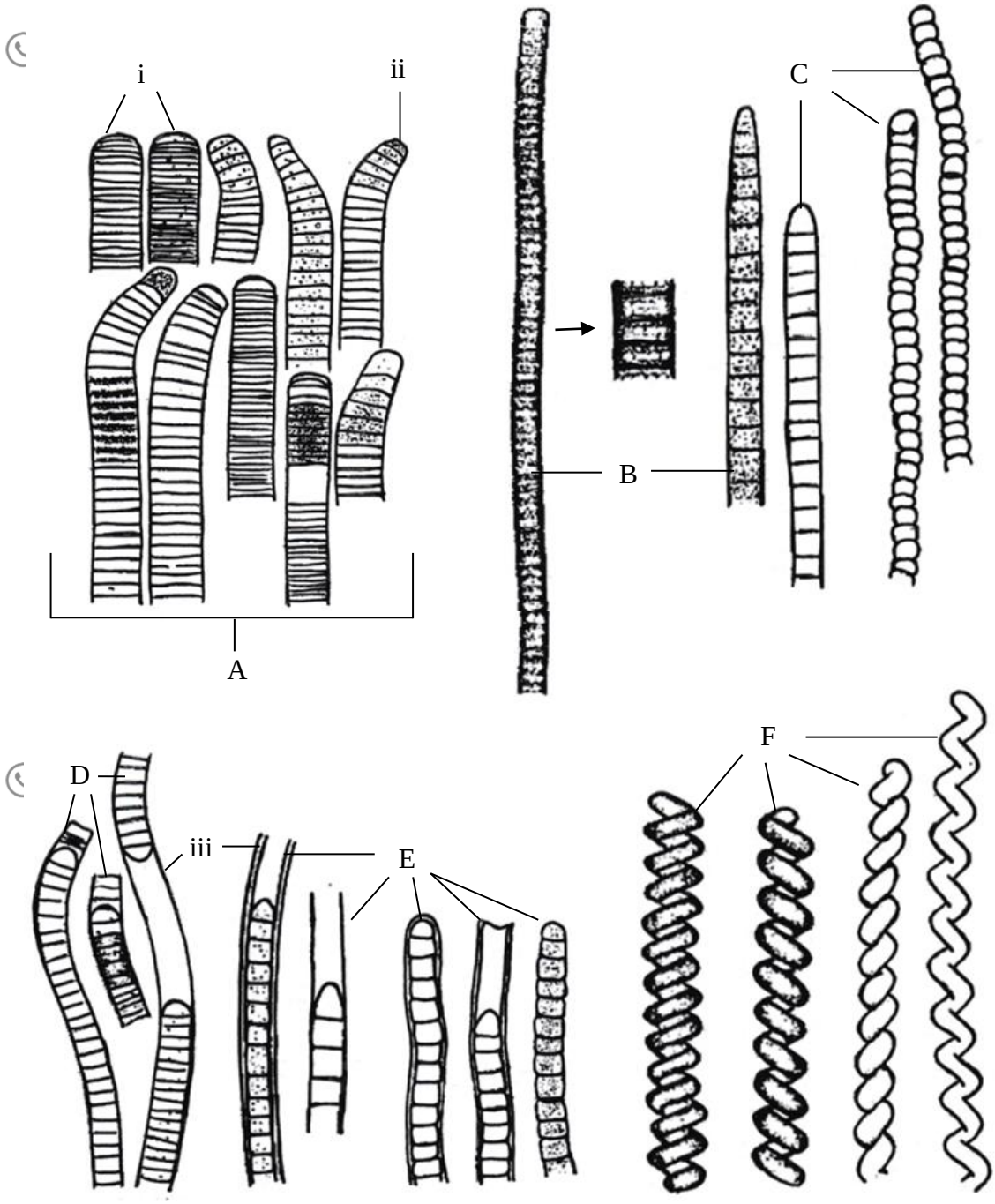

Figure 2.3. Schematic diagrams of Oscillatoriales. (A) *Oscillatoria* spp., (B-C) *Planktothrix* spp., (D-E) *Phormidium* spp., (F) *Spirulina* spp, (i) Widely rounded end cells, (ii) Narrow calyptra, and (iii) Visible sheaths (Komarek & Hauer, 2013)

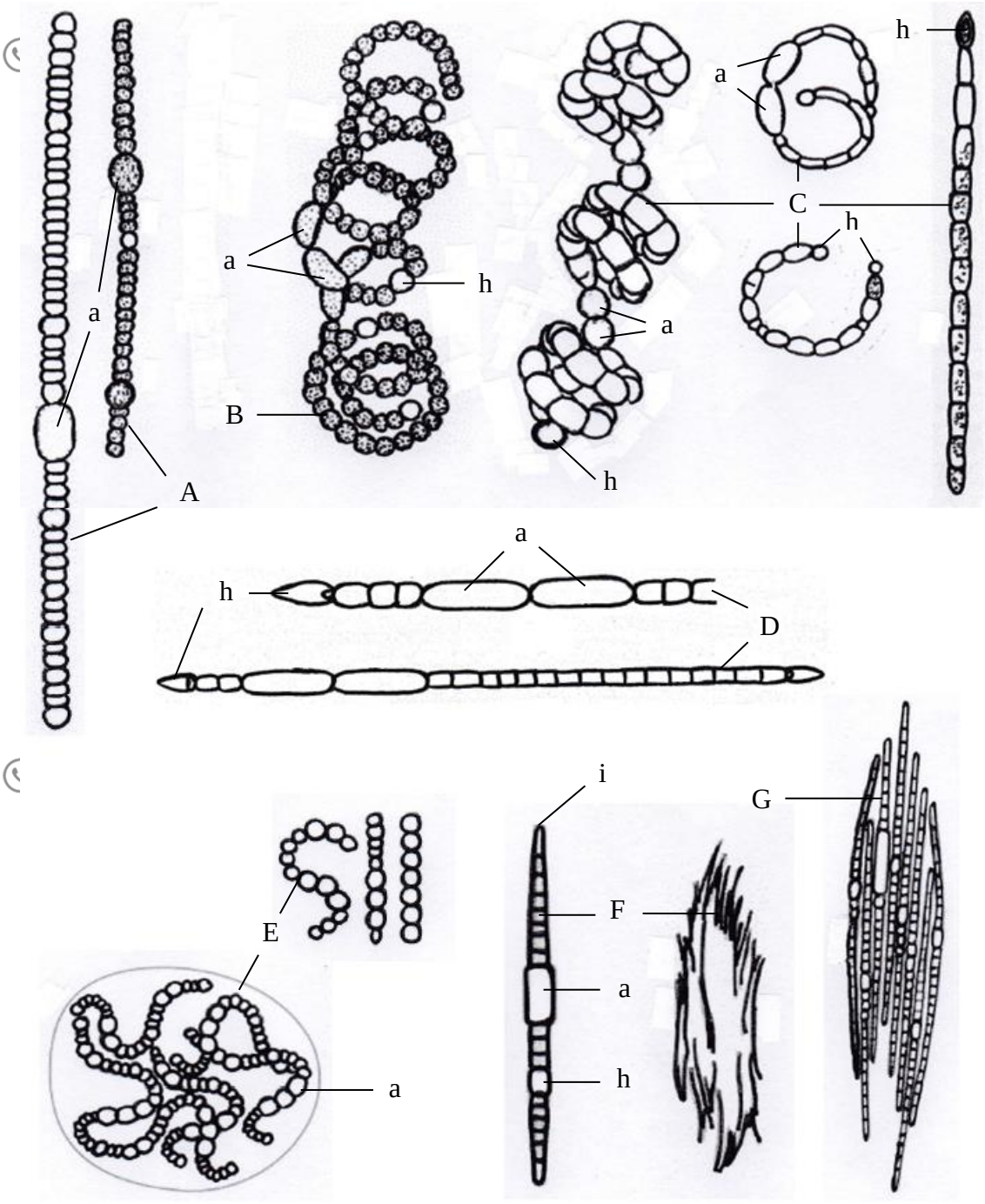

Figure 2.4. Schematic diagrams of Nostocales. (A-B) *Anabaena* spp., (C) *Anabaenopsis* spp., (D) *Cylindrospermopsis* spp. (E) *Nostoc* spp., (F-G) *Aphanizomenon* spp., (a) Akinetes, (h) Heterocytes, and (i) Taper ends (Komarek & Hauer, 2013; Komarek, Kastovsky, Mares, & Johansen, 2014)

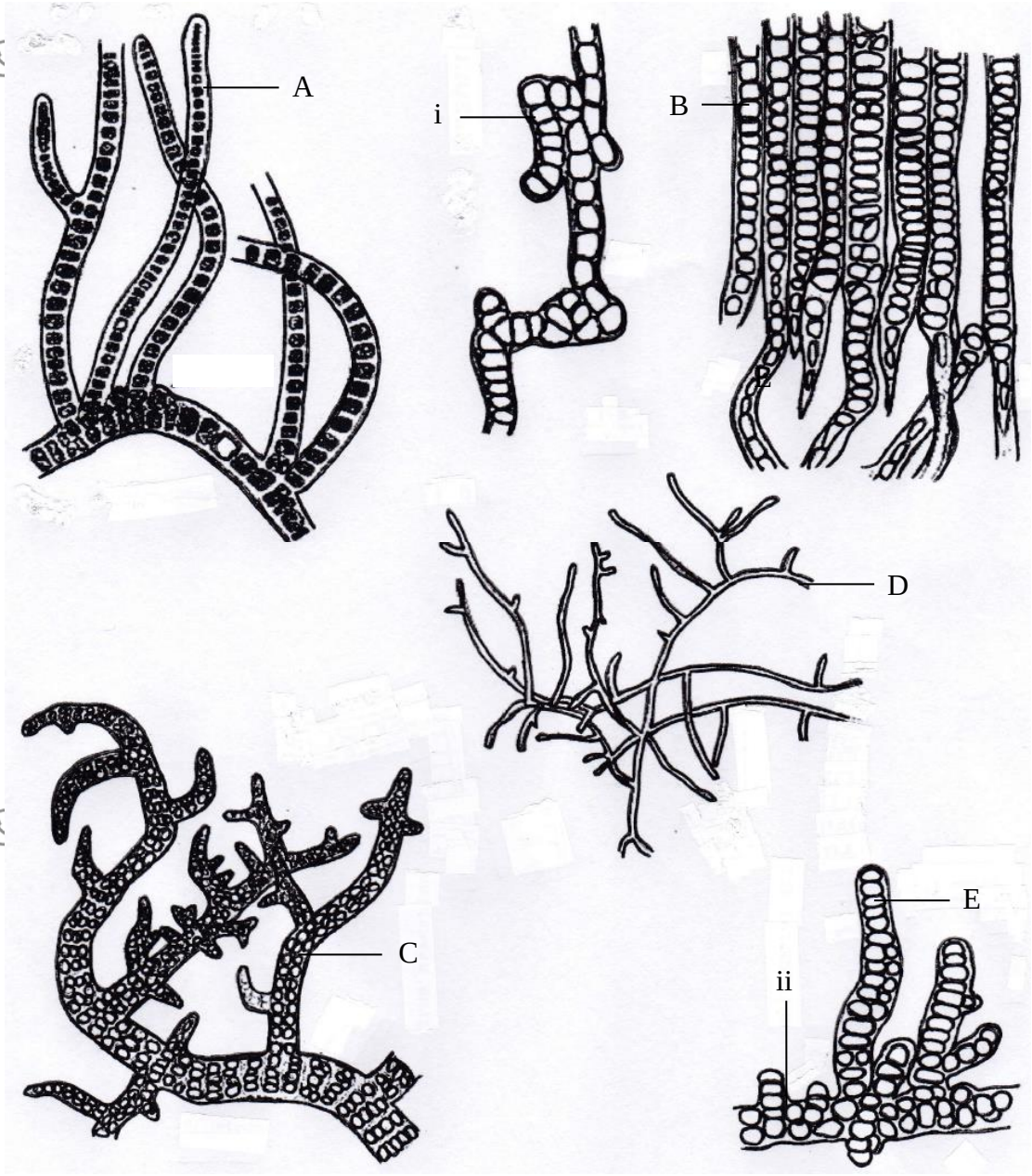

Figure 2.5. Schematic diagrams of Stigonematales. (A) *Fischerella* spp., (B) *Stauromatonema* spp., (C-E) *Stigonema* spp., (i) True branching, and (ii) Multiserial trichomes (Komarek & Hauer, 2013)

Cyanobacteria brings both beneficial and detrimental impacts to human (Chorus & Bartram, 1999). Among the benefits of cyanobateria include its potential in drug discovery, bioremediation, biofertiliser, bioenergy, bioplastic and food supplement (Abed, Dobretsov, & Sudesh, 2009). In spite of that, the extensive growth

of cyanobacteria in freshwater lakes and aquaculture ponds may give negative implications to water supply, recreation activity as well as aquaculture business. Some cyanobacteria are capable to release odorous and / or toxic compounds into the water bodies. In aquaculture ponds, the odorous substances may cause unpleasant taste on fish (Tucker, 2000). The synthesis of toxic compounds known as cyanotoxin on the other hand, has the potential to accumulate in fish tissues and pose health risk to human through fish consumption (Peng et al., 2010; Poste, Hecky, & Guildford, 2011; Schmidt et al., 2013). However, unlike pathogenic bacteria, cyanobacteria only proliferate in abiotic ecosystem but incapable to do so within the human body (Chorus & Bartram, 1999).

2.2 Cyanobacterial Bloom

Under suitable conditions, cyanobacteria multiply themselves to high densities and form visible surface scum known as cyanobacterial bloom (Chorus & Bartram, 1999). Cyanobacterial bloom is not a new phenomenon, instead it has been reported as early as in AD77 (Codd, Steffensen, Burch, & Baker, 1994). In 1878, George Francis documented the first scientific report on animals poisoning due to cyanobacterial bloom (Chorus & Bartram, 1999; Codd et al., 1994). In the report, Francis described the rapid death of stock animals after drinking water from Lake Alexandrina in Australia. During the poisoning, the lake water was observed having two to six inches thick of green scum believed to be *Nodularia spumigera* (Francis, 1878). Subsequent to the work of Francis, many studies on cyanobacterial bloom were reported in

Europe, North America, South America, Australia, Asia and Africa (Chorus & Bartram, 1999).

Cyanobacterial bloom may give deleterious impacts to the water quality, biological communities and ecosystem services, both directly and indirectly. The direct impacts are, for instance the contamination of toxin in fish, invertebrates and other aquatic organisms, while the indirect impacts include the reduction of submerged plants as well as changes in the fish community structure (Havens, 2008). These effects are not surprising as about 60 to 75% of the cyanobacterial blooms are normally toxic (Mitsoura et al., 2013). Besides, the presence of surface scum and unpleasant odours due to cyanobacterial bloom in recreational lakes would also impair its use (Chorus & Bartram, 1999).

2.2.1 Factors Affecting Cyanobacterial Bloom

Cyanobacterial bloom in freshwater systems is believed to be triggered by a range of physicochemical parameters such as nutrient inputs, temperature (Paerl & Huisman, 2008), dissolved oxygen and water pH (Okogwu & Ugwumba, 2009). The environmental variables may sometimes correlated among one another (D. Stone & Bress, 2007), or / and work in combination (Mioni et al., 2011; Okogwu & Ugwumba, 2009) rather than just relying on a single environmental stressor (Mioni et al., 2011).

2.2.1.1 Nutrient Inputs

Human activities such as urbanisation, agricultural practice and industrialisation released excessive nutrients loading into water bodies, leading to eutrophication. Phosphorus and nitrogen are the most common nutrients associated with cyanobacterial bloom. Although it was commonly accepted that cyanobacteria prefers high concentration of phosphorus and nitrogen, the truth is cyanobacteria normally occurred when the availability of dissolved phosphate were lowest (Chorus & Bartram, 1999). For example, the optimum ratio between nitrogen and phosphorus to support the growth of cyanobacteria is about 10 to 16 molecules of nitrogen to one molecule of phosphorus (Schreurs, 1992 as cited in Chorus & Bartram, 1999). Besides, the concentration variations between the two compounds can also regulate the composition of cyanobacterial community in water bodies. Andersson, Höglander, Karlsson, and Huseby (2015) reported that the dominance of Chroococcales, Oscillatoriales and Nostocales were correlated with the low amount of phosphorus, high amount of nitrogen and high amount of phosphorus, respectively.

In addition, nutrient inputs also contain carbonates such as magnesium and calcium that will affect the hardness as well as the alkalinity of water bodies. Hard water is detrimental to the growth of most eutrophic algae (Shaw, Mechenich, & Klessig, 1993). However, cyanobacteria was reported to be able to tolerate hard water and showed improved growth. Carneiro, Beatriz, Pacheco, Maria, and Oliveira (2013) observed significant correlations between magnesium and calcium with the growth of *Cylindrospermopsis raciborskii*, both positively (magnesium) and negatively (calcium).

2.2.1.2 Temperature

The phenomena of climate change and global warming have raised concerns among scientists on the possibility of a greater incidence of cyanobacterial bloom in the future (Paerl & Huisman, 2008; Paerl & Paul, 2012). Increased temperature promotes massive proliferations of cyanobacteria as most species attained the optimum growth rate between 25 °C (Robarts & Zohary, 1987) to 35 °C (Kumar, Kulshreshtha, & Singh, 2011; Lürling, Eshetu, Faassen, Kosten, & Huszar, 2012). Besides, most green algae and diatoms are unable to tolerate high water temperature (Chorus & Bartram, 1999), thus leading to the dominance of cyanobacteria in water bodies (Elliott, 2010). Despite cyanobacterial preference towards warm temperature, most of the species showed cells degradation at temperature above 35 °C (Butterwick, Heaney, & Talling, 2005) suggesting that too high of water temperature could be harmful to cyanobacteria.

Other than that, temperature also plays a part in determining the cyanobacteria compositions in water bodies (Sharma, Rai, & Stal, 2013). For example, both *Anabaena flos-aquae* and *Aphanizomenon flos-aquae* were able to thrive in water at 20 °C onwards. However, when the growths were tested at 35 °C, *Anabaena flos-aquae* showed rapid mortality while *Aphanizomenon flos-aquae* was still capable to sustain its growth. The proliferation of *Microcystis aeruginosa* on the other hand, was only recorded at 25 °C to 30 °C (Butterwick et al., 2005). Some thermophilic cyanobacteria such as *Synechococcus elongatus* was reported to be able to withstand water temperature of up to 60 °C (Miyairi, 1995).

High temperature enhances the accumulation of nutrients in water column, thus promoting cyanobacterial bloom (Mioni et al., 2011). Similar result was also documented by Davis, Berry, Boyer, and Gobler (2009). The study described that elevated water temperature significantly increase the growth rates of *Microcystis* spp. in Lake Champlain, Missisquoi Bay, Long Island, and Lake Ronkonkoma during their two years of study. However, when there was concurrent increase in temperature and phosphorus concentration, higher biomass were recorded (Davis et al., 2009). Both of these studies illustrated the potential occurrence of an even greater incidence of cyanobacterial bloom in relation to future eutrophication and global warming.

In addition, high temperature also reduces the solubility of oxygen in water bodies, leading to a decrease in the concentrations of dissolved oxygen. This is because warmer surface water requires less concentration of oxygen to reach the equilibrium point, which is the 100% air saturation (Verma, 2004). Apart from that, rising temperature may also induces the occurrence of thermal stratification which will separate the water column into three distinct layers based on the water density (Paerl & Huisman, 2008; Paerl & Paul, 2012). Thermal stratification stabilises the water column and restrains vertical mixing (Elçi, 2008; Ibelings, Vonk, Los, van Der Molen, & Mooij, 2003; Paerl & Huisman, 2008), leading to further depletion of dissolved oxygen especially at the bottom layer. This condition will enhance the growth of cyanobacteria especially among the buoyant taxa (Paerl & Huisman, 2008).

2.2.1.3 Dissolved Oxygen

Dissolved oxygen indirectly promotes the proliferations of cyanobacteria through its role in nutrient cycling. The abundance of oxygen in water column provides aerobic conditions that facilitate the decomposition of nutrients essential for the growth of cyanobacteria such as phosphorus, from organic phosphate into the bioavailable form of inorganic phosphate (Hudnell, 2008). High level of bioavailable nutrients stimulates the massive growth of cyanobacteria.

Excessive growth of cyanobacteria also can cause fluctuations in the level of dissolved oxygen in water bodies (Hudnell, 2008). For example, during cyanobacterial bloom, photosynthesis activity increases leading to the escalation of dissolved oxygen (Cuiya, 1998). However, when the bloom collapsed, high amount of oxygen will be used to decompose the microorganism, hence depletes the oxygen level (Hudnell, 2008). Significant correlations between dissolved oxygen and cyanobacterial biomass have also been documented by Cuiya (1998) and Okogwu and Ugwumba (2009).

2.2.1.4 pH

Similar to dissolved oxygen, water pH also helps to promote the growth of cyanobacteria indirectly. High pH enhances the dissociation of bound phosphorus and ultimately increases the bioavailability of the essential nutrient in water systems. This

condition intensifies the proliferation of cyanobacteria and encourage the longevity of the blooms (Hudnell, 2008).

Active photosynthetic activity during cyanobacterial bloom leads to increase in water pH. Phytoplanktons may lose their efficiency to utilise carbon at high pH, hence promoting the growth and dominance of cyanobacteria (Dokulil & Teubner, 2000).

2.2.2 Ecostrategies of Cyanobacteria

Cyanobacteria develop specific ecostrategies depending on the environments they thrive in. The ecostrategies include the behaviour and reactions of cyanobacteria in the environments with limited nutrients, high water temperature, and low light availability. Cyanobacteria ecostrategies cause the species to have competitive advantage over other phytoplanktons, hence ensuring its succession in dominating the water bodies (Chorus & Bartram, 1999).

2.2.2.1 Chromatic Adaptation

Cyanobacteria use light to perform photosynthesis for energy. Just like plant, cyanobacteria have both Photosystem I (PSI) and Photosystem II (PSII) in their photosynthetic apparatus. The photosynthetic pigments of cyanobacteria comprised of chlrophyll-*a*, carotenoids and accessory pigments known as phycobiliproteins or

phycobilins. Phycobiliproteins that normally present in cyanobacteria are phycocyanin, allophycocyanin, and phytoerythricin. Cyanobacteria regulate the composition of its phycobiliproteins based on the quality of light through a process called chromatic adaptation. This enables cyanobacteria to absorb light most efficiently wherever they thrive (Chorus & Bartram, 1999).

2.2.2.2 Efficient Carbon Utilisation

Carbon dioxide (CO_2) provides the main source of inorganic carbon for cyanobacteria and phytoplanktons to perform photosynthesis (Whitton, 2012). Cyanobacteria gains competitive advantage over other phytoplanktons due to its lower half-saturation constant (Shapiro, 1990; Dokulil & Teubner, 2000) and high affinity towards CO_2 (Whitton, 2012). Besides, cyanobacteria are also capable to utilise bicarbonate (HCO_3^-) as an alternative source of carbon for photosynthesis. These features help to promote cyanobacteria succession in carbon limited environments (Shapiro, 1990; Dokulil & Teubner, 2000).

2.2.2.3 Phosphorus Uptake and Storage

The presence of phosphorus in water systems is essential for the growth and survival of phytoplanktons (Correll, 1998). In eutrophic water bodies, cyanobacteria can outcompete other phytoplanktons for phosphorus because of its lower half-saturation constant as well as high affinity for the compound (Padisák, 1997; Amano et al.,

2010). Apart from that, cyanobacteria can also store higher capacity of phosphorus in their cells (internal P storage) as compared to eukaryotic algae, leading to longer survival under phosphorus deficient conditions (Kromkamp, 1987). Cyanobacteria was reported to be able to store sufficient amount of phosphorus for the microorganism to carry out at least two to four cell divisions, which may result in 4 to 32 folds increase in the biomass (Chorus & Bartram, 1999).

2.2.2.4 Nitrogen Fixation

Some cyanobacteria are capable to convert atmospheric nitrogen directly into ammonium. Ammonium is the readily assimilated form of nitrogen (Herrero et al., 2001; Rückert & Giani, 2004) that is required by cyanobacteria for the synthesis of amino acids and proteins (Reynolds, 1984). Nitrogen fixation capability allows the heterocystous cyanobacteria to gain competitive advantage under nitrogen (N) deficient conditions for instance in water bodies with low N:P ratio (Havens, 2008). Among cyanobacterial species that possess the nitrogen fixing feature include *Anabaena* spp., *Aphanizomenon* spp., *Cylindrospermopsis* spp., *Nodularia* spp., and *Nostoc* spp. (Chorus & Bartram, 1999).

2.2.2.5 Siderophore Mediated Iron Uptake

Iron is a trace element essential for the growth and metabolism of all living organisms (Sunda & Huntsman, 2015). In cyanobacteria, iron is needed particularly for the

synthesis of photosynthetic pigments such as chlorophyll-*a* and phycocyanin (Whitton, 2012), as well as for carbon (Sigman & Boyle, 2000) and nitrogen fixation (Gruber, 2008) processes. However, the bioavailable forms of dissolved iron in water bodies are extremely low (less than 1%) (Johnson, Gordon, & Coale, 1997), leading to high competition for the substrate amongst aquatic microorganisms and eukaryotic algae. Some cyanobacteria such as *Anabaena* spp., *Microcystis* spp., and *Planktothrix* spp. gain competitive advantage over other phytoplanktons due to their capability to produce siderophores. Siderophores are compounds that have high affinity towards ferric ions, Fe(III) to facilitate the uptake of iron into the cyanobacteria cells (Kranzler, Rudolf, Keren, & Schleiff, 2013).

2.2.2.6 Buoyancy Regulation

Some cyanobacteria species are able to control their position in water column due to the presence of gas vesicles in their cells. The gas vesicles cause cyanobacteria to have lower density than water, allowing the species to be buoyant. Buoyancy characteristic enables cyanobacteria to outcompete for nutrients as it can move up and down in the water column and migrate to depth with more abundant essential nutrients (Bellinger & Sigee, 2010; Chorus & Bartram, 1999). Besides, buoyancy also allows cyanobacteria to move up to water surface for absorption of light energy and stay a little downward when the light intensity is unfavourable to avoid photoinhibition or permanent cell damage (Bellinger & Sigee, 2010). Under limited light conditions, cyanobacteria are capable to induce themselves to create more gas vesicles to enhance the buoyancy (Chorus & Bartram, 1999). It was reported that

buoyancy characteristic can increase the cyanobacteria photosynthesis rate by nearly two folds (Walsby, Hayes, Boje, & Stal, 1997). Examples of cyanobacteria genera that possess this capability are *Microcystis* spp. (Chorus & Bartram, 1999), *Aphanizomenon* spp. (Walsby et al., 1997), and *Cylindrospermopsis* spp. (Padisák, 1997).

Buoyancy is indeed the most superior capability of cyanobacteria as it can facilitate other ecostrategies to ensure the dominance of the species in aquatic environments. This therefore explains the abundance and dominance of buoyant *Microcystis* spp. in freshwater systems all over the world as reported by numerous literatures including in Malaysia (Sinang et al., 2015).

2.3 Cyanobacterial Toxin

The ability of some cyanobacterial species to produce a wide range of toxins known as cyanobacterial toxins or cyanotoxins has raised public health concern. Cyanotoxin can cause both water-borne and water-contact diseases (Chorus & Bartram, 1999). Fatal poisoning of livestock and wildlife upon ingestion of cyanotoxin have been documented across the globe, however, there was no human fatalities recorded so far due to oral uptake of this toxic compound (Malbrouck & Kestemont, 2006). In 1996, the first human poisoning due to cyanobacterial toxin was recorded in Caruaru, Brazil subsequent to renal dialysis procedure. The tragedy, also known as "Caruaru Syndrome" happened in February 1996 and had caused death to 52 out of 100 affected patients (Azevedo et al., 2002).

Cyanotoxin can be classified into three groups based on the function and target area: (i) neutrotoxin, (ii) hepatotoxin, and (iii) dermatoxin (Rodgers, 2008). Most cyanobacterial toxins are either neurotoxin or hepatotoxin (Codd et al., 2005). Neurotoxin targets the nervous system (D. Stone & Bress, 2007) and causes blockage of voltage-gated sodium channel leading to paralysis and even death in certain cases (Metcalf & Codd, 2004). Anatoxin, anatoxin-*a*, saxitoxin and neosaxitoxin are the examples of neurotoxin. Hepatotoxin attacks the function of liver and leads to liver damage. Examples of hepatotoxin are nodularin, cylindrospermopsin and microcystin. Among all of the mentioned cyanobacterial toxins, microcystin is the most commonly found cyanotoxin in freshwater systems (Bartram, 2015; Chorus & Bartram, 1999).

2.3.1 Microcystin

Being the most widely distributed cyanotoxin in freshwater systems and the predominant toxin of cyanobacterial bloom (Bartram, 2015; Chorus & Bartram, 1999), microcystin was actually named after the first cyanobacterial species found to produce the toxic compound, which was *Microcystis aeruginosa* (Chorus & Bartram, 1999). To date, more than 90 known structural variants of microcystin has been discovered throughout the globe (Su, Xue, Steinman, Zhao, & Xie, 2015), making microcystin the most commonly studied cyanotoxin across the world (Ferrão-Filho & Kozlowsky-Suzuki, 2011; Lawton and Edwards, 2008). Microcystin is usually produced by *Microcystis* spp., *Anabaena* spp., *Anabaenopsis* spp., *Planktothrix* spp., *Oscillatoria* spp., and *Nostoc* spp. (Sivonen & Jones, 1999). Among all of the

microcystin producers, *Microcystis aeruginosa* was documented as the most common species producing the hepatotoxin (Mioni et al., 2011).

Microcystins are cyclic peptides comprised of seven amino acids. Figure 2.6 shows the general structure of all microcystin variants. The variants of microcystin are determined by the amino acids arranged at the second (X) and fourth (Z) positions. For example, microcystin-LR actually contains leucine at the X-position and arginine at the Z-position (Butler et al., 2009). The main chromophore of microcystins is the amino acid located at the fifth position known as ADDA. This molecule absorbs ultraviolet light at 238 nm which is crucial for the identification of microcystin compound (Bogusz, 2000).

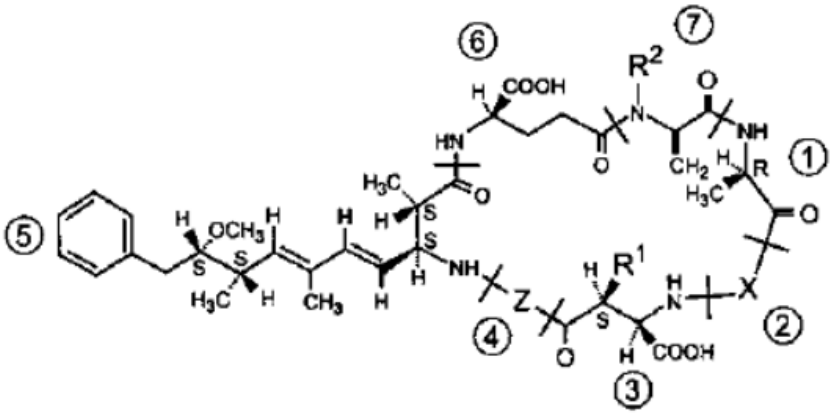

Figure 2.6. General structure of microcystin. (1) D-alanine, (2) Variable L-amino acid, (3) D-methylaspartic acid, (4) Variable L-amino acid, (5) 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, (6) D-glutamic acid, and (7) N-methyldehydroalanine (Butler et al., 2009)

As mentioned earlier, microcystin is a type of hepatotoxin. This toxin attacks the liver of an organism by inhibiting the production of a class of enzymes called protein phosphatases 1 (PP1) and 2A (PP2A) (Butler et al., 2009; Su et al., 2015).

This causes build up of phosphorylated proteins in liver, leading to liver damage (Butler et al., 2009; D. Stone & Bress, 2007). Early indications of liver damage include increases in the serum of liver enzymes and liver weight (Butler et al., 2009). Apart from that, microcystin was also reported to act as tumour promoter by stimulating the proliferation of cancer cells (Butler et al., 2009; Fujiki & Suganuma, 2011). Microcystin-LR, the most toxic, the most abundant (Lone, Koiri, & Bhide, 2015) and the most commonly studied microcystin variant in the world (Butler et al., 2009) was found to be more toxic to male reproductive system (testis) as compared to other organs including liver (Y. Li, Sheng, Sha, & Han, 2008; Lone et al., 2015). Following its potential toxicity, microcystin-LR has been described as possibly carcinogenic to humans by an International Agency for Research on Cancer (Butler et al., 2009; Fujiki & Suganuma, 2011).

In natural environment, microcystins are usually confined within the cyanobacterial cells (intracellular) and rarely being secreted into the surrounding water bodies (Chorus & Bartram, 1999; Meriluoto & Codd, 2005), unless during the rupture of cyanobacterial cell wall upon senescent (Butler et al., 2009). Backer et al. (2008) reported that about 95 to 98% of the toxin remained intact inside the cyanobacterial cells during the active growth of cyanobacteria.

In addition, microcystin is also an extremely stable compound (Bogusz, 2000) and is able to resist common chemical breakdown such as hydrolysis or oxidation processes (Butler et al., 2009). Slow breakdown was only noticed at temperature above 40 °C at either very acidic (pH < 1) or very alkaline (pH > 9) conditions. However, even under such undesirable environments, microcystin still can persist for

several weeks in water. Harada, Tsuji, Watanabe, and Kondo (1996) reported that at 40 °C, the half-life of microcystin was three weeks at pH 1 and 10 weeks at pH 9. Similar to detrimental pH, microcystins only showed slow breakdown in full sunlight (Tsuji et al., 1995) and capable to stand boiling at up to 300 °C (Wannemacher, 1989 as cited in Zhang, Xie, & Chen, 2010). Moreover, Zhang et al. (2010) found that boiling can significantly increase the concentration of microcystins detected in the muscle of bighead carp. This is due to the release of the covalently-bound microcystins from the target molecules such as protein phosphatases upon heating (Zhang et al., 2010).

2.3.2 Factors Affecting Microcystin Production

The synthesis of microcystins by cyanobacteria are highly dependent on the biological and environmental factors with each giving a direct, as well as an indirect effects to the concentrations of microcystins (Pimentel & Giani, 2014). As described in Figure 2.7, both optimum and detrimental environmental factors may results in an increase of microcystin production through the regulation of biological factors.

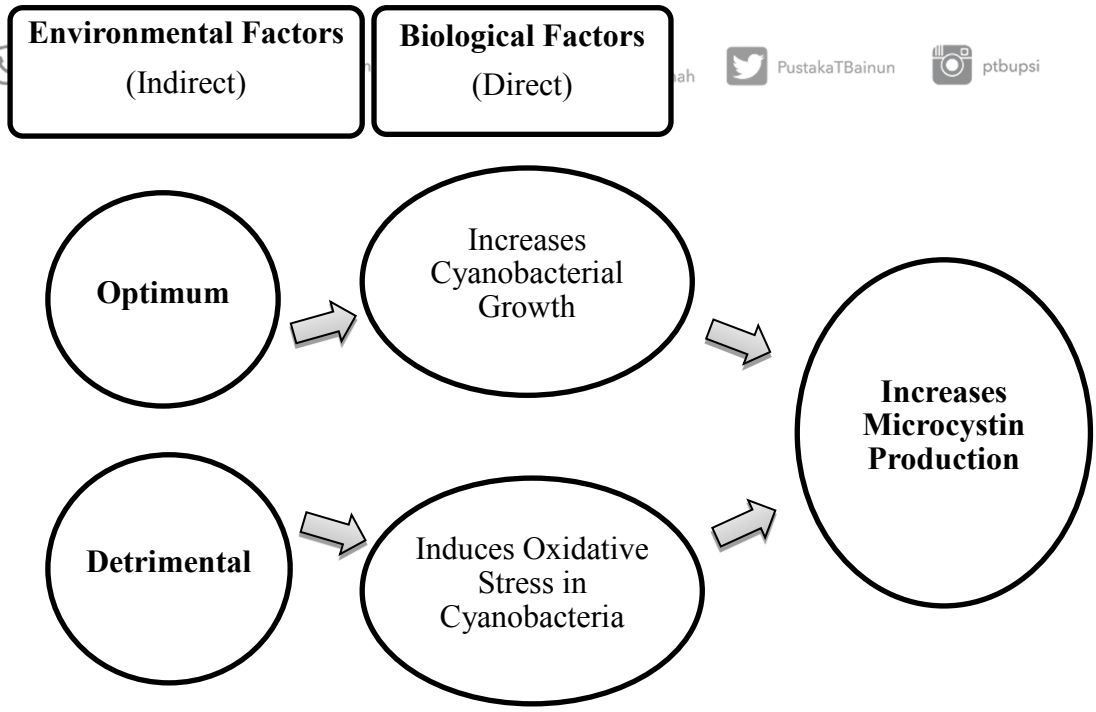

Figure 2.7. Microcystins production under optimum and detrimental conditions

Environmental factors as mentioned in section 2.2.1 provide optimum conditions for the proliferation of cyanobacteria in water bodies. The favourable conditions help to increase the production of microcystins through the quantity as well as quality (toxic strains) of cyanobacteria. Joung, Oh, Ko, and Ahn (2011) observed significant correlations between temperature with the cell density of potentially toxic *Microcystis* spp. and microcystin concentration, but reported insignificant correlation between the parameter with the non-toxic strain of cyanobacteria. Similar finding was also documented by Brutemark, Engström-Öst, Vehmaa, and Gorokhova (2015). In addition, temperature also plays a part in regulating the microcystin variants, whereby higher temperature (> 25 °C) promotes the production of microcystin-RR while lower temperature (< 25 °C) enhances the synthesis of microcystin-LR (Rapala, Sivonen, Lyra, & Niemelä, 1997; Rapala & Sivonen, 1998). Chorus and Bartram (1999)

reported that temperature gradient can cause up to three-folds difference in cellular microcystin production.

Just like temperature, nutrients also regulate the production of microcystin by promoting the growth of cyanobacteria. Microcystin contents in cyanobacteria are highly correlated with both high phosphorus (Jacoby, Collier, Welch, Hardy, & Crayton, 2000; Kotak, Lam, Prepas, & Hrudey, 2000; Rapala et al., 1997; Rinta-Kanto et al., 2009) and high nitrogen (Rapala et al., 1997) in some literatures. Additionally, Vézie, Rapala, Vaitomaa, Seitsonen, and Sivonen (2002) stated that the variations between the macronutrients are essential in the regulation of either toxic or non-toxic cyanobacteria. This was demonstrated in Utkilen and Gjølme (1995) and Lee, Jang, Kim, Yoon, and Oh, (2000) whom reported significant correlations between higher N:P ratio with microcystins. Despite the common belief that optimum environment positively influences the cyanobacterial growth, hence the production of microcystins (Kotak et al., 2000; Rinta-Kanto et al., 2009; Wu et al., 2008), Joung et al. (2011) recorded contradicting finding. The study only observed significant relationship between high phosphorus with cyanobacterial cell density, but not the microcystin concentration (Joung et al., 2011).

Detrimental condition, such as nutrients limited environment may increase the production of microcystin by triggering the oxidative stress in cyanobacteria (Pimentel & Giani, 2014). Oxidative stress enhances the transcription of *mcy* gene in cyanobacteria (Boopathi & Ki, 2014) as a response to protect the cell against stressors (Zilliges et al., 2011). In cyanobacteria cells, microcystins produced are covalently bounded to its cellular proteins such as phycobilins and enzymes. However, when the

cyanobacteria cells are experiencing oxidative stress, the binding becomes stronger (Zilliges et al., 2011). Furthermore, detrimental environments will also help to promote the growth of potentially toxic cyanobacteria due to the strains' tolerance towards hostile environments (Jähnichen, Long, & Petzoldt, 2011; Van de Waal et al., 2011). Significant correlations between microcystin production with low phosphorus (Oh, Lee, Jang, & Yoon, 2000; Pimentel & Giani, 2014) and low nitrogen (Pimentel & Giani, 2014) were demonstrated in several studies.

2.4 Provisional Guidelines for Microcystin

World Health Organisation (WHO) established three guidelines for safe exposure to microcystins: (i) Tolerable Daily Intake (TDI) level, (ii) Drinking Water Quality, and (iii) Safe Practice in Managing Recreational Water Exposure. The TDI and drinking water quality are as stated below, whereas the guidelines for recreational water are as compiled in Table 2.2 (Chorus & Bartram, 1999).

Tolerable Daily Intake (TDI) = 0.04 µg microcystin-LR per kg body weight per day

Drinking Water Quality = 1.0 µg microcystin-LR per L

Table 2.2

World Health Organisation Guidelines for Safe Practice in Managing Recreational Water

Relative probability of adverse health impact	Cell counts (cells/ml)	Chlorophyll-*a* (µg chl-*a*/L)	Microcystin (µg MC-LR/L)
Low	≤ 20,000	≤ 10	≤ 10
Medium	> 20,000 – 100,000	> 10 – 50	> 10 – 20
High	> 100,000 – 10,000,000	> 50 – 5,000	> 20 – 2,000
Very high	> 10,000,000	> 5,000	> 2,000

Note. Guidelines are adapted from Chorus & Bartram (1999).

2.5 Previous Studies on Cyanobacteria in Malaysia Water System

In Malaysia, the earliest scientific study on cyanobacteria is perhaps the literature documented by Fatimah et al. (1984) which studied the composition and production of phytoplanktons in Paya Bungor, Pahang. The study reported that cyanobacteria, composed mainly of *Anabaena* spp., were the most abundant phytoplankton in Malaysian lakes in terms of cell density (Fatimah et al., 1984). Nearly 20 years after the work of Fatimah et al. (1984), animal poisoning which involved the death of several cattle after drinking water from freshwater lakes under cyanobacterial bloom was recorded in the country (Lim et al., 2003). However, there was no thorough investigation carried out on the contaminated lakes.

The occurrence of potentially toxic cyanobacteria, particularly those that can produce microcystin according to Sivonen and Jones (1999) were reported in several studies conducted in Malaysia (see Table 2.3). However, the toxicity of the microorganisms in our freshwater systems is still rarely studied. Research conducted

by Sinang et al. (2015) is probably the only study carried out in Malaysia so far that specifically evaluate the presence of toxic cyanobacteria and its potential toxicity in our freshwater systems. The study recorded relative cyanobacterial biomass of up to 903.1 µg chl-*a* /L and microcystin concentration above the detection limit of 10 µg/L in Tasik Metropolitan Kepong (Sinang et al., 2015), suggesting potential health impact to human.

Prior to Sinang et al. (2015), Jasmina, Samsur, and Ruhana (2010) assessed the toxicity of laboratory cultured cyanobacteria. Despite the fact that the cyanobacteria were actually collected from aquaculture ponds, the toxicity assessment was not carried out on the environmental samples (Jasmina et al., 2010), hence failed to demonstrate the danger of cyanobacterial toxin in our water systems. Since there are insufficient studies conducted to assess the toxicity of cyanobacteria in Malaysia environment, more research on cyanobacterial toxin especially in aquaculture system is needed.

By understanding the influence of cyanobacteria in eutrophication, Malaysia has contributed to the automation of cyanobacteria identification and classification to ease the process (Mansoor, Sorayya, Aishah, & Mosleh, 2011). The system was developed with a combination of image processing techniques and artificial neural network (ANN) algorithms to detect the presence of four most common eutrophication-associated cyanobacterial genera namely *Microcystis* spp., *Oscillatoria* spp., *Anabaena* spp. and *Chroococcus* spp. An accuracy of more than 95% can be achieved with the automatic recognition system (Mansoor et al., 2011).

Table 2.3

Occurrence of potentially toxic cyanobacteria in Malaysia freshwater systems

Location	Cyanobacteria			References
	Genera	Biomass	Microcystin	
Paya Bungor in Pahang	*Anabaena*, *Microcystis*, *Oscillatoria*	380 cells /ml	N/R	Fatimah et al. (1984)
Tasik Chini, Pahang	*Microcystis*	12 cells /ml	N/R	Ahmad-Abas et al. (2001)
Kuching, Sarawak	Anabaena, *Microcystis*, *Oscillatoria*	N/R	N/R	Abang (2003)
Sarawak Lenthic Zones	Anabaena, *Microcystis*, *Oscillatoria*	N/R	N/R	Ramlah (2005)
Rivers in Gunung Stong, Kelantan	*Anabaena*, *Oscillatoria*	N/R	N/R	Merican et al. (2006)
Sungai Pontian Kecil, Johor	*Anabaena*, *Nostoc*, *Oscillatoria*	38.4 µg chl-*a* /L	N/R	Nor Azman (2006)
Aquaculture ponds in Serian, Sarawak	*Anabaena*, *Microcystis*, *Nostoc*, *Oscillatoria*	172.1 µg chl-*a* /L	N/R	Mohd. Nasarudin & Ruhana (2007)
Tasik Bera, Pahang	*Anabaena*, *Microcystis*, *Oscillatoria*	N/R	N/R	Chan (2009)
8 freshwater systems in Serian, Bau and Batang Ai, Sarawak	*Anabaena*, *Anabaenopsis*, *Microcystis*, *Nostoc*, *Oscillatoria*	N/R	N/R	Mohd. Nasarudin & Ruhana, (2011b)
10 freshwater lakes in Selangor	*Microcystis*, *Oscillatoria*, *Planktothrix*	903.1 µg chl-*a* /L	> 10.0 µg/L	Sinang et al. (2015)

Note. Cyanobacterial genera listed are only those producing microcystin; Biomass and microcystin stated are the maximum values quantified in the specified studies; N/R = Not reported

2.6 Toxic Cyanobacteria in Aquaculture Systems and Its Impact on Fish

Human populations in general receive about 15 to 20% of the total animal protein intake from fish (Food and Agriculture Organisation [FAO], 2014). Out of this proportion, nearly half was contributed by aquaculture farming (FAO, 2014). Since the declined in the catches of wild fisheries, aquaculture industry has grown to meet the consumers' demands and now known as the fastest growing animal food sector in the world (FAO, 2014). This includes the shift from extensive farming that relies solely on the natural productivity of water bodies into an intensively operated aquaculture systems (Avault, 1996).

In intensive aquaculture systems, large amount of formulated pellets are introduced into the water bodies in order to achieve high productivity per unit volume of water (Smith, Boyer, & Zimba, 2008). This leads to water eutrophication (Tucker, 1996) as the pellet for growing fish usually contains about 32 to 45% of protein (Pandey, 2013). Abundance of nutrients in aquaculture ponds promotes the proliferation of cyanobacteria and resulted in cyanobacterial bloom.

The occurrence of cyanobacteria especially those that can form bloom is a nuisance in aquaculture industry (Paerl & Tucker, 1995; Stone & Daniels, 2006). Being one of the primary producer in water ecosystems, cyanobacteria was reported to serve as a poor base for aquatic food chains (Paerl & Tucker, 1995). Excessive proliferation of cyanobacteria also inhibits the growth of beneficial phytoplanktons essential in maintaining the balance of aquatic environment. This is because of

cyanobacteria competitive advantage towards nutrients, light, and carbon dioxide (Havens, 2008; Rodgers, 2008).

During cell lysis, some species of cyanobacteria such as *Oscillatoria* spp., *Anabaena* spp., and *Microcystis* spp. can synthesise two types of highly odorous compounds known as geosmin (*trans*-1,10-dimethyl-*trans*-9-decalol) and 2-methylisoborneal (1,2,7,7-tetramethyl-*exo*-bicyclo [2.2.1] heptan-2-ol) (Paerl & Tucker, 1995; Schrader & Dennis, 2005; Tucker, 1996). Geosmin has earthy odour, whereas MIB has musty flavour. The unpleasant scent of these compounds can be detected in water bodies at relatively low concentrations (< 0.05 µg/L). Upon sufficient absorption, geosmin and MIB alter the natural flavour of fish at concentration around 5 µg/kg and 1 µg/kg, respectively (Tucker, 1996).

In addition, cyanobacteria capability to produce a wide range of toxin as described in section 2.3 is not only hazardous to the aquatic organisms, but may present potential health risk to human through fish consumption (Poste et al., 2011). Fish are exposed to the toxic metabolites in aquaculture systems through ingestion of cyanobacterial cells, aquatic food chain (Smith et al., 2008) and absorption of dissolved compounds in water bodies through gills during breathing (Zimba et al., 2001). Ingestion of toxic cyanobacteria and its associated toxin may inhibit the growth of fish (Bury, Eddy, & Codd, 1995; Kamjunke, Mendonca, Hardewig, & Mehner, 2002) whereas in some cases, cause mortality to the aquatic species (Jewel et al., 2003; Landsberg, 2002; Zimba et al., 2001). Bioaccumulation of microcystins in fish cultured in aquaculture systems were reported to be significantly higher than the

TDI recommended by WHO (Mohamed, Carmichael, & Hussein, 2003; J. F. Vasconcelos, Barbosa, Lira, & Azevedo, 2013; Zimba et al., 2001).

2.6.1 Cyanobacterial Bloom in Aquaculture Systems

The occurrence of cyanobacterial bloom in commercialised aquaculture systems has been documented in several scientific literatures (see Table 2.4). Layers of "green paints" (Jewel et al., 2003) and visible brownish (Mohd. Nasarudin & Ruhana, 2007) on water surface were observed during the bloom event. In Bangladesh and United States, cyanobacterial bloom have caused massive fish kills (Jewel et al., 2003; Zimba et al., 2001). Analysis carried out on the gills of dead fish found the presence of cyanobacterial cells on the said organ (Jewel et al., 2003). The study then proposed that gill clogging and low amount of dissolved oxygen due to cyanobacterial decomposition could be the reasons for the fish death (Jewel et al., 2003). Apart from that, both Jewel et al. (2003) and Zimba et al. (2001) agreed that the fish fatality might be related to the production of cyanobacterial toxins in aquaculture ponds.

As summarised in Table 2.4, *Microcystis* spp. were the most frequently occurred cyanobacterial genera in freshwater aquaculture systems. The presence of these taxa was reported in all of the listed scientific literatures comprised of both tropical and temperate regions. The least detected cyanobacterial diversities in global aquaculture freshwater bodies were *Anabaenopsis* spp. and *Nostoc* spp., in which the presence was only documented in one and two out of 11 published articles, respectively. Cyanobacterial biomass of up to 2,060 µg chlorophyll-*a* per liter

(Suneerat, Wichien, Paveena, & Monthon, 2014) and 2.5 x 10^8 cells per liter (Chia et al., 2009), as well as microcystin concentration of up to 78 µg MC-LR per liter (Zimba et al., 2001) and 1,120 µg MC-LR per g dry weight (Mohamed et al., 2003) present potential microcystin contamination in aquaculture system which may pose significant health hazard to human through fish consumption.

05-4506832 pustaka.upsi.edu.my Perpustakaan Tuanku Bainun Kampus Sultan Abdul Jalil Shah PustakaTBainun ptbupsi

05-4506832 pustaka.upsi.edu.my Perpustakaan Tuanku Bainun Kampus Sultan Abdul Jalil Shah PustakaTBainun ptbupsi

Table 2.4

Occurrence of potentially toxic cyanobacteria and microcystins contamination in aquaculture systems

Country	Species	Cyanobacteria			References
		Genera	Biomass	Microcystin	
USA	*Ictalurus punctatus* (Catfish)	*Microcystis*	100.0 µg chl-*a* /L	78.0 µg/L	Zimba et al., (2001)
USA	*Ictalurus punctatus* (Catfish)	*Oscillatoria, Miscrocystis, Anabaena*	N/R	N/R	Schrader & Dennis (2005)
USA	*Litopenaeus vannamei* (White shrimp)	*Anabaena, Microcystis*	N/R	45.0 µg/L	Zimba et al. (2006)
Bangladesh	Mixed-culture	*Microcystis,*	1.3×10^5 cells /L	N/R	Jewel et al. (2003)
Australia	*Penaeus monodon* (Black tiger prawn)	*Oscillatoria, Microcystis*	4.0×10^6 cells /L	1.2 µg/g d.w.	Kankaanpää et al. (2005)
Brazil	*Oreochromis niloticus* (Nile tilapia)	*Microcystis, Planktothrix, Anabaena*	4.1 µg chl-*a* /L	49.8 µg/L	J. F. Vasconcelos et al. (2013)
Egypt	*Oreochromis niloticus* (Nile tilapia)	*Microcystis, Oscillatoria*	N/R	1,120.0 µg/g d.w.	Mohamed et al. (2003)
Malaysia	*Tor tambroides* (Empurau)	*Microcystis, Anabaena, Nostoc, Oscillatoria*	172.1 µg chl-*a* /L	N/R	Mohd. Nasarudin & Ruhana (2007)
Nigeria	Tilapia and African catfish (Species N/R)	*Microcystis, Nostoc, Anabaena, Planktothrix*	2.5×10^8 cells /L	5.9 µg/L	Chia et al. (2009)
China	*Ictalurus punctatus* (Catfish)	*Microcystis, Oscillatoria*	4.0×10^6 cells /L	N/R	Zhong et al. (2011)
Thailand	*Clarias macrocephalus* vs. *C. gariepinus* (Catfish)	*Microcystis, Oscillatoria, Anabaena, Anabaenopsis*	2,060.0 µg chl-*a* /L	N/D	Suneerat et. al, (2014)

Note. Cyanobacterial genera listed are only those producing microcystin; Cyanobacterial biomass and microcystin concentrations stated above are the maximum values quantified in the specified studies; Species referred to the farmed animals for aquaculture productions; N/R = Not reported; N/D = Not detected; d.w.= Dry weight

2.6.2 Microcystins Bioaccumulation in Fish Tissues in Freshwater Systems

Bioaccumulation of microcystins in fish, either in aquaculture systems or natural water bodies, poses health threat to human through fish consumption. This is due to the stability of the toxic compounds which can stand boiling at up to 300 °C (Wannemacher, 1989 as cited in Zhang, Xie, & Chen, 2010). To make it worse, boiling process is not only unhelpful, but was proven to increase the concentration of the detected microcystins from fish tissues (Zhang et al., 2010).

Although the target organ of microcystin is the liver, many studies found the presence of this hepatotoxin in the other parts of fish as well such as kidney, gut, intestine, gill, bile, viscera, gonad, blood, brain and muscle (Cazenave et al., 2005; Mohamed et al., 2003; Papadimitriou, Kagalou, Bacopoulos, & Leonardos, 2009; D. Stone & Bress, 2007; Xie et al., 2005; Zhang et al., 2009). This could be due to overwhelming or bypass of presystematic hepatic elimination in the affected fish during the exposure to microcystins (Mohamed et al., 2003; Papadimitriou et al., 2009). Presystematic hepatic elimination is a process that prevent, or at least minimise the distribution of foreign chemicals into the other parts of the body of an organism (Klaassen & Watkins, 1984).

Table 2.5 shows the summary of microcystins concentration detected in several species of fish collected from aquaculture ponds and natural water bodies significant for fishery as reported in previous literatures. Of all the studies, Ibelings et al. (2005) recorded the highest concentration of microcystin in the tissue of fish namely *Osmerus eperlanus* (874 µg/g) despite detected only a maximum of 10 µg/L

of the toxin in seston samples (intracellular microcystins) throughout their study period. This indicates that low concentration of microcystin in water bodies does not guarantee the safety of previously contaminated aquatic species. In tilapia fish tissues, microcystin concentrations ranged from safe level and up to 804 µg/g as documented by Vasconcelos et al. (2013).

Human in average take about 100 to 200 g of fish per serving (Mohamed et al., 2003). Although muscle is the commonly eaten part of fish, consuming organs or the whole fish is normal in some culture such as in China and Norway ("Offal," n.d.). In addition, accidental consumption of contaminated organs is also possible due to improper cleaning. Connecting this fact, it is important to take into consideration the amount of microcystins accumulated in the entire body of fish when analysing the potential health risk of microcystin to human.

To assess the risk of microcystin intake through fish consumption based on data compiled from previous studies (see Table 2.5), a man with an average Malaysian weight of 62.65 kg (Azmi et al., 2009) whom taking 100 g of the specified fish tissue is potentially exceeding the provisional TDI guideline by up to four orders of magnitude.

Table 2.5

Summary of microcystins bioaccumulation in fish tissues under field conditions and associated health risk to human

Species	Highest microcystin concentration			TDI (Times)	Country	References
	Ambient (µg/L)	Tissues (µg/g)				
Oreochromis niloticus (Nile tilapia)	1,120. µg/g d.w	821.0 ng/g	f.w	33	Egypt	Mohamed et al. (2003)
	N/R	1,479.2 ng/g	f.w	59	Uganda	Nyakairu et al. (2010)
	125.0 (water)	57.7 ng/g	w.w	2	Uganda	Poste et al. (2011)
	49.8	804.0	N/R	32,083	Brazil	J. F. Vasconcelos et al. (2013)
	0.6	~ 4.0 ng/g	w.w	Below	Brazil	Hauser-Davis et al. (2015)
Tilapia rendalli (Redbreast tilapia)	980.0	71.6	f.w	2,857	Brazil	Magalhaes et al. (2001)
Cyprinus carpio (Common carp)	1.4	8.7	d.w	347	China	Zhang et al. (2009)
	0.4	138.7 ng/g	d.w	6	Mexico	Berry et al. (2011)
	5.8	1,082.0 ng/g	d.w	43	Greece	Mitsoura et al. (2013)
	537.0 µg/g d.w	6.3 ng/g	f.w	Below	USA	Schmidt et al. (2013)
Cyprinus sp. (Carp)	37.0 (water)	280.0 ng/g	N/R	11	Portugal	V. M. Vasconcelos (1999)
Ictalurus punctatus (Catfish)	78.0	250.0	N/R	9,976	USA	Zimba et al. (2001)
Coregonus lavaretus L. (Whitefish)	6.5 x 10^{-3} µg/g d.w	35.0	d.w	1,397	Germany	Ernst et al. (2001)
Carassius auratus (Red cap oranda)	N/R	22.6	d.w	902	China	Xie et al. (2005)
	1.4	73.3 ng/g	d.w	3	China	Zhang et al. (2009)
Odontesthes bonariensis (Silverside)	41.6 µg/g d.w	1,010.0 ng/g	f.w	40	Argentina	Cazenave et al. (2005)
Osmerus eperlanus (Smelt)	10.0	874.0	afdw	34,876	Natherlands	Ibelings et al. (2005)
Gymnocephalus cernua (Ruffe)	10.0	194.0	afdw	7,741	Natherlands	Ibelings et al. (2005)
Perca fluviatilis (Perch)	10.0	51.0	afdw	2,035	Natherlands	Ibelings et al. (2005)
Perca flavescens (Yellow perch)	4.3	1,182.0 ng/g	d.w	47	Canada	Wilson et al. (2008)
Fish (Species N/R)	0.8	39.6 ng/g	w.w	2	Brazil	Magalhães et al. (2003)

Note. Ambient microcystin concentrations refer to the concentration of microcystins quantified from cyanobacterial seston (intracellular microcystins) during water sampling, unless stated otherwise; Microcystin concentrations in water bodies and tissue samples are presented in µg/L and µg/g, respectively, unless stated otherwise; "~" refers to estimated value; TDI (times) refer to the maximum potential risk of exceeding the TDI guideline (0.04 µg MC-LR /kg body weight per day) recommended by WHO when a man weighing 62.65 kg consumes 100 g of the specified tissue; N/R = Not reported; d.w.= Dry weight; f.w = Fresh weight; w.w = Wet weight; afdw = Ash free dry weight

2.6.3 Laboratory Exposure of Microcystins on Fish

Many experimental studies have been conducted to investigate the bioaccumulation of microcystins on different fish species upon exposure to certain concentrations of the toxic compound (see Table 2.6). Microcystin exposure methods include feeding of fish with toxic cyanobacterial biomass, immersion with cyanobacterial bloom or crude cyanobacterial extracts, and intraperitoneal (i.p.) injection. The first two approaches are actually the immitation of natural conditions occurred in water bodies (Malbrouck & Kestemont, 2006). Under aquatic environments, fish tend to ingest cyanobacteria cells either accidentally or purely as a source of food (Smith et al., 2008). Immersion of fish in water containing cyanobacterial bloom or crude cyanobacterial extracts mirrors the situation experiencing by the organisms during the active growth of cyanobacteria and the collapsed of cyanobacterial bloom, respectively.

As summarised in Table 2.6, the results of microcystin bioaccumulation reported in past literatures showed up to two orders of magnitude higher potential microcystin ingestion as compared to the recommended TDI level. Among the compiled studies, feeding of hybrid tilapia (*O. niloticus x O. aureus*) with 80 and 410 µg/g microcystin in the diet resulted in the highest maximum accumulation (~ 6,500 ng/g d.w) (Dong et al., 2009). In general, we can see that higher dosage of microcystins led to higher maximum toxic accumulation in fish tissues. Even so, some variations existed between species, method of exposure and duration of experiment. Growth inhibition due to toxic exposure to microcystin was reported by Li, Chung, Kim, and Lee (2004) and Dong et al. (2009).

Table 2.6

Summary of microcystins bioaccumulation in fish tissues under laboratory conditions and associated health risk to human

Species	Exposure design	Microcystin concentration			TDI (Times)	References
		Dosage (µg/L)	Tissue (ng/g)			
Oreochromis niloticus (Nile tilapia)	Fed with cyanobacterial biomass for 12 weeks	316.8 – 1,646.6 ng/fish/day	3,638.9	d.w	145	Zhao et al. (2006a)
	Fed with cyanobacterial biomass for 28 days	4.9, 19.5 µg/g diet	315.0	f.w	13	Ziková et al. (2010)
	Immersed in cyanobacterial bloom for 28 days	17.4 – 25.4	350.0	f.w	14	Palikova et al. (2011)
	Immersed in cyanobacterial bloom for 2 months	19.0 – 20.3	10.8	f.w	Below	Ruangrit et al. (2013)
Tilapia rendalli (Redbreast tilapia)	Fed with cyanobacterial biomass for 15, 28 and 42 days	14.6 – 29.2 µg/fish/day	1.7 µg/g	f.w	68	Soares et al. (2004)
O. niloticus x *O. aureus* (Hybrid tilapia)	Fed with cyanobacterial biomass for 60 days	80, 410 µg/g diet	~6,500.0	d.w	259	Dong et al. (2009)
Cyprinus carpio (Common carp)	Fed with cyanobacterial biomass for 28 days	5.0×10^{-2} µg/g b.w	369.3	f.w	15	Li et al. (2004)
	Immersed in cyanobacterial bloom for 9 weeks	14.2 – 22.7	110.7	f.w	4	Adamovský et al. (2007)
	Immersed in crude cyanobacterial extracts for 5 days	12.0	303.0	f.w	12	Sieroslawska et al. (2012)
Carassius auratus (Goldfish)	A single dose of intraperitoneal injection. Analysis after 8 hours	1.3×10^{-1} µg/g b.w	~0.3 µg/g	N/R	12	Malbrouck et al. (2003)

Note. Microcystin concentrations in the fish tissues stated above are the maximum values detected at the end of the experiment as described in exposure design; The dosage of microcystins introduced to the fish and microcystin concentrations quantified from the tissue samples are presented in µg/L and ng/g, respectively, unless stated otherwise; "~" refers to estimated value; TDI (times) refer to the maximum potential risk of exceeding the TDI guideline (0.04 µg MC-LR /kg body weight per day) recommended by WHO when a man weighing 62.65 kg consumes 100 g of the specified tissue; N/R = Not reported; d.w.= Dry weight; f.w = Fresh weight.

2.7 Aquaculture and Tilapia (*Oreochromis* spp.) Farming in Malaysia

Fishery industry, which comprised of marine capture, inland fishery and aquaculture have contributed to 1.1% or RM 7.91 billion to the total Gross Domestic Product (GDP) of Malaysia in 2013. Of this, about 15% was contributed by aquaculture sector (DoF Malaysia, 2013b). Aquaculture practise in Malaysia can be divided into two culture systems namely brackish water and freshwater. Tilapia is one of the most common fish cultured in freshwater systems in the country.

Tilapia farming in Malaysia has started from a humble beginning in 1952 with the introduction of *Oreochromis mossambicus*. Since then tilapia was regarded as poor man's food and grown only at small scales in rural areas (Mazuki, 2015). In 1980's, tilapia started to gain popularity amongst Malaysians when the red hybrid tilapia (*O. mossambicus albino* x *O. niloticus*) which was first produced in Taiwan, was introduced into the country (Gupta et al., 2004; Mazuki, 2015).

In Malaysia, many researches have been conducted to further improve the tilapia strains in terms of growth performance, survival rate, environmental tolerance and disease resistance. These include the development of genetically improved farmed tilapia (GIFT) strain through selective breeding by a collaboration between World Fish Center and the Department of Fishery Malaysia (WorldFish, 2015). Monosex culture technique was also adopted in commercialised fish farms to ensure optimum yields (Gupta et al., 2004). However, despite the advantages of GIFT strain, Red hybrid tilapia is still preferred in Malaysia (Hamzah, Nguyen, & Ponzoni, 2008; Mazuki, 2015) and accounted for approximately 80% of the total tilapia production in

the country (see Figure 2.8). Earth pond is so far the most common method used to cultivate the fish at both small and big scales. Of all the states in Malaysia, Perak became the largest producer of freshwater aquaculture products and have contributed to nearly 37% to the total freshwater harvest in the country (DoF Malaysia, 2013a).

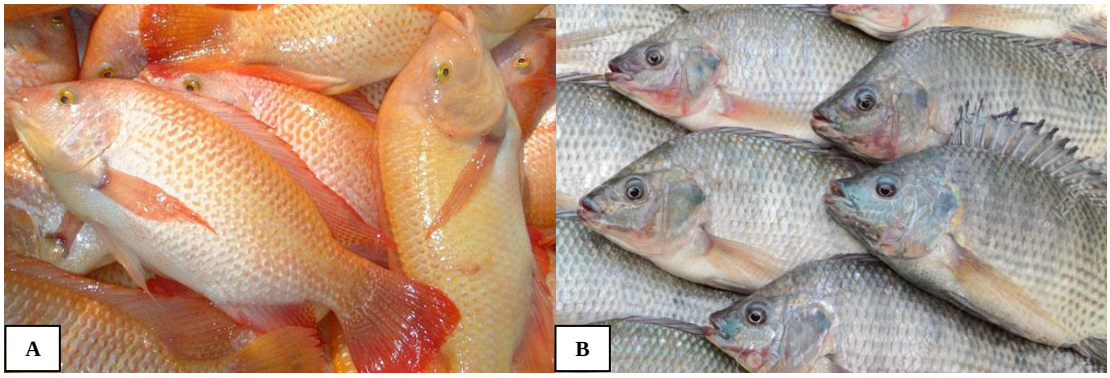

Figure 2.8. Commonly grown tilapia in Malaysia. (A) Red tilapia and (B) Black tilapia. (Sources of photos:https://ccresaquaponics.wordpress.com/2011/10/10/tilapia-fish-by-ccres-aquaponics/; http://www.123rf.com/photo_28073288_nile-tilapia.html)

Tilapia productions in Malaysia have increased over the years. Statistics from 2008 to 2013 showed an average annual increase of 4.31% in terms of metric tonnes and 16.67% in terms of market value. The six years data also indicated that tilapia alone have contributed to about 23.03 to 35.01% to the total freshwater aquaculture productions in Malaysia (see Table 2.7) (DoF Malaysia, 2008; 2009; 2010; 2011; 2012; 2013a). In 2013, tilapia accounted for the second largest freshwater farmed species (32.19%) in the country after catfish (keli) (38.03%) (see Figure 2.9). Tilapia is traded at various prices in different states of Malaysia. For example, the retail price of Red tilapia was between RM 7.00 – RM 13.00 per kg, whereas Black tilapia was sold between RM 2.00 – RM 10.00 in 2013 (DOF Malaysia, 2013).

Table 2.7

Tilapia productions in Malaysia from 2008 to 2013

Year	Production (MT)	Value (RM)	Tilapia (%)
2008	34,633.78	199,090.60	36.13
2009	35,148.11	274,708.68	23.03
2010	38,643.27	347,589.36	24.87
2011	42,786.23	409,430.77	35.01
2012	51,555.00	513,980.79	31.48
2013	42,774.18	430,444.49	32.19
Average annual growth rate (%)	4.31	16.67	N/A

Note. Average annual growth rate (%) = $[(\text{Data 2013} / \text{Data 2008})^{1/n} - 1] \times 100$, n = 2013 - 2008; Tilapia (%) indicates the % of tilapia productions in MT out of total aquaculture productions from freshwater culture systems; N/A = not applicable. (DoF Malaysia, 2008; 2009; 2010; 2011; 2012; 2013)

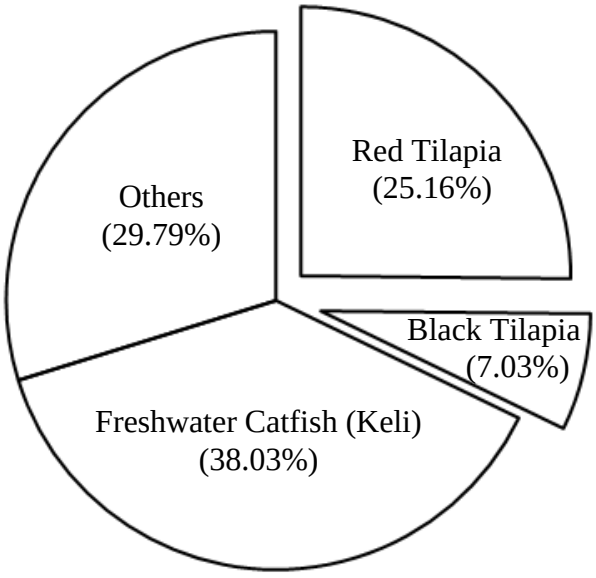

Figure 2.9. Proportions of aquaculture productions from freshwater culture system in Malaysia for year 2013. Others include feather black (belida), snakehead (haruan), jade perch, goby (ketutu), *Glossoma macropomum* (pacu), climbing perch (puyu), labeo rohita (rohu), snakeskin grouramy (sepat siam), freshwater sea bass (siakap air tawar), catfish (baung and tapah), giant snakehead (toman), giant freshwater prawn (udang galah), carps (jelawat, big head, grass, kelah, kerai, lampam jawa, lampam sungai, lee koh, mrigal, patin, sebarau, and terubul), and unstated species. (DoF Malaysia, 2013)

Malaysia is optimistic to achieve 1.76 million metric tonnes in total aquaculture productions by 2020 to meet both local and global demands ("Malaysia's aquaculture production target 'to hit 1.76 million metric tonnes in six years,'" 2014).

Of this, tilapia is expected to contribute a significant proportion to the productions due to high demands for the fish in United States and European countries (FAO, 2015). Food and Agriculture Organisation (2015) projected tilapia's market to continue to grow at a steady pace. This present a promising future for tilapia businesses across the globe, including in Malaysia. To be able to compete sustainably in this industry, food safety is extremely crucial to gain and preserve the trust of consumers. Unexpected event such as the occurrence of toxic cyanobacterial bloom in aquaculture ponds may not only lead to severe economic losses to fish farmers (Rodgers, 2008), but will also compromise with the safety (Poste et al., 2011) of the tilapia deliver to the public.

CHAPTER 3

METHODOLOGY

3.1 Location and Description of Study Ponds

Forty aquaculture ponds from a total of 10 locations in Perak, Malaysia were selected and sampled in this study. In more specific, the ponds are located in Behrang, Tapah, Temoh, Chenderiang and Air Kuning (see Figure 3.1 – 3.2). The Global Positioning System (GPS) coordinates of sampling locations are shown in Table 3.1. The aquaculture ponds comprised of earth ponds in use for fish production business which ranged from small, medium and up to big farms with about 100 ponds. Red tilapia (*Oreochromis* spp.) was found to be the most common fish selected for the business. Water source was obtained from the nearby natural water bodies.

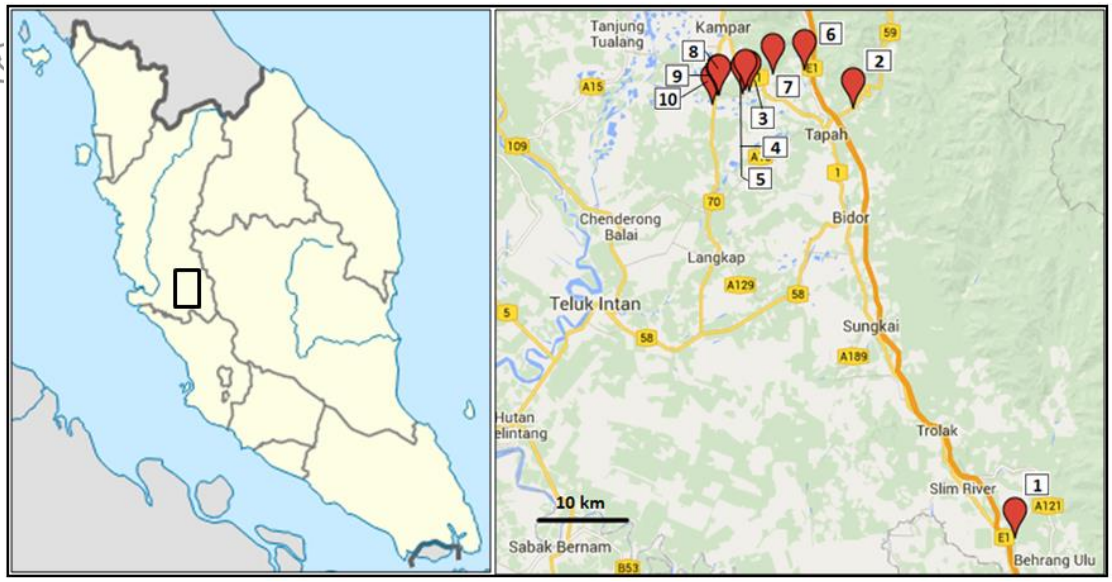

Figure 3.1. Map of 10 study locations around Perak, Malaysia. (1) Behrang; (2) Tapah; (3) Temoh I; (4) Temoh II; (5) Temoh III; (6) Chenderiang I; (7) Chenderiang II; (8) Air Kuning I; (9) Air Kuning II; and (10) Air Kuning III. (Bar = 10 km)

Table 3.1

Global Positioning System (GPS) coordinates of sampling location

Location	GPS Coordinate
1	3.783455, 101.458448
2	4.225602, 101.290220
3	4.244946, 101.177267
4	4.244775, 101.173662
5	4.245974, 101.182073
6	4.267876, 101.238398
7	4.263939, 101.205611
8	4.238325, 101.148491
9	4.238753, 101.149800
10	4.229358, 101.142354

Note. Sampling locations : (1) Behrang; (2) Tapah; (3) Temoh I; (4) Temoh II; (5) Temoh III; (6) Chenderiang I; (7) Chenderiang II; (8) Air Kuning I; (9) Air Kuning II; and (10) Air Kuning III.

Figure 3.2. Aquaculture ponds in Tapah

3.2 Water Sampling and Analysis

Sampling was carried out in each aquaculture system between December 2013 to March 2014. For each location, a total of four ponds were sampled randomly. Water temperature, DO and pH were measured on-site with a portable probe (YSI 550A) at a depth of 0.5 m from the water surface. Water sample was then grabbed from 0.15 m below the water surface to avoid scum. Approximately 2 ml of the grabbed water sample was placed in a cuvette to measure the instantaneous chlorophyll fluorescence (F_T) of cyanobacteria with AquaPen-C (AP 100 from Photon System Instrument), and were expressed in relative unit (r.u.). The remaining water was stored immediately into High-Density Polyethylene (HDPE) bottles, labelled accordingly and placed in containers containing ice. Water samples were brought back to laboratory for subsequent analysis; (i) nutrients analysis, (ii) chlorophyll-*a* extraction and quantification, and (iii) microcystin extraction and quantification (see Figure 3.3).

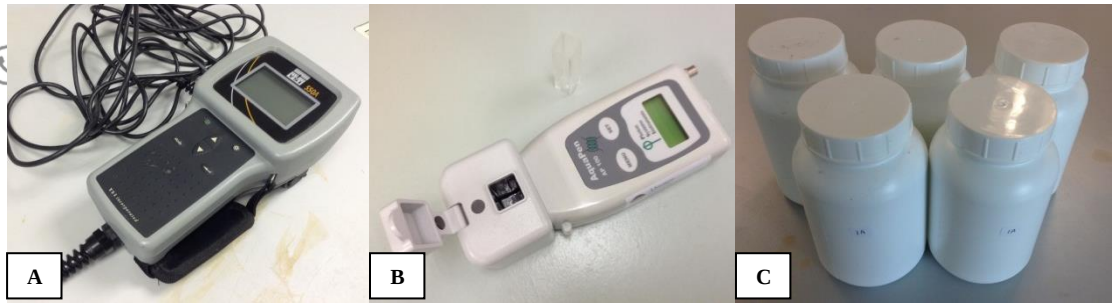

Figure 3.3. Water sampling equipments. (A) YSI 550A portable probe, (B) AquaPen-C AP-100 and (C) HDPE bottles

Phytoplankton samples were collected with a plankton net (mesh size:37µm). These samples were used to determine the presence of potentially toxic cyanobacterial diversity in the aquaculture systems through microscopic analysis. The phytoplanktons were rinsed off from the plankton net by using water from the same pond, stored in an amber bottle and preserved with 2 – 3 ml of Lugol's iodine per 100 ml of sample (see Figure 3.4). Lugol's iodine was prepared beforehand by dissolving 150 g of potassium iodide and 50 g of iodine with 980 ml of distilled water. When the solution was fully dissolved, 20 ml of glacial acetic acid was added into it (Bellinger & Sigee, 2010).

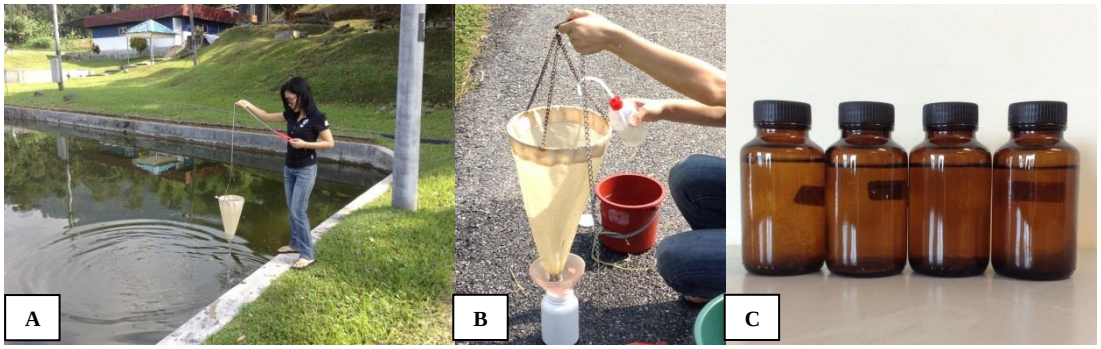

Figure 3.4. Phytoplanktons sampling. (A) Concentration of phytoplantons with plankton net, (B) Rinsing off the phytoplanktons from plankton net, and (C) Storage of phytoplanktons in amber bottles.

3.2.1 Microscopic Analysis of Cyanobacteria

The potentially toxic cyanobacterial diversity were identified to the taxonomic level of genera according to phytoplankton taxonomic guidelines (Komarek and Hauer, 2013) by using an inverted microscope (Nikon Eclipse TE 2000-U, software: NIS-Elements BR3.0) (see Figure 3.5). The water sample was gently mixed through agitation with hand prior to microscopic analysis. Then, 0.1 ml of the water sample was pipetted out with a micropipette, placed on a microscope slide and allowed for air dry. The cyanobacterial diversity was characterised based on morphological appearance under 40X magnification. Three slides were prepared for each sample. Photos were captured and combined for each sampling pond. The relative frequency, *f* of each cyanobacterial genera were then calculated according to equation modified from (Goettsch & Hernández, 2006) as stated below:

Relative frequency, *f* (%)

= (Number of ponds in which the genera occurs / Total sampled ponds) x 100

whereby,

Total sampled ponds : 40

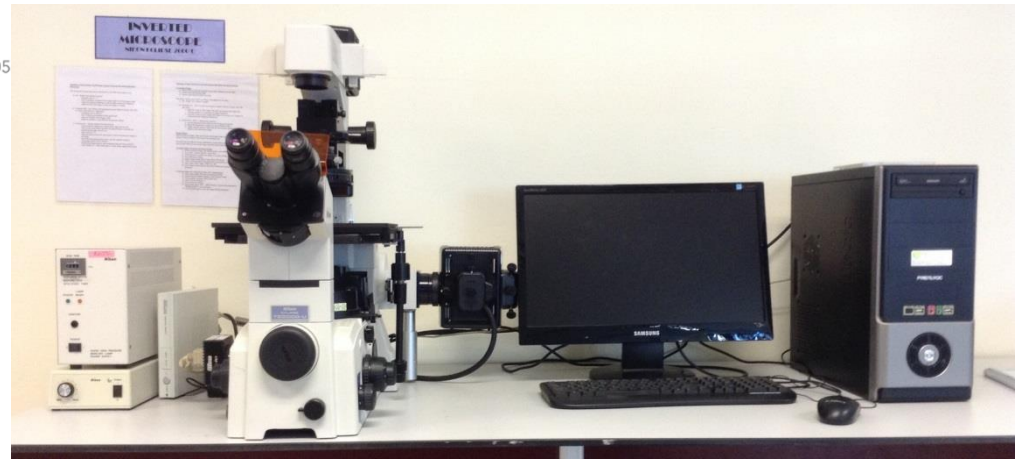

Figure 3.5. Inverted microscope

3.2.2 Nutrients Analysis

Nutrients were analysed with ion chromatography (DIONEX ICS-1100 RFIC, ASDV) (see Figure 3.6) within two days after the sample collection (Jackson, 2000). In this study, six types of nutrients which can be further classified into two groups were quantified: (i) **Anions:** nitrate (NO_3^-), phosphate (PO_4^{3-}), and nitrite (NO_2^-); and (ii) **Cations:** magnesium (Mg^{2+}), calcium (Ca^+), and ammonium (NH_4^+). The specifications for ion chromatography analysis are as shown in Table 3.2. Anions and cations for each sample were analysed separately.

Table 3.2

Specifications for nutrient analysis with ion chromatography

Ion	Anion	Cation
Column	RFIC IonPac AS23	RFIC IonPac CS12A
Suppressor	RFIC ASRS 300 4mm	RFIC CSRS 300 4mm
Mobile phase	9.0 ml 0.5M sodium carbonate + 1.6 ml 0.5M sodium bicarbonate in 1 L ultra-pure water	1.3 ml methanesulfonic acid in 1 L ultra-pure water
Current	100 mA	100 mA
Flow rate	1.0 ml/min	1.0 ml/min
Conductivity	18 – 22 µS	< 1 µS
Pressure	1800 - 2000	~ 1400
Duration/sample	30 minutes	15 minutes

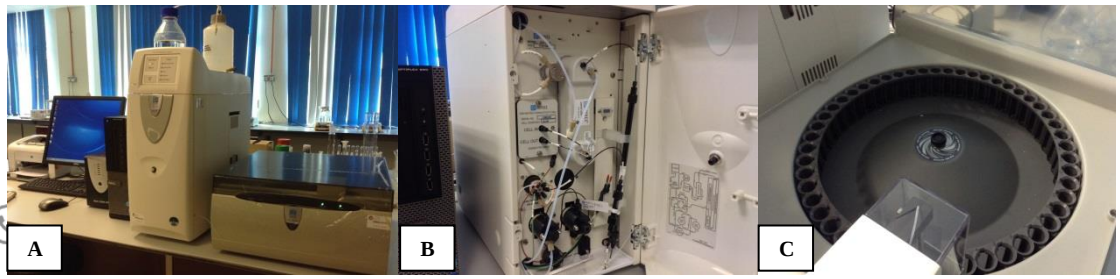

Figure 3.6. Nutrient analysis. (A) Ion chromatography system, (B) Interior part of ion chromatography machine whereby suppressor and column are placed, and (C) Autosampler.

Ion chromatography system needs to be flushed when changing the column and suppressor between anion and cation. System flushing was carried out with fresh ultra-pure water (Sartorius Stedim Biotech) for 15 minutes. After the flushing process, system priming was performed immediately with the desired mobile phases at 0.1 ml/min until there were no air bubbles present on the pump tubes. The pump flow rate was then gradually increased to 1.0 ml/min (see Table 3.2). Priming procedure was carried out each time before sample analysis in order to stabilise the chromatography instrument.

To start the analysis, 5 ml of fresh ultra-pure water was first injected for at least three times through autosampler. This procedure is essential to get a good chromatogram, as well as to detect any impurities that might be present in the ultra-pure water. Once the chromatogram gets stabilised, ion standards were run. In this study, the ion standards for both cation and anion were prepared and calibrated with four concentrations. The concentrations of ion standard used in this study are as shown in Table 3.3.

Table 3.3

Standard concentration

	Standard concentration (mg/L)			
Standard No.	1	2	3	4
(i) Anions				
- Nitrate	0.1	0.5	2.5	5.0
- Nitrite	0.1	0.5	2.5	5.0
- Phosphate	0.2	1.0	5.0	10.0
(ii) Cations				
- Magnesium	0.1	0.5	2.5	5.0
- Calcium	0.2	1.0	5.0	10.0
- Ammonium	0.1	0.5	2.5	5.0

Pre-filtered water samples were then injected into the ion chromatography system upon completion of the standard calibration (> 99.9% Coeff. Det.). To prepare the samples for ion chromatography analysis, HDPE bottles containing water sample were first shaken vigorously to ensure that the sample was well-mixed. By using a 15 ml syringe, the water sample was collected into the syringe and then filtered through 0.45 µm membrane filter into an ion chromatography vial (see Figure 3.7). The sample vials were then placed on the autosampler to queue for analysis.

Chromatogram analysis was carried out with Chromeleon Console version 7.1.0.898 software.

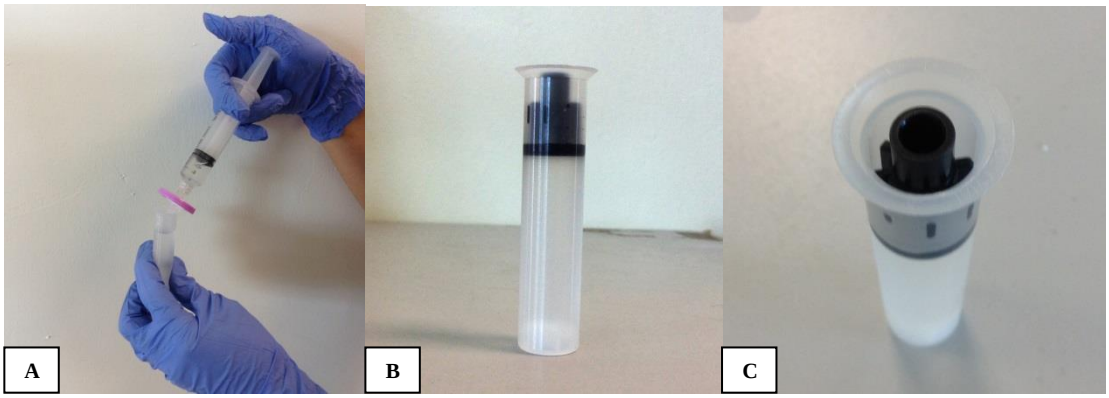

Figure 3.7. Sample preparation for ion chromatography analysis. (A) Filtration of the water sample with syringe filter, (B) Filtered water sample in a chromatography vial, and (C) View of a capped ion chromatography vial from the top.

3.2.3 Chlorophyll-*a* Extraction and Quantification

Chlorophyll-*a* was extracted and quantified according to chlorophyll-*a* Standard Methods (American Public Health Association [APHA], 1998). The analysis can be divided into three sections: (i) sample filtration, (ii) chlorophyll-*a* extraction, and (iii) chlorophyll-*a* quantification. This analysis was carried out under a subdued light in order to minimise the chlorophyll-*a* degradation (see Figure 3.8).

3.2.3.1 Sample Filtration

Water samples collected from aquaculture ponds were filtered through glass fiber filter paper (GF/C 47 mm Whatman Glass Microfibre filters paper). A filter paper was first placed on the filter holder by using a forceps. A desired volume of water sample was then poured into a measuring cylinder. In this study, water sample used was between 60 ml to 650 ml. Suction was applied to the filter flask and the filter paper was sealed onto the filter holder with a small amount of distilled water. Then, the measured water sample was poured gradually into the filter flask until water movement was observed to decelerate. The volume of water sample filtered was recorded.

The suction was continued for another three minutes to ensure complete filtration of water sample. By using a forceps, the filter paper was removed from the filter holder and placed onto a piece of aluminium foil. Then, the filter paper with algal cells was carefully folded into two, wrapped with aluminium foil and stored at - 20 °C. The filtrate from the filtration procedure was discarded.

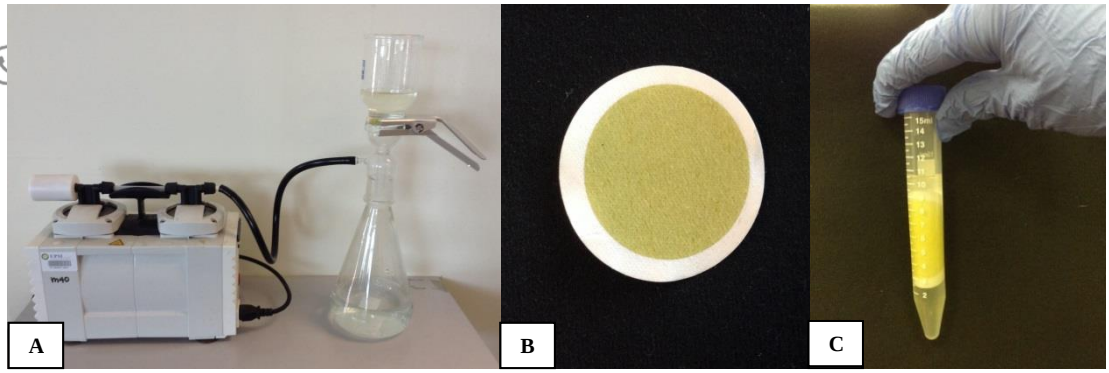

Figure 3.8. Chlorophyll-*a* extraction and quantification procedure. (A) Filtration of water sample on glass fiber filter paper with a filtration apparatus, (B) Filtered algal cell on a glass fiber filter paper, and (C) Extraction of chlorophyll-*a* with 90% v/v acetone.

3.2.3.2 Chlorophyll-*a* Extraction

Prior to chlorophyll-*a* extraction, freeze-thaw was carried out to physically lyse the algal cells. The frozen phytoplankton cells wrapped with aluminium foil (see section 3.2.3.1) were taken out of the freezer and allowed to thaw at room temperature for two hours. After two hours, the filter papers were placed back into the freezer (- 20 °C) to freeze for another two hours. This process was repeated for three times.

Upon completion of freeze-thaw cycle, the aluminium foil was unfolded, and the filter paper containing filtered algal cells was carefully grabbed with a forceps and placed into a 15 ml centrifuge tube. The filter paper was ensured to be as open as possible to allow effective extraction (see Figure 3.8c). After that, 10 ml of 90% v/v acetone was added into the centrifuge tube. The centrifuge tubes were then placed into a sonicator (Ultronics) containing cold water bath with ice and sonicated for 10

minutes to break the algal biomass. After sonication process, the samples were kept overnight in a refrigerator.

On the following day, the sample extracts were removed from the refrigerator and shaken well prior to centrifugation. Centrifugation process was carried out with a centrifuge machine (Sigma) for 5 minutes at 3,800 rpm to separate particulate materials from chlorophyll suspension. The sample extracts were allowed to settle down at room temperature before chlorophyll-*a* quantification.

3.2.3.3 Chlorophyll-*a* Quantification

Chlorophyll-*a* extracts were pipetted out from the centrifuge tube and transferred into a glass cuvette. The cuvette was then placed onto a spectrophotometer (PRIM 1835 by SECOMAM CE), and the absorbance of the sample extracts was measured at 750 nm ($E750_o$) and 665 nm ($E665_o$) against 90% v/v acetone blank. After that, 0.2 ml of 1% v/v hydrochloric acid (HCl) was added into the cuvette, mixed well and allowed to be still for two minutes. The absorbance of the sample extracts was measured again at 750 nm ($E750_a$) and 665 nm ($E665_a$) against 90% v/v acetone. Total chlorophyll-*a* was calculated with revised Lorenzen (1967) equation as stated below:

Chlorophyll-*a* (mg/m^3)

$$= 11.4 * K * [(E665_o - E750_o) - (E665_a - E750_a)] * [V_e / (L * V_f)]$$

whereby,

L : Cuvette light path in cm = 1 cm for a glass cuvette

V_e: Extraction volume in L
V_f: Filtered volume in m^3
R : Maximum absorbance ratio of $E665_o$ / $E665_a$ in the absence of pheopigments = 1.7
K : R / (R-1) = 2.43

The obtained chlorophyll-*a* value (mg/m) was then converted to µg/L.

$$1.0 \text{ mg/m}^3 = 1.0 \text{ µg/L}$$

3.2.4 Microcystin Extraction and Quantification

Intracellular microcystins were extracted and quantified according to Lawton, Edwards, and Codd (1994) method which consisted of four steps: (i) sample filtration, (ii) microcystin extraction, (iii) sample clean-up, and (iv) microcystin quantification. Water samples were filtered through 47 mm glass fibre filter paper as described in section 3.2.3.1 to collect the cyanobacteria intracellular cells. Then, the filter papers were folded into two, wrapped with aluminium foil and stored at - 20 °C. High performance liquid chromatography (HPLC) grade chemicals were used for the analysis.

3.2.4.1 Microcystin Extraction

Microcystin extraction was initiated with the freeze-thaw procedure for two times, each with three hours cycle as described in section 3.2.3.2. At the end of the freeze-thaw process, the samples were frozen back in a freezer at - 20 °C. Microcystin

extraction was carried out on the following day by first thawing the samples at room temperature.

Aluminium foil containing thawed sample was then unfolded, and the filter paper was grabbed with a forceps and placed into a 15 ml centrifuge tube. The intracellular cells on the filter papers were allowed to expose as much as possible as shown in Figure 3.8(c). Then, 5 ml of 75% (v/v) methanol was added into each of the centrifuge tubes and the samples were sonicated in cold water bath with ice for 25 minutes. After sonication process, the centrifuge tubes were placed on a horizontal shaker, and the samples were shaken for another 25 minutes. The filter papers were then taken half-out from the centrifuge tubes and squeezed with a spatula to remove the remaining extracts from the filter paper. After that, the squeezed filter paper was transferred into another centrifuge tube containing 5 ml of 75% (v/v) methanol and sonicated for the second time. The sample extract was subjected to centrifugation for 25 minutes at 3800 rpm under room temperature. Microcystin extraction procedures were repeated three times for each sample. Sample supernatants from each extraction round were then pooled into a 100 ml beaker. At the end of the microcystin extraction experiment, the final volume for each sample extract was about 13.5±1.0 ml. The pooled extracts were then diluted with ultra-pure water to reach 20% (v/v) methanol in the final concentration.

3.2.4.2 Sample Clean-Up

Sample extracts in 20% (v/v) methanol were purified with solid phase extraction (SPE) cartridge (Waters Sep-Pak Vac 6cc / 500 mg). Filtration apparatus was modified to act as the SPE manifold in this study (see Figure 3.9). SPE cartridge was first conditioned with 5 ml of 100% (v/v) methanol and followed by 5 ml of ultra-pure water at a loading speed of < 10 ml/min. After conditioning the SPE, the sample extract (see section 3.2.4.1) was applied into the cartridge drop by drop until finish. To get rid of the impurities that might be present in the sample extract, the cartridge was then rinsed off with 10 ml of 10%, 20%, and 30% (v/v) methanol. When the washing process completed, the air flow was continued for another four minutes to decrease the water elution in the cartridge. After four minutes, the pump was switched off, and 20 ml of a sample storage bottle was placed at the bottom of the SPE cartridge to collect the purified microcystin elution. At low vacuum, the cyanobacterial toxin was then eluted with 5 ml of 100% (v/v) methanol + 0.1% (v/v) trifluoroacetic acid (TFA).

The microcystin elution was now subjected to drying process by using a rotary evaporator at 40 °C. When the sample has completely evaporated, resuspension was carried out with 360 µl of 100% (v/v) acetonitrile until the hepatotoxic compound was fully dissolved. After that, 840 µl of ultra-pure water was added into the suspension. The suspension was then transferred into an HPLC vial and stored in a freezer at - 20 °C.

Figure 3.9. Purification of sample extracts. (A) Modified filtration apparatus as SPE manifold and (B) SPE cartridge.

3.2.4.3 Microcystin Quantification

Intracellular microcystins were quantified by using HPLC with photodiode array (PDA) detector (Agilent Technologies 1260 infinity) and an Atlantis T3 3 µm column (Waters). Mobile phases used were 100% (v/v) acetonitrile + 0.05% (v/v) TFA and ultra-pure water + 0.05% (v/v) TFA. Microcystin peaks were separated using linear gradient method (see Table 3.4) for a duration of 37 minutes per sample injection, and the column temperature was maintained at 37.5±2.5 °C throughout the analysis. The spectra were acquired between 200 nm to 300 nm and recorded at 238 nm. Microcystin peaks were identified based on the characteristics of UV spectra according to Lawton et al. (1994), Chorus and Bartram (1999), Meriluoto and Codd (2005), Purdie et al. (2009), and Indabawa (2010). Commercially available microcystin-LR standard (Abraxis, USA) was used to quantify the microcystin concentration.

Table 3.4

Microcystin separation with HPLC

Time (min)	Flow rate (ml/min)	Mobile Phases	
		Acetonitrile (%)	Ultra-pure water (%)
-	0.6	30	70
5	0.6	35	65
28	0.6	100	0
31	0.6	100	0
32	0.6	30	70
37	0.6	30	70

Instrument setting and method were captured into HPLC software (Chemstation version 2011). The purified microcystin samples (see section 3.2.4.2) were then placed onto HPLC autosampler, and the sample name, as well as injection location were keyed-in into the sequence table. The sequence was run only when the HPLC machine is stable, which was indicated by a straight blue colour baseline at 0 mAU. Machine stabilisation could take up to 30 minutes.

Chromatograms generated from microcystin analysis were integrated manually from valley to valley, and the peak spectrums were then analysed. The area of all peaks with microcystin UV-spectra characteristics per sample were total up and compared against MC-LR standard. In this study, microcystin concentration was expressed in µg (microcystin-LR equivalent) per L of water.

3.3 Bioaccumulation of Microcystin in Red Tilapia (*Oreochromis* spp.) Tissue

Microcystin is able to accumulate in fish tissues and pose health risk to human through fish consumption (Peng et al., 2010; Poste et al., 2011). This study was conducted to determine the bioaccumulation of microcystin in *Oreochromis* spp. tissues after seven days of exposure to the toxic microcystin. Schliewen (2008) recommended to perform regular partial water changes on a weekly basis to maintain the water quality of fish aquarium. Four times of pre-trial experiment conducted in this study showed the presence of ammonia in the culture water after 7 days should there was no water changes performed. Hence, to avoid the accumulation of harmful substances other than microcystin in the fish tank and the reduction of microcystin concentration due to water changes procedure, the experiment was only carried out for seven days. Furthermore, Zíková et al. (2010) reported accumulation of microcystins in fish tissues even after a day of exposure to the toxic metabolites. Natural bloom extract containing *Microcystis* spp. was selected to conduct this experiment in order to mimic the natural conditions.

3.3.1 Preparation of Crude Cyanobacterial Extracts

Crude cyanobacterial extracts were prepared according to Ghazali et al. (2009) methods which composed of three steps: (i) cyanobacterial bloom sampling and lyophilisation, (ii) microcystins detection and quantification, and (iii) preparation of crude cyanobacterial extracts. In this study, crude cyanobacteria were collected from Aquaculture Development Centre, Tapah.

3.3.1.1 Crude Cyanobacteria Sampling and Lyophilisation

The presence of green scums on water surface which commonly indicates the occurrence of cyanobacterial bloom was observed on selected aquaculture ponds in Tapah. Water samples were then collected from the ponds suspected to be under bloom and observed under light microscope to determine the presence of *Microcystis* spp. Green scums from fish ponds confirmed to have *Microcystis* spp. were afterward collected and concentrated with a plankton net (mesh size: 37 µm). Water from the same pond was used to rinse the scums from the plankton net into an HDPE bottle. This process was repeated several times until the HDPE bottle was fully filled. Cyanobacteria scums were collected as much as possible. The bottles with scums were then placed in a cooler container to maintain the freshness.

Upon reaching the laboratory, the scum samples were poured into plastic container until half-filled and froze in a freezer at - 20 °C. Freeze dryer was used to lyophilise the natural bloom sample at - 110 °C until completely dry (see Figure 3.10). The lyophilised crude cyanobacteria cells were then mixed in a container and stored in a freezer at - 20 °C.

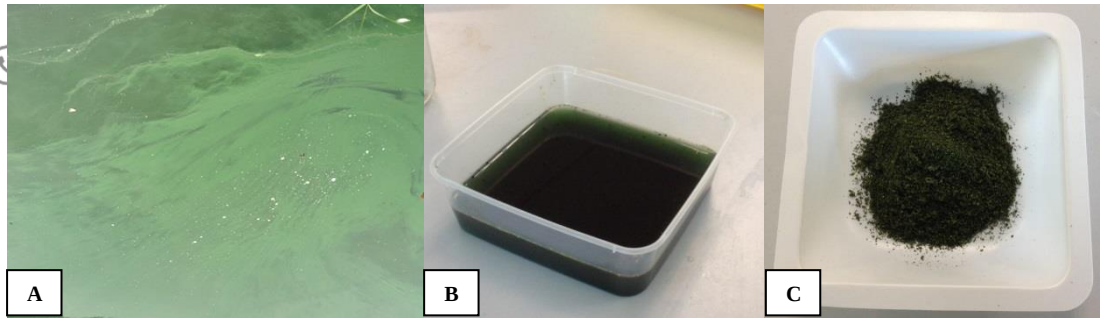

Figure 3.10. Crude cyanobacteria sampling and lyophilisation process. (A) Cyanobacterial scums on aquaculture water surface, (B) Cyanobacterial scums in a plastic container ready for freeze-dry, and (C) Lyophilised cyanobacteria cells.

3.3.1.2 Microcystin Detection and Quantification

Lyophilised cyanobacteria cells were taken out of the freezer and allowed to cool down at room temperature. The crude cells were then mixed evenly to ensure the uniformity. Then, 250 mg of the crude cells was weighed and poured into a 15 ml centrifuge tube. Three replicates were prepared. About 1.25 ml of ultra-pure water was added into each of the centrifuge tubes and froze overnight at - 20 °C. On the following day, the samples were taken out from freezer and freeze-thaw cycle was carried out as described in section 3.2.4.1 prior to extraction. Microcystin extraction from the lyophilised cells was conducted on the day after the freeze-thaw process.

Microcystins were extracted from the crude cyanobacteria cells for three times with 5 ml of 75% (v/v) methanol. At the beginning of the extraction, 3.75 ml of 100% (v/v) methanol was added into each of the centrifuge tubes with thawed samples. This was due to the addition of 1.25 ml of ultra-pure water beforehand for the freeze-thaw procedure which contributed to 25% of the solution volume. The samples were then

sonicated in cold water bath with ice for 25 minutes. After sonication, the samples were subjected to centrifugation for another 25 minutes at 3,800 rpm. The supernatants were then pipetted out with a micropipette and stored in storage tubes.

The crude cells were further extracted with an addition of 5 ml 75% (v/v) methanol into the centrifuge tubes. Cell pellets that settled down at the bottom of the centrifuge tubes were broken down with micropipette tips and manually agitated with hand prior to sonication. Supernatants from each replicate were stored in their respective storage tubes. The bloom extracts were then diluted with ultra-pure water to reach 20% (v/v) methanol in the final concentration prior to SPE clean-up. Sample purification and quantification of microcystin were conducted as described in section 3.2.4.2 and section 3.2.4.3. Microcystins concentration in this experiment is the average of three quantified replicates and was expressed in µg (microcystin-LR equivalent) per g of cell.

3.3.1.3 Preparation of Crude Cyanobacterial Extract

Microcystins were extracted from the lyophilised cyanobacteria cells (see section 3.3.1.1) according to Ghazali et al. (2009) method with some modification on the methanol concentration and volume. In this study, 75% methanol was used to extract microcystins from the cyanobacteria cells instead of 50% methanol in order to standardise the solvent concentration used throughout the experiment. The selected methanol extraction volume of 24 ml on the other hand was derived from the permitted 0.1% methanol in the culture tanks.

Cyanobacterial bloom extracts with the concentration of 17.96, 40.66 and 78.32 µg microcystin-LR equivalent /L were prepared. The microcystins concentration were taken from the first quartile (Q1), second quartile / median (Q2) and the third quartile (Q3) of the naturally occurring microcystins during sampling period from a total of 40 aquaculture ponds in Perak, Malaysia (see section 3.2.4.3). To prepare the extracts, 207.95 mg, 470.84 mg, and 906.94 mg of cyanobacteria cells with known concentration of microcystins (1,554.47 µg MC-LR equivalent /g) (see section 3.3.1.2) were weighed and transferred into 50 ml centrifuge tubes, each with three replicates. Microcystins extraction were then carried out with 24 ml of 75% (v/v) methanol for 10 minutes in ice. After that, the suspension were subjected to centrifugation at 23,000 rpm for another 10 minutes at 4 °C.

Supernatants produced were filtered using 0.2 µm sterile syringe filter to exclude the cell debris. The filtered extracts were then pooled into three amber glass bottles. Each bottle was labelled with the microcystins concentration and stored in a fridge at 4 °C (see Figure 3.11). At this stage, each bottle would have about 72 ml of cyanobacteria extracts or less due to evaporation of methanol.

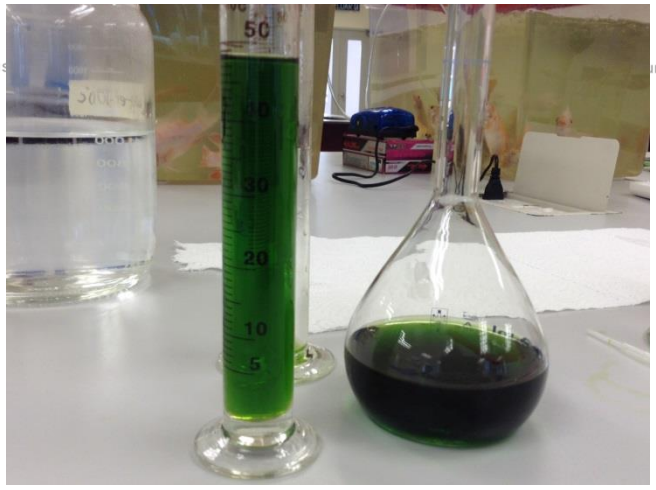

Figure 3.11. Filtered crude cyanobacterial extracts

3.3.2 Acclimatisation of *Oreochromis* spp. in the Laboratory

Red tilapia were acclimatised prior to the experiment. In this study, fish acclimatisation was conducted according to Ibrahem, Khairy, and Ibrahim (2012) and Jos et al. (2005) methods. *Oreochromis* spp. fingerlings (mean weight: < 10 g) were obtained from a fish hatchery in Chenderiang, Perak. The acclimatisation was carried out in plastic aquariums (top: 59 cm x 30 cm; bottom: 45 cm x 26 cm; h: 32 cm) with 40 L of conditioned tap water. The fish aquariums were set up with continuous aeration and water filtration system. Tanks with conditioned water were left for three days with the aeration on before introducing the fish. Seven tanks were prepared for the acclimatisation process, each with 40 L of water. A total of 10 Red tilapias were then introduced into each of the tank. Acclimatisation was carried out for 14 days at room temperature (25±2 °C) with eight hours light. Water quality was monitored daily and maintained at the optimum level according to Mjoun, Rosentrater, and Brown (2010) (see Table 3.5). The fishes were fed twice a day with commercialised

fish pellet (see Table 3.6) and starved for 24 hours before the experiment to empty the gut of the fish (Dong et al., 2009).

Table 3.5

Limits and optimum range of water quality parameters for tilapia

Parameter	Range	Optimum	Device
Salinity, ppt	Up to 36	Up to 19	
Dissolved oxygen, mg/L	Down to 0.1	> 3	YSI portable probe
Temperature, ^{o}C	8 - 42	22 - 29	
pH	3.7 - 11	7 - 9	
Ammonia, mg/L	Up to 7.1	< 0.05	Ammonia test kit

Table 3.6

Proximate composition of the experimental diet

Nutrient	Percentage in Feed (%)
Crude protein	≥ 32
Moisture	≤ 10
Crude fat	≥ 5
Crude fibre	≤ 5
Crude ash	≤ 10

3.3.3 *Oreochromis* spp. Culture

This experiment aimed to investigate the bioaccumulation of microcystin in *Oreochromis* spp. tissues after seven days of immersion exposure. Twelve plastic aquariums (top: 59 cm x 30 cm; bottom: 45 cm x 26 cm; h: 32 cm) were prepared with 18 L of conditioned tap water and labelled with the name of treatment. Aquarium air pumps and bubble stones were used to provide aeration to the fishes. The experiment was carried out after three days of water preparation.

Crude cyanobacterial extracts (see section 3.3.1.3) were taken out from the chiller and measured with measuring cylinder. As mentioned previously, the final volume for each extract concentration should be 72 ml. However, the actual volume was lower due to evaporation of alcohol. Hence, 75% (v/v) methanol was used to top up the cyanobacterial extracts to 72 ml, and the toxin was then mixed well. Every concentration of microcystin extracts was afterward divided into three portions, each with 24 ml of cyanobacterial toxin. Then, the measured extracts were poured into the culture tanks. For control tanks, 24 ml of 75% (v/v) methanol was added into each of the aquarium. All of the culture tanks were now having 0.1% (v/v) methanol in 18 L of water.

Each fish tank was then introduced with three fingerlings of Red tilapia. The fishes were weighed before being introduced into the tanks to get the initial wet weight. The experiment was carried out at room temperature (25±2 °C) for eight hours photoperiod per day. Water parameters were monitored daily and maintained within the desired ranges (see Table 3.5). The fishes were fed three times a day at 2% wet body weight per day with commercialised fish pellet with a minimum of 32% protein (see Table 3.6). Unconsumed pellets after 5 minutes of feeding were immediately removed from the fish tank to avoid ammonia build-up (Hargrove & Hargrove, 2011). Fish mortality was observed throughout the culture period and recorded. The dead fish was then weighed, kept in labelled plastic containers and froze at - 20 °C.

The Red tilapias were starved for 24 hours before the final day of experiment to completely clear the fish gut. Fish fingerlings that were still alive were weighed

again to get the final weight and placed in labelled plastic containers. The fishes from the same aquarium were pooled together and each group was treated as one sample in latter analysis. This includes the fish that died during the experiment. The containers were then sealed and froze at - 20 °C.

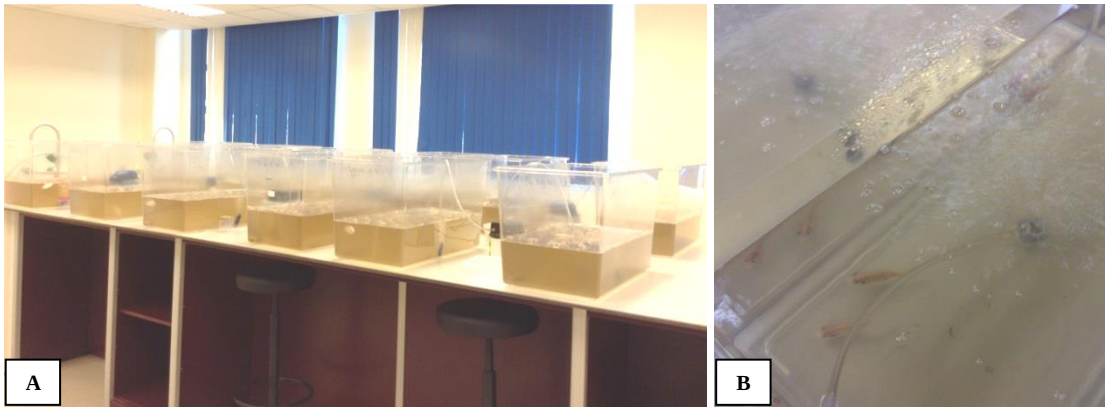

Figure 3.12. Oreochromis spp. culture. (A) Fish tanks during experiment and (B) View of a fish tank during experiment from the top.

The survival and growth parameters of the cultured fish were then calculated with equations as described below:

Survival (%)

= (Total number of fish survived on the 7^{th} day of experiment / 9) x 100

whereby,

9 : Total number of fish introduced on the first day of experiment for each treatment

Growth (%)

= [(Total final weight – Total initial weight) / Total inititial weight] x 100

3.3.4 Microcystin Analysis on *Oreochromis* spp. Tissues

Microcystin was extracted from the entire body of fish according to modified Nyakairu et al. (2010) method. Frozen fish samples (see section 3.3.3) were lyophilised with freeze dryer at - 110 °C for three days. The dried tissues were then homogenised with a dry mill (see Figure 3.13). To start the extraction, 0.5 g of lyophilised tilapia tissues were weighed and poured into 15 ml centrifuge tubes. After that, 2.5 ml of ultra-pure water was added into each of the tubes and froze at - 20 °C overnight. On the following day, freeze-thaw was carried out on the samples as described in section 3.2.4.1. After the freeze-thaw process, the fish tissues were frozen at - 20 °C for another night.

Figure 3.13. Oreochromis spp. tissues. (A) Fresh tissue, (B) Lyophilised tissue, and (C) Homogenised tissue.

Microcystins extraction from fish tissues were conducted three times with 75% (v/v) methanol as described in section 3.3.1.2 with some modifications. A total of 7.5 ml of 100% (v/v) methanol was added during the first extraction while, for the subsequent extractions, 10 ml of 75% (v/v) methanol was used. Microcystin extracts for each sample were pooled together in 50 ml centrifuge tube. At the end of the

extraction process, every sample would have about 25 to 28 ml of microcystin extracts.

Hexane was then added into the centrifuge tubes containing sample extracts in the ratio of 1:1 (hexane:75% methanol) and subjected to centrifugation for 10 minutes at 1,000 rpm. Centrifugation process led to the development of three distinct layers: (i) hexane layer (top), (ii) lipid layer (middle), and (iii) microcystin extract layer (bottom) (see Figure 3.14). The top and middle layers were gently discarded by pipetting with a micropipette. Then, another equal volume of hexane was added again into the centrifuge tubes. The delipidation process was repeated for three times per sample.

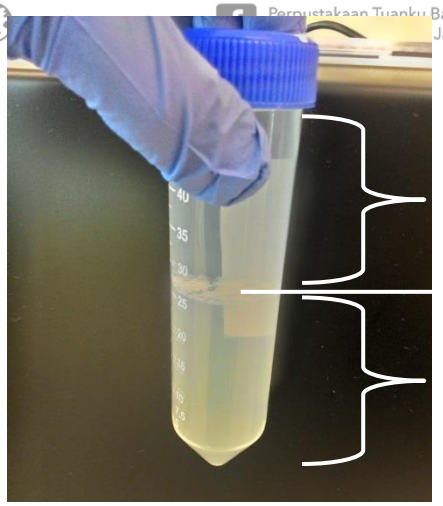

Figure 3.14. Delipidation of *Oreochromis* sp. tissue extracts

After removal of lipids from the organic samples, the fish extracts were diluted with ultra-pure water to 20% (v/v) methanol in final concentration. Sample clean-up and microcystin quantification were then carried out as described in section

3.2.4.2 and section 3.2.4.3. Microcystin concentration was expressed in µg (microcystin-LR equivalent) per g of tissue.

3.4 Statistical Analysis of Data

Data collected were analysed with IBM SPSS Statistics version 20. To meet the assumption of normality, data were first log-transformed should necessary. Bivariate correlation with Pearson's test was used to (i) validate the relationship between chlorophyll-*a* with instantaneous chlorophyll fluorescence of cyanobacteria, (ii) analyse the correlation between cyanobacterial biomass with intracellular microcystin concentration, and (iii) identify the relationships between temperature, DO, pH, and nutrients with both cyanobacterial biomass and microcystin concentration.

Multiple linear regression with forward selection on the other hand was used to identify the environmental variable that could best explain the variability of cyanobacterial biomass and microcystin concentration in Perak aquaculture systems. One-way ANOVA with Tukey's HSD post-hoc test was used to compare the means and to find the statistical difference of instantaneous chlorophyll fluorescence of cyanobacteria, cyanobacterial biomass, and microcystins concentration between sampled locations, as well as microcystins concentration extracted from Red tilapia tissues and fish growth between treatments. The survival distribution of the cultured fish were analysed with Kaplan-Meier method and the equality between treatments was compared with Log-Rank (Mentel-Cox) test.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Presence of Potentially Toxic Cyanobacteria in Perak Aquaculture Systems

The presence of cyanobacteria in Perak aquaculture systems were detected on-site with a portable fluorometer (AquaPen-C) and analysed microscopically in the laboratory with a light microscope (inverted microscope). Results for this part of study are as documented in section 4.1.1 and section 4.1.2.

4.1.1 On-Site Detection of Cyanobacteria

AquaPen-C (AP 100) was used for on-site detection of cyanobacteria in this study. This fluorometer measures the instantaneous chlorophyll fluorescence (F_T) of

cyanobacteria through excitation of phycocyanin pigment at 620 nm (red-orange excitation light). Phycocyanin fluorescence method has long been used to detect the presence of cyanobacteria in water column as it provides rapid and real-time status of cyanobacteria (Asai et al., 2001; Campbell, Hurry, Clarke, Gustafsson, & Oquist, 1998; Gregor, Maršálek, & Šípková, 2007). This makes it a useful tool to provide an early warning to the occurrence of potentially toxic cyanobacteria in water bodies (Gregor et al., 2007).

The presence of cyanobacteria was detected in all of the selected aquaculture ponds during water sampling and cyanobacterial fluorescence varied significantly between locations, $F(9,30) = 17.188$, $p < .001$. The highest average cyanobacterial fluorescence was recorded in location 6 (2084.50 ± 2201.76 r.u.), followed by location 9 (2077.50 ± 1160.34 r.u.), and location 5 (1741.13 ± 1418.95 r.u.). Significantly lower cyanobacterial fluorescences on the other hand were quantified in location 10 (39.35 ± 67.63 r.u.) and location 7 (13.00 ± 2.33 r.u.) (see Figure 4.1). Although the ranges of F_T measured in this study were quite high which was between 2.67 r.u. to 5,308.00, 72.5% of the ponds were detected having F_T of less than 1,500.00 r.u. The result indicated spatial variability of cyanobacterial intensity in aquaculture systems which was also described by Rahman and Jewel (2008). Gregor et al. (2007) monitored the cyanobacterial fluorescence in raw water samples collected from a water treatment plant in Czech Highland. Although the study measured phycocyanin fluorescence of up to over 12,000 r.u. during the study period at 10 m depth, the fluorescence readings were observed to be more concentrated at below 4,000 r.u. (Gregor et al., 2007).

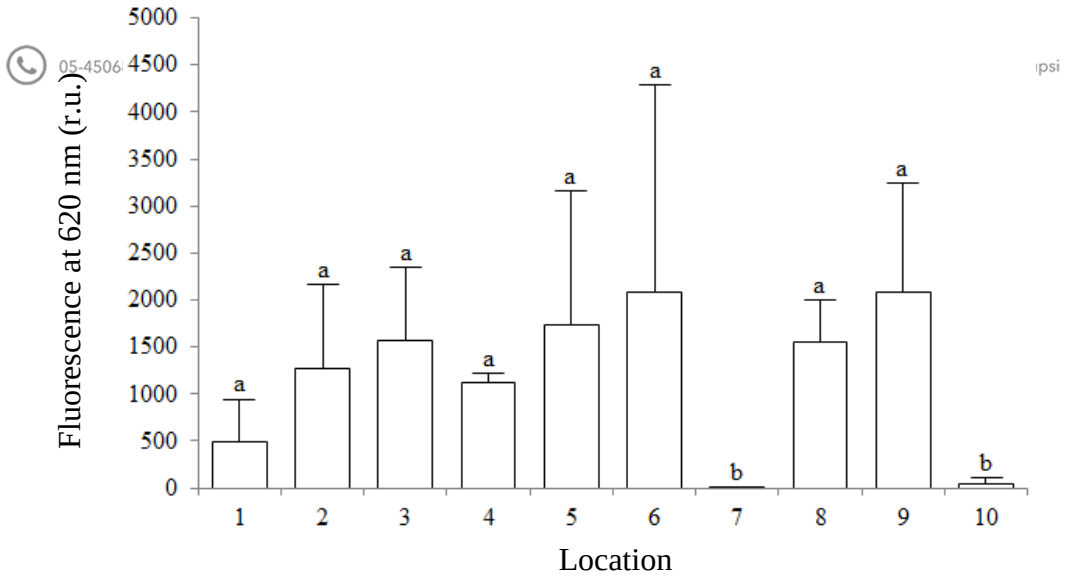

Figure 4.1. Instantaneous chlorophyll fluorescence (F_T) of cyanobacteria at 620 nm in 10 selected study locations around Perak, Malaysia during sampling period. (1) Behrang; (2) Tapah; (3) Temoh I; (4) Temoh II; (5) Temoh III; (6) Chenderiang I; (7) Chenderiang II; (8) Air Kuning I; (9) Air Kuning II; and (10) Air Kuning III. Data were expressed as the mean value and standard deviations of four sampled ponds. Locations with common letters were not statistically significant different ($p > .05$).

4.1.2 Microscopic Analysis of Cyanobacteria

Cyanobacteria were distinguished between genera based on their morphological appearance. Figure 4.2 to Figure 4.5 showed the photos of potentially toxic cyanobacteria captured under 40X magnification. Identification of cyanobacteria to the genera level is adequate for preliminary assessment of potential hazard of this noxious bacteria in water systems (Lawton, Marsalek, Padisák, & Chorus, 1999). In this study, *Microcystis* spp. was observed in the form of irregularly arranged cells to densely packed colonies. *Anabaena* spp. was detected in coiled, tangled and spiral structures. The presence of heterocyst and akinete on the filaments were hardly recognised, while the trichomes were noticeable in some photos captured. *Oscillatoria*

spp. was observed to appear solitary or in groups with straight filaments. *Nostoc* spp. showed the presence of false branching on their filaments which made this genus easily distinguishable. Trichomes can be seen in some photo, however, the presence of heterocyst and akinete on the filaments were unrecognisable.

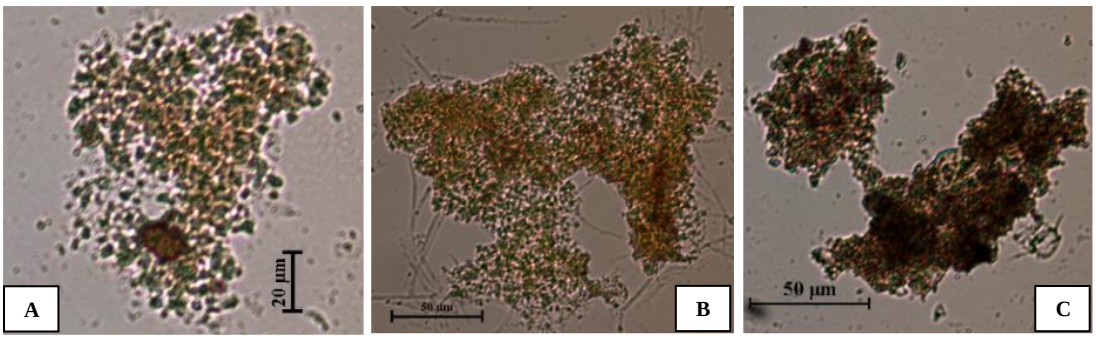

Figure 4.2. Microcystis spp. (a) Irregularly arranged cells forming a colony, (b) Few colonies clustered together, and (c) Irregular mucilaginous colonies with densely arranged cells.

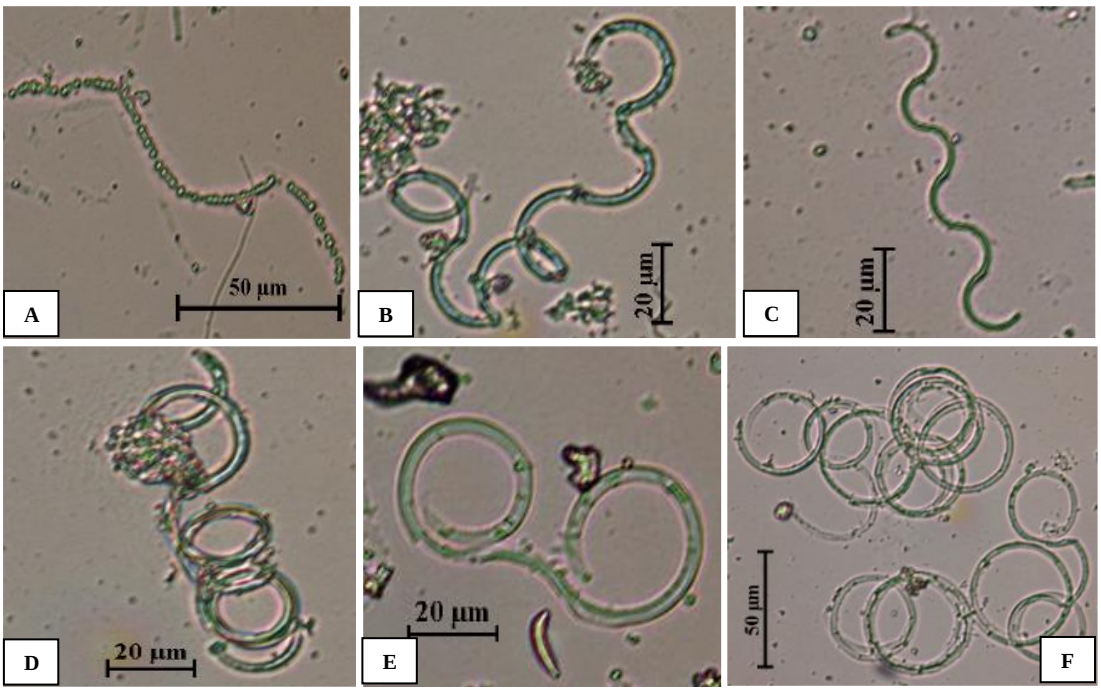

Figure 4.3. Anabaena spp. (a) Irregularly coiled filament with visible trichomes, (b) Irregularly coiled filament and almost spiral, (c) Regularly coiled filament,

(d) Spirally-coiled, screw-like filament, (e) Solitary arcuated filament, and (f) arcuated filament in tangled.

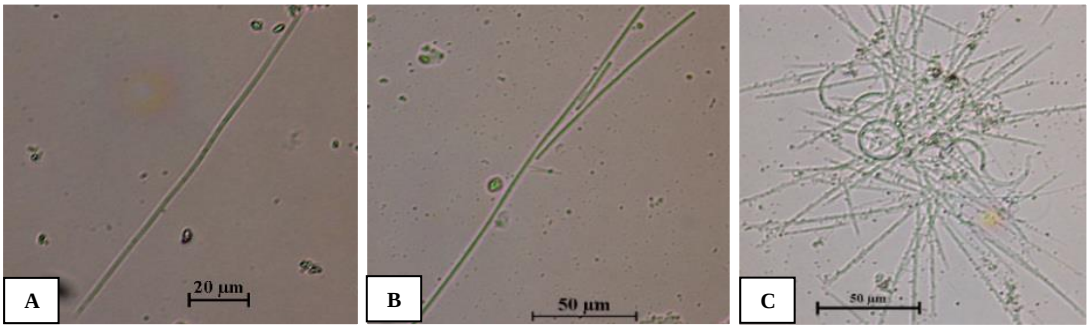

Figure 4.4. Oscillatoria spp. (a) Solitary, fine, thin and sheathless straight filament, (b) Filaments forming a small group, and (c) Filaments forming disintegrating fascicles.

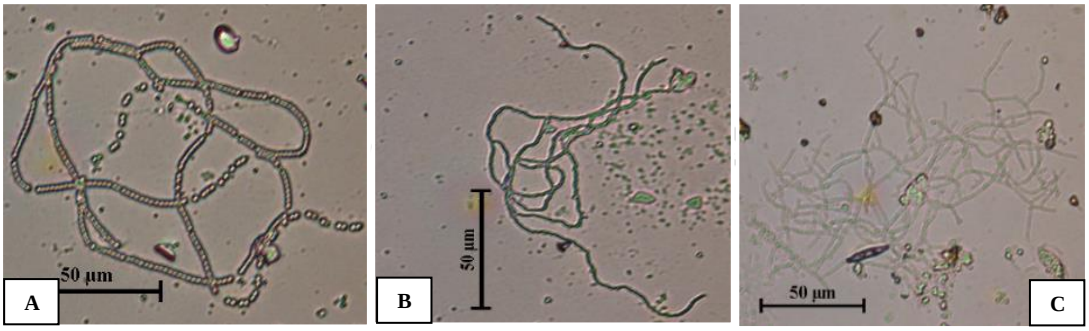

Figure 4.5. Nostoc spp. (a) False-branched filament with visible trichomes, and (b-c) False-branched and densely agglomerated filaments.

Microscopic analysis of water samples found the presence of *Microcystis* spp., *Anabaena* spp., and *Oscillatoria* spp. in all of the selected study locations in Perak, Malaysia during the sampling periods. *Nostoc* spp. was found to be the least detected in Perak aquaculture systems as this taxa was only occurred in four out of 10 sampled locations (see Table 4.1).

Table 4.1

Presence of potentially toxic cyanobacteria in selected study locations in Perak, Malaysia

Cyanobacterial genera	Location									
	1	2	3	4	5	6	7	8	9	10
Microcystis spp.	+	+	+	+	+	+	+	+	+	+
Anabaena spp.	+	+	+	+	+	+	+	+	+	+
Oscillatoria spp.	+	+	+	+	+	+	+	+	+	+
Nostoc spp.	+	+	-	+	+	-	-	-	-	-

Note. Sampling locations : (1) Behrang; (2) Tapah; (3) Temoh I; (4) Temoh II; (5) Temoh III; (6) Chenderiang I; (7) Chenderiang II; (8) Air Kuning I; (9) Air Kuning II; and (10) Air Kuning III. "+" = present; "-" = absent

The occurrence of *Microcystis* spp., *Anabaena* spp., *Oscillatoria* spp., and *Nostoc* spp. have been reported in freshwater systems, including lakes and aquaculture ponds (Bellinger & Sigee, 2010; Dokulil & Teubner, 2000; Paerl & Tucker, 1995). Furthermore, *Microcystis* spp., *Anabaena* spp., and *Oscillatoria* spp. were observed to be few of the most common genera responsible for cyanobacterial bloom in aquaculture ponds (Jewel, Affan, & Khan, 2003; Paerl & Tucker, 1995). This is consistent with the finding of this study as the presence of *Microcystis* spp., *Anabaena* spp., and *Oscillatoria* spp. were detected in all of the studied locations. An assessment of cyanobacteria diversity carried out in aquaculture ponds in Sarawak, Malaysia also recorded the presence of *Microcystis* spp., *Anabaena* spp., *Oscillatoria* spp., and *Nostoc* spp. (Mohd. Nasarudin & Ruhana, 2007).

Among all of the analysed cyanobacteria, *Microcystis* spp. was the most commonly observed genera in this study as the presence was detected in all of the 40 sampled ponds. This was followed by *Anabaena* spp. (87.5%) and *Oscillatoria* spp. (70%). *Nostoc* spp. remained as the least occurred cyanobacterial taxa in the studied aquaculture ponds (10%) (see Table 4.2).

Table 4.2

Relative frequency of potentially toxic cyanobacteria in selected study locations in Perak, Malaysia

Cyanobacterial genera	Relative frequency, *f* (%)
Microcystis spp.	100.0
Anabaena spp.	87.5
Oscillatoria spp.	70.0
Nostoc spp.	10.0

Note. Relative frequency, *f* (%) = (no. of ponds in which the genera occurs / total sampled ponds) x 100, whereby total sampled ponds = 40

Many countries have reported the dominance of *Microcystis* spp. in freshwater systems such as lakes, water reservoirs and aquaculture ponds. These include United States (Zimba et al., 2001), Philippines (Baldia, Conaco, Nishijima, Imanishi, & Harada, 2003), Kenya (Ballot, Pflugmacher, Wiegand, Kotut, & Krienitz, 2003), Algeria (Nasri, Bouaïcha, & Fastner, 2004), Sri Lanka (Jayatissa, Silva, McElhiney, & Lawton, 2006), Bulgaria (Pavlova, Babica, Todorova, Bratanova, & Maršálek, 2006), Central Spain (Carrasco et al., 2006), Czech Republic (Znachor et al., 2006), New York (Hotto, Satchwell, Berry, Gobler, & Boyer, 2008), Portugal (Valério, Faria, Paulino, & Pereira, 2008), China (Ren et al., 2014; Wu et al., 2008), Uganda (Okello, Portmann, Erhard, Gademann, & Kurmayer, 2010), Saudi Arabia (Al-Shehri, 2010), Australia (Sinang, 2012a), Canada (Davis et al., 2014), and Malaysia (Sinang et al., 2015). Since *Microcystis* spp. is the primary producer of microcystins (Jiang, Yu, Chai, Song, & Li, 2013; Joung et al., 2011; Mioni et al., 2011), it is unsurprising that this particular hepatotoxin becomes the most commonly found cyanotoxin in freshwater systems all around the world (Bartram, 2015; Chorus & Bartram, 1999).

4.2 Relative Abundance and Toxicity of Cyanobacteria in Perak Aquaculture Systems

Relative abundance of cyanobacteria, which is also described as cyanobacterial biomass in this study was measured with the total chlorophyll-*a* concentration extracted and quantified according to Standard Methods provided by APHA (1998). The total chlorophyll-*a* was first validated against phycocyanin fluorescence of cyanobacteria measured with AquaPen-C fluorometer to determine its suitability to represent the cyanobacterial biomass. Cyanobacterial toxicity namely the microcystin concentration was quantified with HPLC and expressed in MC-LR equivalent. Results for this part of study are as documented in section 4.2.1 to section 4.2.4.

4.2.1 Validation of Total Chlorophyll-*a* for Cyanobacterial Biomass Estimation

The validity of total chlorophyll-*a* to be used to estimate cyanobacterial biomass was confirmed through correlation established between total chlorophyll-*a* with the F_T of cyanobacteria (see Figure 4.6). In this study, a strong positive correlation was obtained between F_T of cyanobacteria with the total chlorophyll-*a* of phytoplanktons ($p < .001$, $r = .91$). This was in line with results reported by Gregor and Maršálek (2005) and Gregor et al. (2007) who found strong correlations between phycocyanin fluorescence with the concentration of chlorophyll-*a*. Figure 4.6 also shows that the variability of cyanobacterial fluorescence at 620 nm explained 82% of the variability in total chlorophyll-*a*.

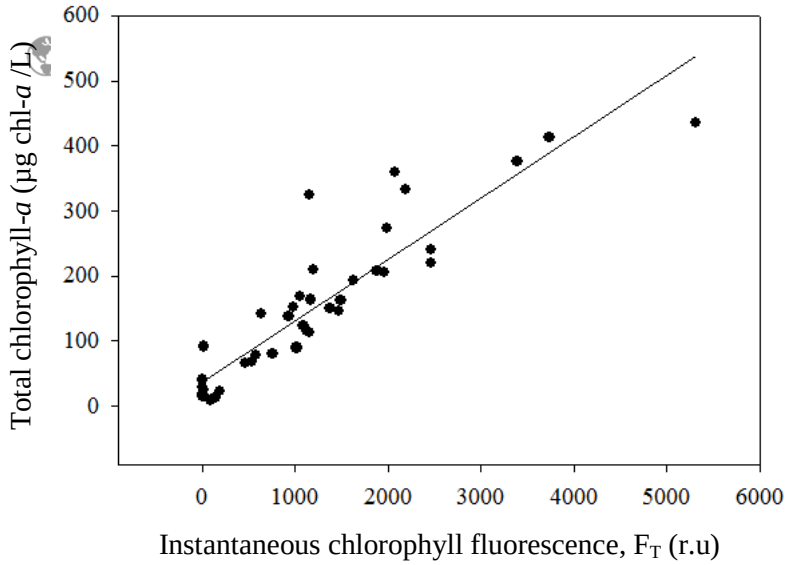

Figure 4.6. Correlation coefficient analysis for relationship between total chlorophyll-*a* and instantaneous chlorophyll fluorescence (F_T) of cyanobacteria at 620 nm (y = 0.09x + 38.08; r^2 = .82).

A strong significant correlation observed in this study suggesting that cyanobacteria is a significant component of phytoplanktons community in 40 aquaculture ponds in Perak. This indicates that the variability of chlorophyll-*a* can be used as a proxy to estimate the variability of cyanobacterial biomass. Chlorophyll-*a* is an indirect measure (Nagarkar & Williams, 1997) and accepted indicator (Meriluoto & Codd, 2005) of cyanobacterial biomass. It has been used widely to estimate the biomass of cyanobacteria especially during cyanobacterial bloom (Lawton et al., 1999). Thus, for the rest of this manuscript, the term "cyanobacterial biomass" was used to describe the relative abundance of cyanobacteria based on the total chlorophyll-*a* concentration.

05-4506832 pustaka.upsi.edu.my Perpustakaan Tuanku Bainun Kampus Sultan Abdul Jalil Shah PustakaTBainun ptbupsi

4.2.2 Cyanobacterial Biomass in Water Body of Aquaculture Ponds

Cyanobacterial biomass quantified in this study varied significantly between locations, $F(9,30) = 3.496$, $p < .01$. During the sampling period, 75% of the ponds were recorded to be under cyanobacterial bloom as the chlorophyll-*a* concentrations were above the level of bloom definition of 50 µg chl-*a* /L (Chorus & Bartram, 1999). The highest average concentration of cyanobacteria was detected in location 9 (237.21 ± 135.93 µg chl-*a* /L), followed by location 4 (212.61 ± 79.17 µg chl-*a* /L) and location 6 (208.53 ± 160.72 µg chl-*a* /L). Aquaculture ponds in location 10 on the other hand showed significantly lower biomass of cyanobacteria as compared to other studied locations (25.21 ± 12.14 µg chl-*a* /L) (see Figure 4.7).

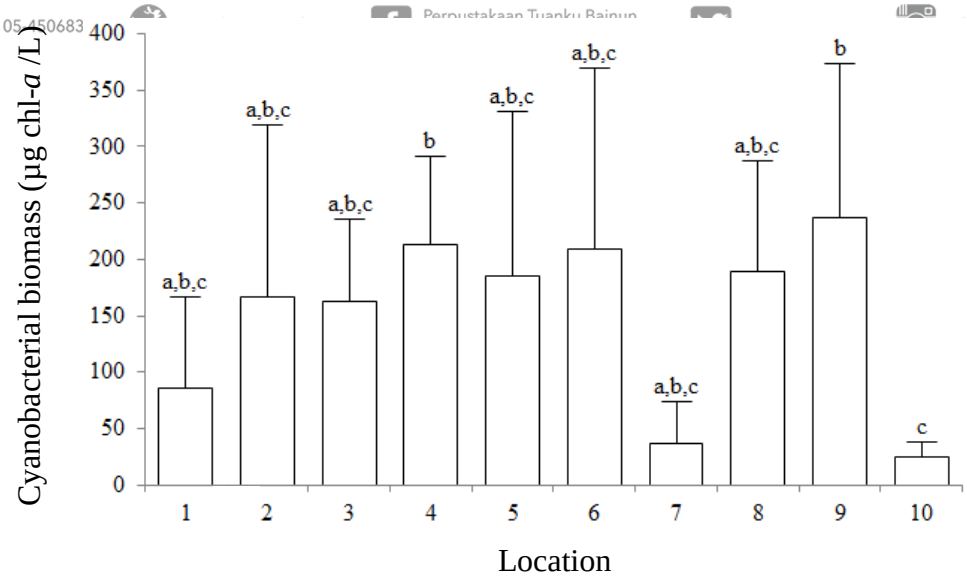

Figure 4.7. Cyanobacterial biomass in 10 selected study locations around Perak, Malaysia during sampling period. (1) Behrang; (2) Tapah; (3) Temoh I; (4) Temoh II; (5) Temoh III; (6) Chenderiang I; (7) Chenderiang II; (8) Air Kuning I; (9) Air Kuning II; and (10) Air Kuning III. Data were expressed as the mean value and standard deviations of four sampled ponds. Locations with common letters were not statistically significant different ($p > .05$).

In this study, cyanobacterial biomass of up to 436.31 µg chl-*a* /L were measured throughout the sampling period. Excessive proliferation of cyanobacteria in water system may lead to negative impacts on water quality. This includes the tendency of some cyanobacterial species to produce toxic metabolites into the water bodies (Barros et al., 2010; Rita, Valeria, Silvia, Pasquale, & Milena, 2014). Four months analysis of water samples collected from three freshwater lakes significant for fisheries in China revealed that chlorophyll-*a* concentrations of up to 463.7 µg/L had the potential to produce up to 2.52 (0.04) µg/L dissolved microcystins and up to 0.23 (0.01) mg/g of microcystin-LR into the water systems (Peng et al., 2010).

A productive aquaculture pond normally has about 50 to 200 µg/L chlorophyll-*a* (Boyd, 1998). In this study, 45% of the sampled ponds were having biomass within the productive range, while 35% of them had above 200 µg chl-*a* /L. In comparison to other studies conducted in aquaculture ponds in Malaysia, these results pose an alarming sign. A research conducted at Indigenous Fisheries Research and Production Centre (IFRPC) in Sarawak reported that 83% of the quantified chlorophyll-*a* were below 20 µg chl-*a* /L and the highest recorded biomass was 172.12 µg chl-*a* /L (Mohd. Nasarudin & Ruhana, 2007).

4.2.3 Microcystin Concentration in Water Body of Aquaculture Ponds

The occurrence of microcystin in the water samples was identified based on the characteristics of its UV-spectra. Figure 4.8 showed the examples of microcystin spectra containing tyrosine residues as well as the typical microcystin spectra with

maximum absorbance at 238.4 nm detected in this study. The presence of tyrosine residues in microcystin compound, possibly MC-YR congener, can be recognised by flatter absorbance maximum at 230 nm to 240 nm (Meriluoto & Codd, 2005; Purdie, Young, Menzel, & Codd, 2009). Microcystin containing tryptophan residue, potentially from WR or LW variants, was not identified during the HPLC analysis. These variants supposed to show maximum absorbance at 222 nm or 223 nm along with a shoulder at 238 nm to 240 nm (Lawton et al., 1994; Meriluoto & Codd, 2005; Purdie et al., 2009).

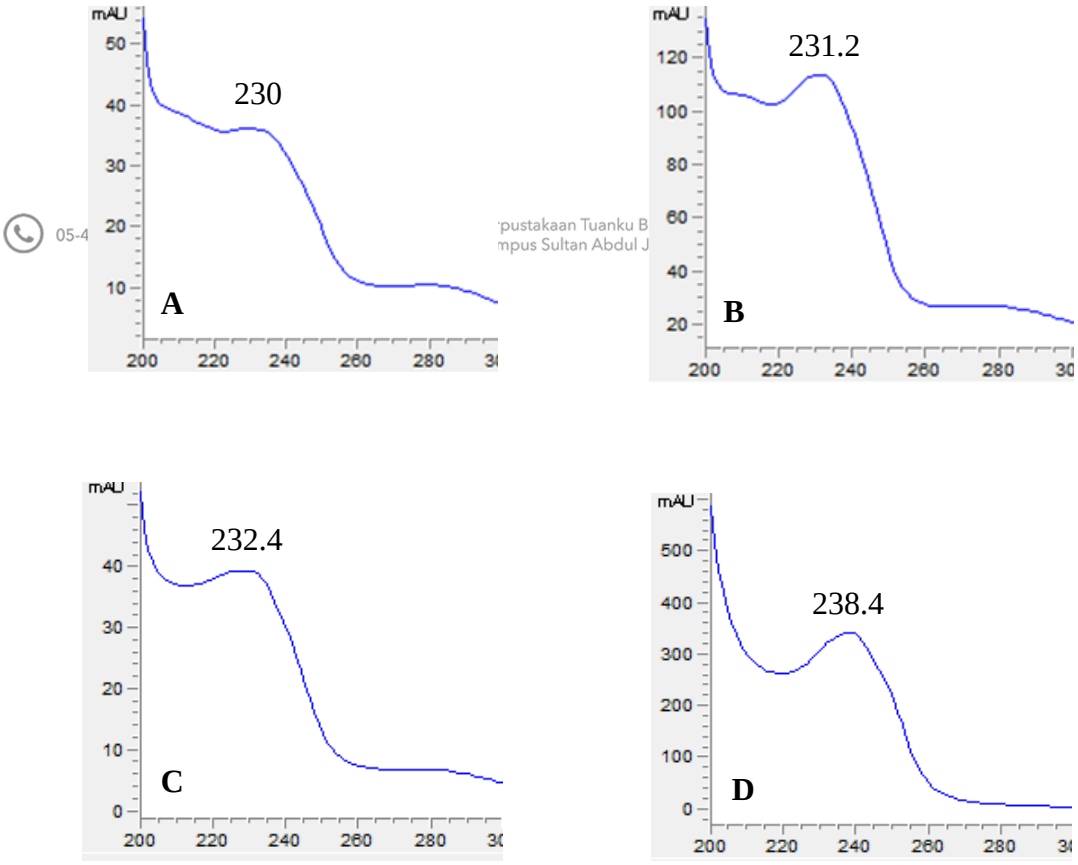

Figure 4.8. Microcystin UV spectra. (A-C) Microcystin containing tyrosine residue and (D) Typical microcystin spectra.

During the sampling period, microcystins were analysed in all selected aquaculture ponds in Perak, Malaysia and the concentration varied significantly between locations, $F(9,30) = 2.945$, $p < .05$. The highest average concentration of microcystins was recorded in location 6 (175.63 ± 105.69 µg MC-LR equivalent /L), followed by location 1 (101.81 ± 93.90 µg MC-LR equivalent /L) and location 9 (97.36 ± 79.28 µg MC-LR equivalent /L). Location 10 on the other hand showed significantly lower microcystins concentration as compared to other sampled locations (13.95 ± 1.81 µg MC-LR equivalent /L) (see Figure 4.9).

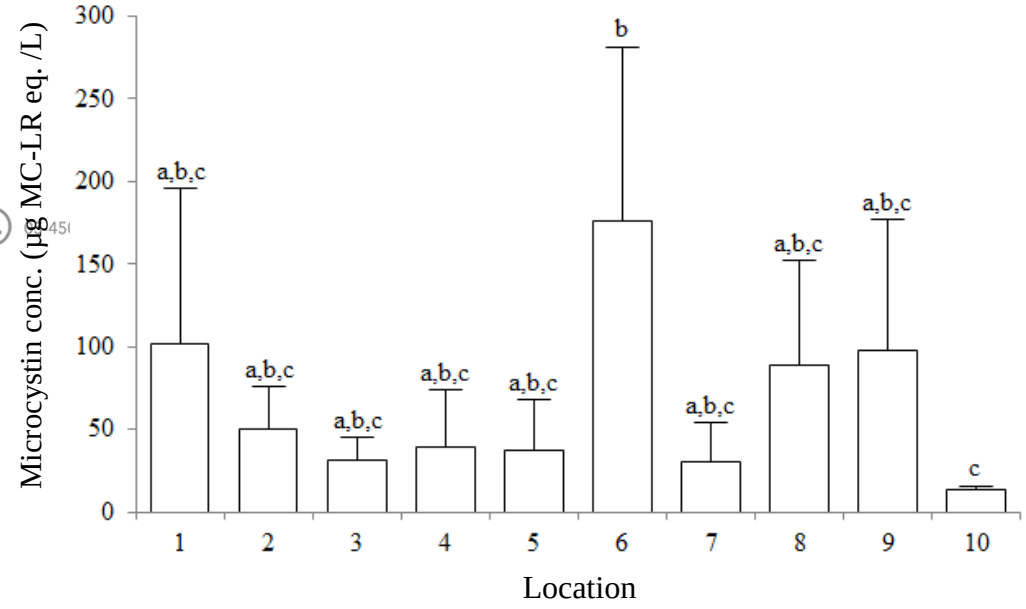

Figure 4.9. Microcystin concentrations in selected study locations around Perak, Malaysia during sampling period. (1) Behrang; (2) Tapah; (3) Temoh I; (4) Temoh II; (5) Temoh III; (6) Chenderiang I; (7) Chenderiang II; (8) Air Kuning I; (9) Air Kuning II; and (10) Air Kuning III. Data were expressed as the mean value and standard deviations of four sampled ponds. Locations with common letters were not statistically significant different ($p > .05$).

In comparison to WHO guidelines for recreational water exposure, most of the sampled ponds were having microcystins concentration at a stage that could pose moderate (27.5%) and high (75%) probability of adverse health effect particularly to

aquaculture farm workers. According to Chorus & Bartram (1999), the water is considered posing medium risk of health impact when the microcystins concentration are between 10 to 20 µg/L MC-LR equivalent, and described as high risk (unsafe) when the toxins concentration are above the medium risk level of 20 µg/L MC-LR equivalent (see Table 2.2). Furthermore, this study recorded microcystins concentration of up to 295.86 µg MC-LR equivalent /L throughout the sampling period. These findings illustrate potential deleterious impacts on cultured fish as well as health risk associated with the toxic cyanobacteria metabolites in the selected aquaculture systems in Perak. Poste et al. (2011) measured microcystins concentration of up to 57.1 (67.9) µg/L in water samples collected from several tropical and temperate lakes and concluded that fish harvested from these lakes might be unsafe for consumption.

4.2.4 Relationship between Cyanobacterial Biomass with Microcystin Concentration

A significant correlation ($p < .001$, $r = .612$) was obtained between cyanobacterial biomass with microcystins concentration. Despite having significant correlation, linear relationship revealed that the variability of chlorophyll-*a* only explained 37.5% ($r^2 = .375$) of the variability in intracellular microcystins concentration (see Figure 4.10).

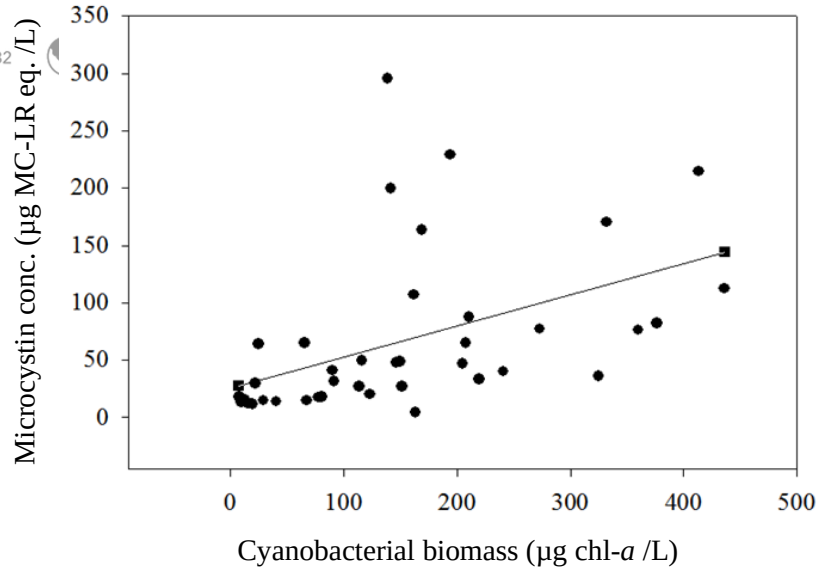

Figure 4.10. Relationship between cyanobacterial biomass with microcystins concentration in selected aquaculture ponds in Perak, Malaysia ($y = 0.27x + 25.47$; $r^2 = .375$; $p < .001$).

Positive correlations between these two parameters were also described in other literatures (Kotak et al., 2000; Okello, Portmann, Erhard, Gademann, & Kurmayer, 2009; Rinta-Kanto et al., 2009; Wu et al., 2008) including studies carried out at spatial and temporal scales (Singh et al., 2015; Su et al., 2015). Although strong positive correlation between cyanobacterial biomass and microcystin concentration is common, weak relationship (Sinang, Reichwaldt, & Ghadouani, 2013) or insignificant correlation between the parameters (Carrasco et al., 2006; Jacoby et al., 2000; H. Yang et al., 2006) remained possible.

4.3 Environmental Trigger of Toxic Cyanobacteria in Perak Aquaculture Systems

The relationships between cyanobacterial biomass, microcystin concentration and selected physicochemical parameters of water, namely temperature, pH, DO, and dissolved nutrients e.g. nitrate, nitrite and phosphate, magnesium, calcium and ammonium were investigated to identify the main trigger of toxic cyanobacterial occurrence in Perak aquaculture systems. Results for this part of study are as documented in section 4.3.1 to section 4.3.3.

4.3.1 Physicochemical Characteristics of Water

Physicochemical characteristics of water sample varied between locations and ponds (see Table 4.3). The temperature ranged between 28.9 °C to 34.4 °C, and most of the ponds were having temperature below 32 °C. Water temperature between 25 °C to 32 °C was reported to be optimum for the growth of freshwater species (Boyd, 1998). Dissolved oxygen (DO), the most crucial water parameter in aquaculture pond (Boyd, 1998) ranged between 5.57 mg/L to 11.54 mg/L in this study. This range of DO concentration is classified as best condition for aquatic growth (Boyd, 1998). The water pH during sampling ranged between 7.19 and 9.74. Aquaculture water with pH 7 to 9 is categorised as an ideal pH for the growth of fish as well as crustaceans whereas pH 9 to 11 can cause slow development to aquatic species (Boyd, 1998). Among all of the analysed dissolved inorganic nutrients in water sample, only nitrate fell in the range of desired concentration (0.2 – 10 mg/L) (Boyd, 1998). Magnesium

concentration was much lower than the preferred concentration of 100 mg/L. Some of the collected water samples had ammonium and calcium below and within the acceptable ranges. No data recorded for phosphate and nitrite as the concentrations were below the detection limit.

05-4506832 pustaka.upsi.edu.my Perpustakaan Tuanku Bainun Kampus Sultan Abdul Jalil Shah PustakaTBainun ptbupsi

05-4506832 pustaka.upsi.edu.my Perpustakaan Tuanku Bainun Kampus Sultan Abdul Jalil Shah PustakaTBainun ptbupsi

Table 4.3

Physicochemical characteristics of the selected aquaculture ponds around Perak, Malaysia during sampling period

Location	Temperature (°C)	DO (mg/L)	pH	Nutrients (mg/L)					
				NO_3^-	PO_4^{3-}	NO_2^-	Mg^{2+}	Ca^+	NH_4^+
1	29.7 (0.4)	5.57 (0.93)	7.19 (0.34)	0.34 (0.02)	N/D	N/D	0.84 (0.22)	8.86 (4.68)	0.0079 (0.0010)
2	29.4 (0.8)	9.44 (4.38)	8.05 (1.56)	0.58 (0.56)	N/D	N/D	0.64 (0.22)	5.70 (1.62)	0.0497 (0.0905)
3	29.1 (0.7)	5.63 (1.51)	7.75 (0.76)	0.28 (0.02)	N/D	N/D	1.03 (0.58)	4.02 (1.78)	0.0006 (0.0000)
4	28.9 (0.6)	7.59 (0.71)	7.25 (0.12)	0.34 (0.05)	N/D	N/D	0.23 (0.09)	2.26 (1.39)	0.0031 (0.0024)
5	32.1 (1.3)	9.25 (3.16)	8.89 (0.98)	0.28 (0.03)	N/D	N/D	0.93 (0.90)	3.54 (2.66)	0.0478 (0.0083)
6	29.2 (0.8)	9.71 (1.56)	8.27 (1.25)	0.36 (0.16)	N/D	N/D	0.59 (0.46)	3.70 (3.57)	0.0106 (0.0103)
7	31.8 (0.2)	5.69 (1.48)	7.97 (0.76)	0.35 (0.07)	N/D	N/D	1.37 (0.89)	5.64 (3.91)	0.4103 (0.8166)
8	30.8 (0.5)	11.54 (1.73)	9.74 (0.37)	0.34 (0.05)	N/D	N/D	2.87 (0.73)	13.69 (3.48)	0.0091 (0.0069)
9	31.4 (0.5)	10.98 (4.57)	9.53 (0.98)	0.29 (0.05)	N/D	N/D	3.37 (0.26)	17.88 (4.75)	0.2204 (0.3781)
10	34.4 (0.3)	7.61 (1.95)	8.20 (0.74)	0.31 (0.09)	N/D	N/D	1.63 (0.18)	16.79 (4.40)	0.5533 (1.0721)

Note. Data above were expressed in Mean (Standard deviation); N/D = Not detected.

4.3.2 Relationship between Physicochemical Parameters of Water with Cyanobacterial Biomass and Microcystin Concentration

Bivariate correlations with Pearson's test revealed that temperature was significantly correlated ($p < .05$) with both cyanobacterial biomass and microcystin concentration. Significant correlations were also observed between DO and pH with cyanobacterial biomass. However, there were no significant relationships ($p > .05$) detected between any of the analysed nutrients with cyanobacterial intensity and intracellular microcystin in water column, as well as DO and pH with microcystin concentration (see Table 4.4).

Table 4.4

Relationship between physicochemical parameters of water with cyanobacterial biomass and microcystin concentration in selected aquaculture ponds in Perak, Malaysia

Parameter	Cyanobacterial Biomass	Microcystin Concentration
(a) Temperature (°C)	-0.434**	-0.320*
(b) Dissolved oxygen (mg/L)	0.424**	0.291 N/S
(c) pH	0.321*	0.257 N/S
(d) Nutrients (mg/L)		
- Nitrate	-0.147N/S	0.068 N/S
- Magnesium	-0.113 N/S	0.007 N/S
- Calcium	-0.239 N/S	-0.066 N/S
- Ammonium	-0.138 N/S	-0.242 N/S

Note. Data were expressed in Pearson product-moment correlation coefficient, *r.* $^{*}p < 0.05$, $^{**}p < 0.01$, N/S = Not significant

The water temperature in all sampled ponds was quite high, and most of them were around 30 °C during sampling (see Table 4.3). Most species of cyanobacteria attained optimum growth rate within the temperature range of 25 (Robarts & Zohary, 1987) to 35 °C (Kumar et al., 2011; Lürling et al., 2012). Other studies reported that

some species of cyanobacteria such as *Microcystis* spp. can out-compete other phytoplanktons at 30 °C and above (Fujimoto, Sudo, Sugiura, & Inamori, 1997). As we can see from the result (see Figure 4.11), temperature has negative correlation with both cyanobacterial biomass as well as microcystin concentration. Additionally, significant decline in biomass and microcystin content were also observed at temperature above 32 °C suggesting that cyanobacteria may be able to thrive to a certain extent of high temperature in freshwater system. However, the trends were only general observation and not applicable to all sampled ponds in this study. For example, a pond in location 5 with recorded water temperature of 33.9 °C contained relatively high biomass of cyanobacteria (67.41 µg chl-*a* /L). The high biomass could be due to the composition of phytoplanktons in water bodies such as the presence of eukaryotic algae as this study measured the total chlorophyll-*a* instead of cyanobacterial concentration. Low amount of microcystins were also quantified in ponds with water temperature under 32 °C such as in a pond in location 4 (4.34 µg MC-LR equivalent /L) despite having 163.44 µg chl-*a* /L of biomass. This confirms that not all types of cyanobacteria are toxic (Chorus & Bartram, 1999).

Similar finding was also reported by a research conducted in Sarawak whereby a significant negative correlation was observed between cyanobacterial cell density with temperature when the range of temperature was between 27.10 °C to 32.30 °C, however, no correlation detected at higher temperature (26.98 °C – 34.41 °C) (Mohd. Nasarudin & Ruhana, 2007). This result was contradicting with the finding demonstrated by Su et al. (2015) which detected strong positive correlation between cyanobacterial biomass and microcystin concentration with temperature. While Su et al. (2015) only measured water temperature of up to 32 °C during their

study, the probability for reduction of cyanobacterial intensity as well as toxicity at above 32 °C is possible.

Paerl and Huisman (2009) stated that cyanobacteria compete more effectively with eukaryotic algae as the water temperature increases and resulted in higher growth rate of cyanobacteria. However, when the cyanobacterial growth achieves its optimum point at certain temperature, the growth started to decline. For instance, the growth of *Microcystis aeruginosa* and *Anabaena flos-aquae* was observed to be active at 25 to 30 °C, however, at 35 °C the cells showed rapid mortality (Butterwick et al., 2005).

Besides temperature, this study recorded insignificant correlations between microcystin content with other selected environmental parameters (see Table 4.4). This result is similar with finding reported by Joung et al. (2011). On the other hand, Wu et al. (2008) found inconsistent relationship between microcystin concentration with environmental factors during field assessment of eight lakes in Yangtze River. The study finally concluded that microcystin has better correlation with biological factors than environmental factors. Hence, this explained the direct effect of cyanobacteria and indirect influence of environmental factors to cyanobacterial toxicity in water systems (Boopathi & Ki, 2014).

A significant correlation was found between cyanobacterial biomass with DO (see Figure 4.8). Dissolved oxygen was reported to have a link with chlorophyll-*a* concentration (Odum, 1956) especially in a reservoir with low water exchange or closed fish farms (Cuiya, 1998). This is because of the direct effect of algal photosynthesis on DO concentration (Cuiya, 1998). Apart from algal photosynthesis,

aquatic respiration is also contributing to this effect and was reported to show diurnal variations in eutrophic water (Zang et al., 2011). Table 4.3 shows that DO measured in the selected aquaculture ponds in Perak varied significantly from 3.41 mg/L to 14.98 mg/L. Similar result was also reported by a research conducted on *Tor tambroides* ponds in Serian, Sarawak (Mohd. Nasarudin & Ruhana, 2007).

Water pH was detected to have a positive correlation with cyanobacterial biomass in this study (see Figure 4.11) which is consistent with result documented in other literatures (Su et al., 2015; Te & Gin, 2011). Cyanobacterial activity and intensity in water column were reported to influence the water pH (You, Cui, Liu, Yang, & Huang, 2007). During intense bloom, photosynthetic activities of phytoplanktons increase and cause depletion of free carbon dioxide. This subsequently leads to increase in pH value which favours the dominance of cyanobacteria (Dokulil & Teubner, 2000).

At high pH, not all types of phytoplanktons are capable to utilise carbon as efficient as cyanobacteria. The dominance of cyanobacteria was due to its lower half-saturation constants (Ks) for CO_2 and its competitive advantage over other phytoplanktons as cyanobacteria can use both free CO_2 as well as bicarbonate (HCO_3^-) as source of carbon during photosynthesis (Dokulil & Teubner, 2000; Jacoby et al., 2000; Shapiro, 1990). In addition, buoyancy characteristic in some cyanobacteria also allow this species to move up and down for the carbon and further reduce the carbon dioxide to a level below the utilisation limit of other phytoplanktons (Jacoby et al., 2000; Shapiro, 1990).

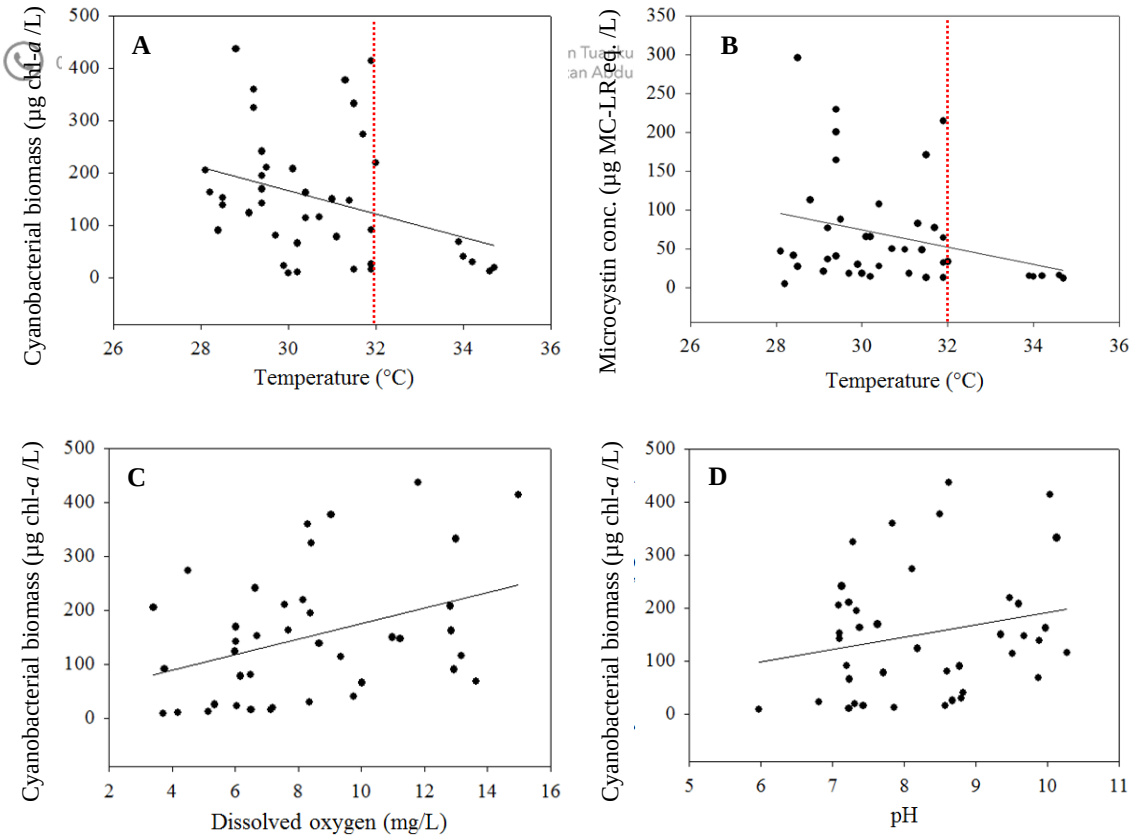

Figure 4.11. Correlation coefficient analysis for relationships between cyanobacterial biomass and microcystin concentration with temperature, DO and pH in selected aquaculture ponds in Perak, Malaysia. (A) Temperature (y = -22.47x + 840.18; r^2 = .188), (B) Temperature (y = -11.09x + 406.64; r^2 = .102), (C) DO (y = 14.43x + 31.19; r^2 = .179), and (D) pH (y = 23.27x – 41.81; r^2 = .103).

Many scientific literatures stated that cyanobacterial proliferation is closely related to nutrient concentration in water bodies (Chorus & Bartram, 1999), particularly nitrogen and phosphorus (Su et al., 2015). This phenomenon was also reported in aquaculture ponds (Kankaanpää et al., 2005). On the contrary, some studies documented that cyanobacterial abundance in aquaculture ponds might be affected by a combination of multiple environmental factors and not solely due to nutrients (Mohd. Nasarudin & Ruhana, 2007; 2011a). This was also proven in this study which found no correlation between any of the analysed nutrients with cyanobacterial proliferation in aquaculture ponds (see Table 4.4). This suggests that

temporal and spatial variations may exist between nutrients and cyanobacterial biomass likewise their compositions as well as toxicity (Chorus & Bartram, 1999).

Moreover, analysis of dissolved (bioavailable) nutrients instead of total nitrogen (TN) and total phosphorus (TP) in this study could also contribute to the insignificant correlation between the parameters. Håkanson, Bryhn, & Hytteborn (2007) demonstrated that "bioavailable" form of nutrients have high coefficient of variation (CV) values, hence are not a reliable indicator to predict the occurrence of cyanobacteria. However, contradicting result was reported by Su et al. (2015) that observed significant correlations ($p < .01$) between all of the analysed dissolved nitrogen and phosphate with both cyanobacterial biomass and microcystins production.

Phosphate measured in the water samples were below the detection level (see Table 4.3). The main reason for this could probably due to minimal input of phosphorus into the aquaculture water body since the concentration of phosphorus in most fish feeds are relatively quite low. Unlike plant fertilizer, fish manure normally contains insignificant amount of phosphorus. Phosphorus is one of the 20 inorganic minerals which composed about 1.0 - 2.5% of fish diet (Pandey, 2013). Undetected phosphate concentration could also be because of the ion chromatography analysis that only quantifies soluble reactive phosphate (SRP) in the form of orthophosphate instead of the total phosphorus itself (Jackson, 2000). Good control system in terms of isolated location and aquaculture pond management plays an important role as well in minimizing external eutrophication from disrupting the water quality. This finding indicated that orthophosphate may or may not give impact on cyanobacterial

proliferation in aquaculture ponds. Although it was assumed that cyanobacteria favours high concentration of phosphorus and nitrogen, there was also report stated that cyanobacterial bloom often taking place when the concentration of SRP is the lowest (Chorus & Bartram, 1999). Insignificant relationship between orthophosphate with cyanobacteria cell density and chlorophyll-*a* concentration was also observed in both studied aquaculture ponds in Serian, Sarawak (Mohd. Nasarudin & Ruhana, 2007; 2011a).

Similar to phosphate, nitrite was also not detected in this study (see Table 4.3). Nitrite present in aquaculture water is commonly the intermediate product of nitrification process (Hargreaves & Tucker., 1996). In aquaculture pond, large amount of nitrogen is introduced into the water body through fish feeds (Boyd, 1998) as most of the fish pellets for growing fish contains about 32 – 45% of protein (Pandey, 2013). Despite so, nitrite normally did not accumulate in water column as it will be quickly converted into nitrate (Boyd & Tucker, 1998). Besides, ammonia assimilation by phytoplanktons and other aquatic plants also limits the concentration of ammonia available for nitrification, hence further reduce the production of nitrite (Boyd & Tucker, 1998). Nitrite concentration in aquaculture water was also reported to be very low, and the acceptable range is below 0.1 mg/L (Boyd, 1998). Water analysis conducted in freshwater fish (*Tor tombroides*) ponds in Sarawak detected between 0.001 mg/L to 0.007 mg/L nitrite in the water sample throughout the study period and reported that this parameter has no significant relationship with both cyanobacteria cell density as well as chlorophyll-*a* concentration in aquaculture water body (Mohd. Nasarudin & Ruhana, 2007).

4.3.3 Environmental Trigger of Cyanobacterial Biomass and Microcystin Concentration

Although there were significant correlations between temperature with cyanobacterial biomass and microcystin concentration, as well as DO and pH with cyanobacterial biomass (see Table 4.4), multiple linear regression analysis with forward selection revealed that a combination of temperature and pH statistically predicted both cyanobacterial biomass, as well as microcystin concentration in aquaculture systems in Perak. The model explained 46.2% of the variability in cyanobacterial biomass, $F(2,27) = 11.593$, $P < .001$, [log(CB) = -10.971*****log(temperature) + 3.928*log(pH) + 14.680], and 30.4% of the variability in microcystin concentration, $F(2,27) = 5.883$, $P = .008$, [log(MC) = -8.038*log(temperature) + 2.878*log(pH) + 10.958]. There was no correlations observed between temperature and pH ($p = .80$), suggesting that either parameter will not control one another. In addition, beta values showed that the effects of temperature were greater on cyanobacterial biomass (.584) and microcystin (.473) as compared to pH (CB: .495; MC: .401). This indicates that temperature was a more powerful predictor of both cyanobacterial biomass, as well as microcystin concentration in the studied aquaculture ponds in Perak.

The finding of this study is parallel with many scientific literatures that relate the occurrence of toxic cyanobacteria with climate change and global warming phenomena (Paul, 2008; Paerl & Huisman, 2008; 2009; Paerl & Otten, 2013; Paerl & Paul, 2012). Global warming promotes the dominance of cyanobacteria over other phytoplanktons in water bodies as these noxious bacteria are able to tolerate high water temperature (Paerl & Huisman, 2008; Paerl & Otten, 2013; Reynolds, 2006;

Robarts & Zohary, 1987). Warming of water surface induces (Brookes et al., 2013), intensifies and lengthens the vertical stratification of water particularly in temperate regions (Paerl & Huisman, 2008; Paerl & Paul, 2012). Buoyant cyanobacteria exploits the stratified water condition and subsequently forming dense blooms on water surface (Elliott, 2010; Paerl & Huisman, 2008; Paerl & Paul, 2012).

The model of this study also confirmed the influence of pH on cyanobacterial biomass and toxicity. Unlike temperature, water pH is normally associated to the direct impact of cyanobacterial proliferation instead of a controlling factor. The pH of water is inversely proportionate to the concentration of carbon dioxide in the water bodies (Wurts & Durborow, 1992). Through active photosynthetic activity, cyanobacterial bloom enhances the absorption of CO_2, hence increases the water pH (Paerl & Paul, 2012). Elevated water pH leads to the dominance of cyanobacteria due to decreased efficiency among phytoplanktons to utilise carbon at high pH (Dokulil & Teubner, 2000).

4.4 Bioaccumulation of Microcystin in *Oreochromis* spp. Tissue and Its Effect on the Survival and Growth of Fish

Bioaccumulation of microcystin in fish tissues was investigated in the laboratory through immersion exposure of *Oreochromis* spp. to the natural bloom extracts with 17.96 µg/L, 40.66 µg/L, and 78.32 µg/L microcystins for seven days. The concentrations of microcystin selected to conduct this experiment represented the

naturally-occurring concentration of microcystins in Perak aquaculture systems as documented in section 4.2.3.

As shown in Table 4.5, microcystins accumulated in *Oreochromis* spp. tissues increased with the concentration of microcystins in the water. The highest survival percentage was recorded in control tanks whereby none of the fish died throughout the experiment. Even so, Red tilapia in the control tanks showed the lowest growth rate as compared to treatment tanks with cyanobacterial extracts. Tilapia cultured in water tanks treated with 17.96 µg/L microcystins surprisingly showed the highest growth rate after seven days despite having the lowest survival percentage. The details of this study are as discussed in section 4.4.1 and section 4.4.2.

Table 4.5

Microcystins accumulated in fish tissues, growth and survival of Oreochromis spp. after 7 days of culture under 8 h light at 25±2 °C

Treatment	Initial wt. (g)	Final wt. (g)	Microcystins Accumulated (µg/g d.w.)	Survival (%)	Growth (%)
Control	7.28 (1.12)	7.55 (1.05)^{a}	0.84 (0.60)^{a}	100.00	3.79
T1	6.03 (1.73)	6.39 (1.68)^{a}	17.55 (10.10)^{ab}	66.67	5.97
T2	5.54 (1.08)	5.77 (1.14)^{a}	35.21 (10.74)^{bc}	77.78	4.15
T3	5.61 (1.77)	5.82 (2.10)^{a}	41.58 (7.93)^{c}	88.89	3.80

Note. Data above were expressed in Mean (Standard deviation). Treatments with common letters were not statistically different ($P > 0.05$). T1: 17.96 µg MC-LR equivalent /L, T2: 40.66 µg MC-LR equivalent /L, and T3: 78.32 µg MC-LR equivalent /L.

4.4.1 Bioaccumulation of Microcystin in *Oreochromis* spp. Tissue

Immersion exposure of *Oreochromis* spp. fingerlings (5.5±1.5 g) to crude cyanobacterial extracts for seven days recorded the bioaccumulation of microcystins in fish tissues in all of the treatment tanks. Microcystins concentration extracted from fish tissues were observed to increase with the concentration of microcystins in the water. In addition, one-way ANOVA revealed that bioaccumulation of microcystins in *Oreochromis* spp. tissues were statistically significant different, $F(3,8) = 14.428$, $p = .001$, between treatments (see Table 4.5).

Low levels of microcystins accumulation (0.84±0.60 µg/g d.w.) were observed in cultured tilapia in the control tanks. This suggests that microcystin might be readily present in fish tissues prior to the experiment, indicating possible microcystin contamination in the fish hatchery. Tilapias used in this experiment were subjected to two weeks acclimatisation in the laboratory before they were exposed to microcystin. As reported in microcystin depuration studies on common carp and silver carp (Adamovský et al., 2007), as well as in goldfish (Malbrouck et al., 2003), significant decline in microcystins concentration were observed when the fish were transferred into clean water. Complete elimination of microcystin within one to two weeks was also recorded (Adamovský et al., 2007). However, this was not the case with Nile tilapia, whereby only minor reduction in microcystin accumulation was reported after four weeks of depuration procedure (Palíková et al., 2011). This suggests that microcystin elimination from fish tissue is highly species-specific (Adamovský et al., 2007; Palikova et al., 2011) and just like Nile tilapia, Red tilapia potentially capable to retain microcystin longer than other species.

Bioaccumulation of microcystins in aquatic species are relatively dynamic (Ferrão-Filho & Kozlowsky-Suzuki, 2011; Martins & Vasconcelos, 2009). The immersion exposure of Red tilapia to crude cyanobacterial extracts at 17.96 µg/L, 40.66 µg/L, and 78.32 µg/L (quantified in MC-LR equivalent) in this study recorded average accumulation of 17.55 µg/g, 35.21 µg/g, and 41.58 µg/g microcystins, respectively in fish tissues after seven days. This finding showed that bioaccumulation of microcystins was rather dose-dependent to the microcystins concentration available in the surrounding water. Feeding of Nile tilapia with fish pellet containing toxic cyanobacteria found that microcystins accumulated in the fish muscle, liver, spleen and gall bladder increased with microcystins concentration in the diet (Zhao et al., 2006a).

In comparison to microcystin accumulation results in Nile tilapia tissues as reported by Palikova et al. (2011) and Ruangrit et al. (2013) (see Table 2.6), this study recorded much higher accumulation although the exposure was only carried out for a shorter duration as compared to the other studies. Furthermore, all of these studies applied the natural environmental approach which was immersion technique. High variation in microcystin bioaccumulation between the studies can be explained with the difference in source of microcystin, size of fish used to conduct the experiment, duration of microcystin exposure, and microcystin extraction target organ.

In natural water environment, fish exposed to microcystin through two ways: ingestion of cyanobacterial cell and epithelium absorption of microcystin dissolved in water (Malbrouck & Kestemont, 2006). The sources of microcystin in the second method are either microcystin released during the active growth or senescent of

cyanobacteria. As mentioned earlier, this study exposed the fish to crude cyanobacterial extracts, while both Palikova et al. (2011) and Ruangrit et al. (2013) used cyanobacterial bloom. These approaches were reported to induce significant toxicity in fish, however, more pronounced effect was usually observed with crude aqueous extract of cyanobacteria (Palíková et al., 2007). This can be explained by the nature of cyanobacterial toxin that remain intact inside the cyanobacterial cell during the active growth of cyanobacteria (Meriluoto & Codd, 2005). Normally, the intracellular cyanotoxin accounted for more than 95% of the total cyanobacterial toxin produced by cyanobacteria (Chorus & Bartram, 1999). The remaining cyanotoxin was released into the surrounding water as dissolved toxin (intercellular) and being accumulated by aquatic organisms. However, natural biodegradation process often decrease the concentration of dissolved cyanobacterial toxin in water bodies (Chorus & Bartram, 1999), hence further reducing the potential of cyanotoxin bioaccumulation in fish exposed to cyanobacterial bloom.

In this study, tilapia fingerlings were selected to conduct the experiment. Early-life stage developments are particularly critical to determine the succession of any species. However, younger fish are generally more sensitive to the toxic microcystin as compared to fish at the juvenile or adult stages (Malbrouck & Kestemont, 2006). This is probably because smaller fish possess thin epithelial layer, large body surface area and high metabolism rate (Von Westernhagen, 1988), hence are capable to accumulate more microcystins in their body.

Zíková et al. (2010) studied the microcystin accumulation in Nile tilapia fed with microcystin containing diets for four weeks. The finding showed that even

though microcystin accumulation might have increased with exposure time, the concentration may dropped to even lower than the concentration of microcystin measured on the first day of exposure at the end of experiment. The study then proposed that at a prolonged period of exposure, the tilapia might have developed metabolic adaptation to reduce the uptake of microcystin into their body. This is added with the fish capability to detoxify microcystin from their bodies through the activity of glutathione-S-transferase (GST) enzyme (Adamovský et al., 2007; Hao et al., 2008). In this study, although microcystin accumulated in fish body could be relatively high in the first seven days of exposure; this does not guarantee the toxic accumulation on a ready-to-harvest fish would be exponent of the current result.

Microcystin accumulation has always been associated to fish liver in many literatures (Ferrão-Filho & Kozlowsky-Suzuki, 2011; Malbrouck & Kestemont, 2006; Palikova et al., 2011; Sieroslawska et al., 2012). Although microcystin is commonly known with its hepatotoxic effect, it was reported to accumulate in other organs as well such as stomach, intestines (Meriluoto & Spoof, 2008), kidney (Hao et al., 2008), gallbladder (Mohamed & Hussein, 2006), muscle (Adamovský et al., 2007) and bile (Sahin, Tencalla, Dietrich, & Naegeli, 1996). Sahin et al. (1996) discovered that the concentration of microcystins in the fish bile were several folds higher than those detected in the liver. Since microcystins were extracted from the entire body of fish in this study, high microcystins concentration recorded could be contributed by all of these affected organs especially the bile.

By assuming that the bioaccumulation of microcystin is directly proportionate to the fish size as well as exposure time, this study found that an adult with Malaysian

average weight of 62.65 kg (Azmi et al., 2009) who consume 100 g of whole body of Red tilapia is potentially to exceed the TDI level recommended by WHO (0.04 µg MC-LR / kg body weight per day). In specific, the estimated daily intakes (EDIs) are 700 times, 1,405 times and 1,659 times higher than the proposed TDI in fish exposed to 17.96 µg/L, 40.66 µg/L, and 78.32 µg/L of microcystins, respectively (see Figure 4.12). This finding is alarming, particularly to Red tilapia consumers in Malaysia.

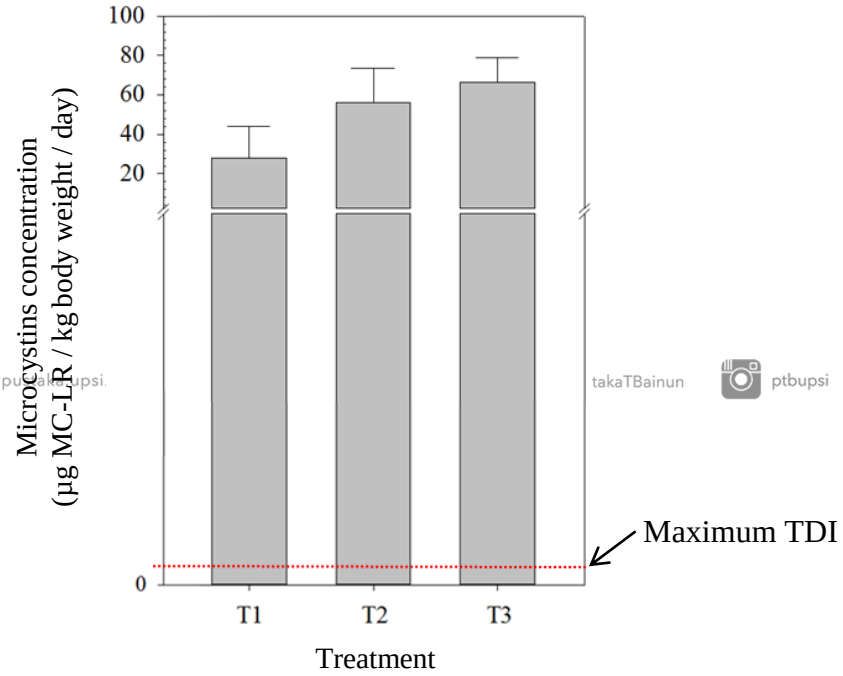

Figure 4.12. Estimated daily intake (EDI) of microcystin by an adult weighing 62.65 kg and ingested 100 g of *Oreochromis* spp. tissue. The red horizontal line indicates the maximum TDI. Data were expressed as the mean value and standard deviation of three replicate determinations. (T1: 17.96 µg MC-LR equivalent /L, T2: 40.66 µg MC-LR equivalent /L, and T3: 78.32 µg MC-LR equivalent /L)

Human risk of exceeding the TDI limit through fish consumption has been documented in many past literatures that focused on the evaluation of environmental samples (see Table 2.5). Some studies even reported microcystin bioaccumulation in fish tissues that could be four orders of magnitude higher than the recommended TDI

(Ibelings et al., 2005; J. F. Vasconcelos et al., 2013). Although these findings can be terrifying, it might not guarantee the actual situation since there are no human poisoning recorded so far due to microcystin contamination in food chain (Malbrouck & Kestemont, 2006).

In addition, the edible part of fish which is the muscle, was reported to accumulate the least concentration of microcystin as compared to other organs (Malbrouck & Kestemont, 2006; Singh & Asthana, 2014) and was estimated to be around 200 to 550 times lower than microcystins accumulated in the fish liver (Zhao et al., 2006a). Microcystin monitoring was carried out in Canada to determine the safety of farmed fish (raw, frozen, fresh, whole and fillet) sold in the retail markets across the country. The study then confirmed that the samples collected were free from microcystins contamination (Niedzwiadek, Scott, & Lau, 2012). So far, there was no known literature that assesses the presence of microcystins in farmed fish supplied to the public in Malaysia.

4.4.2 Effect of Microcystin on *Oreochromis* spp. Survival and Growth

As documented in Table 4.5, the growth percentage of Red tilapia showed inversed relationship with fish survival. This could be due to lesser competition for food and space amongst the survived fish when fatality occurred in the culture tanks. During the experiment, fish mortalities were observed in all of the treatment tanks except in control. The event was first observed on the third day of experiment in culture tanks immersed with 17.96 µg/L and 40.66 µg/L of microcystins, while water tanks with

78.32 µg MC-LR equivalent /L showed first fatality on the fifth day of culture. On the last day of experiment, highest survival rate was recorded in the control (100%), followed by treatments with 78.32 µg/L (88.89%), 40.66 µg/L (77.78%), and 17.96 µg/L (66.67%) microcystins (see Table 4.5).

Although there was no fatality observed in the control tanks throughout the experiment, this study failed to statistically demonstrate the influence of microcystin on fish mortality. This was proven with the Kaplan-Meier analysis with Log-Rank test which revealed no statistically significant ($p > .05$) result between survival curves, indicating equal survival distributions amongst treatments (see Figure 4.13).

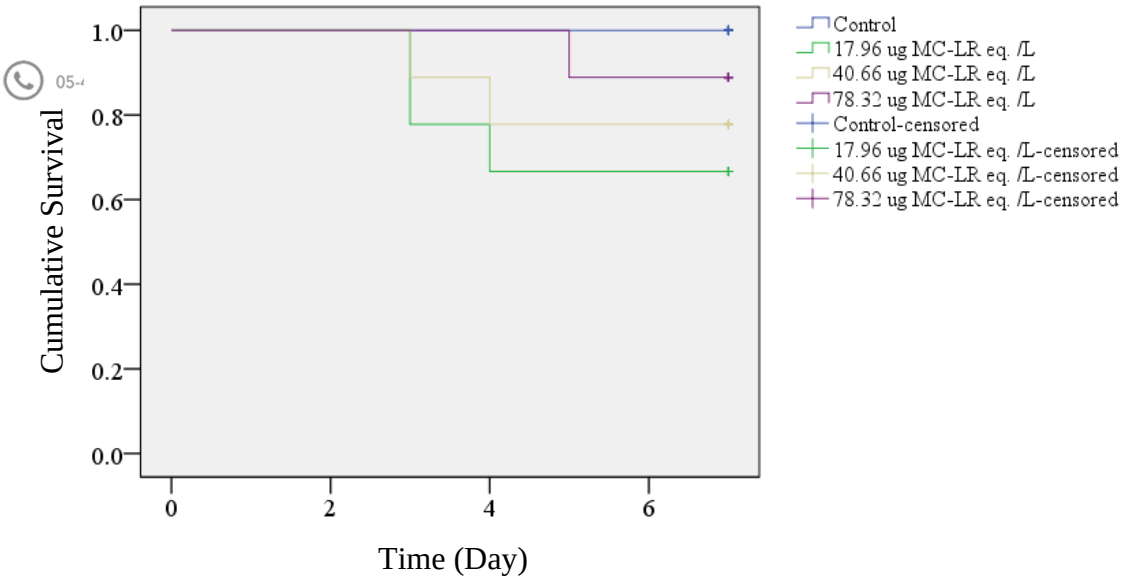

Figure 4.13. Kaplan-Meier survival curve of *Oreochromis* spp. throughout 7 days of culture under 8 h light at 25±2 °C.

Magalhaes et al. (2001) carried out three years study to investigate the bioaccumulation of microcystin in fish in Jacarepaguá Lagoon. Throughout the study period, no fish mortality was documented, even during the highest recorded

microcystin concentration (979 µg/L). The study then suggested that fish death in aquaculture ponds might not be related to the occurrence of cyanobacterial toxin in aquaculture ponds as well as its bioaccumulation in fish, but rather the effect of other factors. This is consistent with the finding of this study.

Snyder et al. (2002) stated that low concentration of dissolved oxygen was the main reason for the mass mortality of channel catfish during cyanobacterial bloom instead of cyanobacteria-associated toxin. However, this might not be the case for this study as dissolved oxygen concentrations as well as other water quality parameters were monitored and ensured to be in the optimum range throughout the study period. Hence, this study proposed that Red tilapia death might be related to the nature of tilapia fingerlings and the physiological stress response amongst the fish during experiment.

Fish fingerlings are naturally weaker and more susceptible to disease as compared to those in the juvenile and adult stages (Tucker & Hargreaves, 2004). In this study, the size of fish selected to conduct the experiment was between 4.46 g to 8.40 g. Sell and Aakre (2004) stated that tilapia fingerlings weighing lesser than 1 ounce (28.35 g) usually suffered from 10% of death loss under normal conditions. Even so, the selection of fingerlings to conduct this experiment is crucial as the survival of fish in early life stage determines the succession of fish to adulthood.

Ziková et al. (2010) documented stress response through the measurements of plasma cortisol, plasma glucose and hepatic glycogen on both groups of Nile tilapia fed with microcystin-containing diet, as well as on fish fed without microcystin. The

study then concluded that the stress response in fish was nearly independent of microcystins content in the fish diet (Ziková et al., 2010) which was in line with this study. Stress causes breakdown in the immune system, making fish more vulnerable to disease (Hargrove & Hargrove, 2011).

According to Postlethwaite (2010), the first sign of stress in fish is the loss of appetite. This is a common symptom shown by fish when they are introduced into a new "home" (Schliewen, 2008), which in this study might refer to the experimental tanks. During the conduct of this experiment, most of the tilapia fingerlings were observed showing reduced response towards fish pellet as the study progressed. This includes tilapia cultured in the control tanks. Hence, this study suggested that the 100% survival recorded in the control could be just by chance. Tilapia fingerlings that died during the experiment were most likely much weaker, thus were unable to handle the physiological stress than the rest of the fish. Although all of the cultured tanks were introduced with 0.1% (v/v) methanol in the beginning of the experiment, this was probably not the trigger or enhancer to the stress response as no significant increase in plasma cortisol was recorded on fish cultured in water treated with up to 1.0% alcohol (Oliveira et al., 2013).

In February 2012, a mass mortality of hybrid Red tilapia was recorded in Como River in Lake Kenyir, Malaysia (DoF Malaysia, 2012b as cited in Najiah et al., 2012). During the incidence, Najiah et al. (2012) analysed the water qualities (temperature, pH and DO) of the affected river and reported that all of the quantified parameters were within the optimum ranges. The study then found the presence of *Streptococcus agalactiae* and *Burkholderia cepacia* in the liver, and *Staphylococcus*

aureus in the eyes of the collected sick fish. Najiah et al. (2012) described *Burkholderia cepacia* and *Staphylococcus aureus* as 'opportunistic pathogens' that can attack stressed fish, whereas *Streptococcus agalactiae* was described as 'true pathogen' that is able to cause disease in healthy fish (Najiah et al., 2012). Hence, fish mortality in this study might be related to bacterial infections especially the opportunistic pathogens. The bacteria, perhaps have attacked the young and stressed fingerlings, and have caused further breakdown in the immune defence systems, leading to fish mortalities in the present study. Common sign of bacterial infection such as inactivity ("Common Symptoms," n.d.) was also observed in this experiment.

Similar to fish survival, the growth of Red tilapia was also found to be unrelated to the concentration of microcystins. This was supported by one-way ANOVA that revealed no statistically significant difference, $F(3,8) = 0.266$, $p > .05$, in the growth of *Oreochromis* spp. between treatments (see Table 4.5).

The effect of microcystin on fish growth documented in past literatures seems to be inconsistent. Some researchers reported growth inhibition (Bury, Eddy, & Codd, 1995; El Ghazali et al., 2010; Malbrouck & Kestemont, 2006; Zhao et al., 2006b) while some studies observed better growth rate with microcystin (Zhao et al., 2006a; Ziková et al., 2010). On the other hand, this study found no clear relationship between the growth of Red tilapia with microcystin concentrations ($p > .05$) even though the growth seemed improving in the presence of microcystin as compared to the control (see Table 4.5). The disagreement that exists was probably due to the presence of undigested pellet in the gut of fish that died during the experiment.

Just like other animals, stress leads to constipation in fish (Hargrove & Hargrove, 2011), making them unable to completely clear the digestive systems. The waste that was not expelled from the dead tilapia added extra weight to the fish and interfere with the result of growth measurement. Reduced appetite and inactivity, the most common symptoms showed by constipated fish (Hargrove & Hargrove, 2011) were observed during this study.

In summary, this study demonstrated *Oreochromis* spp. capability to accumulate microcystin in their tissues when the fish are exposed to the naturally-occurring microcystin concentration in Perak aquaculture systems. Despite showing high bioaccumulation, microcystin did not influence the survival and growth of *Oreochromis* spp. This study also revealed that microcystin accumulated in *Oreochromis* spp. tissues could be two to three orders of magnitude higher than the recommended TDI guideline provided by WHO. The result poses an alarming sign particularly to Malaysian, which may present a significant health risk of microcystin to human through aquaculture fish consumption.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Background

This research was conducted with aims to investigate the presence and abundance of toxic cyanobacteria in aquaculture systems, as well as its toxic accumulation in fish tissues. After thorough discussion, the conclusions and recommendations for the first part and second part of the study (see Table 1.1) are as documented in section 5.1.1 and section 5.1.2, respectively.

5.1.1 Presence and Abundance of Toxic Cyanobacteria in Perak Aquaculture Systems

The presence of toxic cyanobacteria was detected on-site during water sampling, as well as in the laboratory through microscopic analysis. High variations of instantaneous chlorophyll fluorescence were quantified in this study, which ranged between 2.67 r.u to 5,308 r.u. This indicates spatial distribution of cyanobacterial intensities in aquaculture ponds in Perak. Potentially toxic cyanobacteria, namely *Microcystis* spp., *Anabaena* spp., *Oscillatoria* spp. and *Nostoc* spp. were observed in the collected water samples. Of these, *Microcystis* spp. was the most common cyanobacterial taxa present in Perak aquaculture systems, whereby its occurrence was recorded in all of the studied ponds. This was followed by *Anabaena* spp. (87.5%) and *Oscillatoria* spp. (70%).

During the sampling period, 75% of the selected aquaculture ponds were under cyanobacterial bloom (> 50 µg chl-*a* /L) and containing unsafe level of microcystin (> 20 µg MC-LR equivalent /L). Cyanobacterial biomass of up to 436 µg chl-*a* /L and microcystins concentration of up to 295.86 µg MC-LR equivalent /L detected in this study highlighted the need for regular monitoring of cyanobacterial abundance in aquaculture systems. This is essential to avoid possible economic losses, as well as microcystin contamination in the aquaculture harvests deliver to consumers. Since the variability of instantaneous chlorophyll fluorescence of cyanobacteria (F_T) at 620 nm explained 82% of the variability in cyanobacterial biomass, this study suggests that AquaPen-C can be used as a monitoring tool to provide an alert to the occurrence of toxic cyanobacteria in aquaculture systems.

Positive relationship was observed between cyanobacterial biomass with microcystin concentration in this study. Despite having significant correlation, the variability of cyanobacterial biomass only explained 37.5% of the variability in microcystin concentration. The discrepancies in variability present potential significant threat to the management of toxic cyanobacterial bloom which might lead to underestimation of possible cyanobacterial toxicity through indirect microcystin assessment. In order to truly understand the relationship between cyanobacterial biomass with microcystin concentration, more studies should be carried out to assess the correlation between the parameters at both spatial and temporal scales.

Although the occurrence of cyanobacteria in water bodies has always been associated to the event of eutrophication, this study found no correlations between any of the analysed nutrients with both cyanobacterial biomass and microcystin production. This confirmed that nutrients are not the only factors affecting the abundance and toxicity of cyanobacteria, but rather combination of multiple environmental parameters. Environmental variables such as temperature, dissolved oxygen, and pH were found having significant correlations with cyanobacterial biomass in the present study. Microcystin concentration, on the other hand only showed significant relationship with temperature.

Multiple linear regression with forward selection revealed that a combination of temperature and pH was the key environmental variable that trigger the proliferation of cyanobacteria, as well as its toxicity in the selected aquaculture ponds in Perak. This showed that temperature does not work alone, but act with other environmetal factors to promote the cyanobacterial bloom. The model statistically

explained 46.2% of the variability in cyanobacterial biomass and 30.4% of the variability in microcystin concentration. Comparison between beta values revealed that temperature was a more powerful predictor of both cyanobacterial biomass and microcystin concentration in the studied ponds. The finding of this study illustrated the impact of global warming to the occurrence of toxic cyanobacterial blooms in our freshwater systems. The excessive growth of cyanobacteria enhances the water pH, which will further promoting cyanobacterial proliferation in water bodies.

Despite the fact that temperature is responsible for the proliferation of toxic cyanobacteria, negative relationships between temperature with both cyanobacterial biomass and microcystin concentration were recorded in this study. This demonstrated that the influence of global warming to the growth of cyanobacteria could be true to only a certain extent of high temperature. Significant decline in cyanobacterial biomass, as well as microcystin concentration were observed in the present study at temperature above 32 °C, indicating potential photoinhibition of cyanobacterial cells at elevated temperature. Even so, the shift in cyanobacterial composition from the non-toxic to the toxic strains as a response to environmental stressor (high temperature) must not be neglected. This situation might even amplify the production of microcystin in aquatic systems and present greater risk to human through fish consumption in the future.

5.1.2 Bioaccumulation of Microcystin in *Oreochromis* spp. Tissue and Its Effect on the Survival and Growth of Fish

Fingerlings of *Oreochromis* spp. are potent to accumulate microcystins in their tissues when exposed to the naturally-occurring microcystins concentration in our aquaculture systems. This study found that the amount of microcystin bioaccumulated in fish tissues was positively related to the amount of microcystin in the surrounding water. Despite showing high bioaccumulation, microcystin did not influence the survival and growth of fish. These findings illustrated the potential health risk of microcystin contamination in our food chain particularly through the consumption of aquaculture products. Microcystin accumulated in the entire body of fish could be two to three orders of magnitude higher than the TDI guideline recommended by WHO.

However, since the experiment was only conducted under laboratory environment, at short duration of time, and limited number of fish, further studies are required to determine the safety of the ready-to-harvest fish in our aquaculture systems. This study suggests that acclimatisation and fish culture should be carried out in the same tanks to reduce the physiological stress amongst fish during experiment. Microcystin contamination in fish hatchery is possible, hence proper selection of fish source prior to the experiment is crucial. This should be done straight upon receiving the fish from hatchery by sacrificing several percentage of them to determine the microcystin level.

Incomplete digestion amongst fish that died during the experiment may lead to error in growth measurement. This study suggests that the remaining pellet should be

removed from the fish gut prior to final weight quantification to increase the accuracy of growth parameter. Since microcystin depuration from fish tissue is possible, microcystin exposure should be carried out for a longer period in the future to fully understand the effect of the toxic compound to fish in aquaculture systems. To increase our present knowledge on the potential health risk of microcystin in our food, microcystin assessment should be carried out on Red tilapia supplied to the public as well. This can be done by collecting fish samples from several nearby markets. Boiling was reported to increase the concentration of detected microcystin, thus it is wise to determine the presence of the hepatotoxin on both raw and cooked fish tissues.

In conclusion, the findings of this research highlighted the need to tackle the root cause of cyanobacterial bloom and microcystin contamination in aquaculture systems – global warming and climate change. Although microcystin bioaccumulation in fish tissues might not lead to mortality of the aquatic species, it could be lethal enough to pose health threat to human through fish consumption. Therefore, it is crucial to realise that microcystin poisoning is only one of the implication that we and the next generations would face in the future due to unsustainable decisions that we are making today.

REFERENCES

Abang, M. Z. A. D. (2003). *Taburan alga biru-hijau di persekitaran akuatik di Kuching, Sarawak* (Unpublished final year project). Universiti Malaysia Sarawak, Kota Samarahan, Sarawak.

Abed, R. M. M., Dobretsov, S., & Sudesh, K. (2009). Applications of cyanobacteria in biotechnology. *Journal of Applied Microbiology*, *106*(1), 1–12. doi:10.1111/j.1365-2672.2008.03918.x

Adamovský, O., Kopp, R., Hilscherová, K., Babica, P., Palíková, M., Pasková, V., … Bláha, L. (2007). Microcystin kinetics (bioaccumulation and elimination) and biochemical responses in Common carp (*Cyprinus carpio*) and Silver carp (*Hypophthalmichthys molitrix*) exposed to toxic cyanobacterial blooms. *Environmental Toxicology and Chemistry / SETAC*, *26*(12), 2687–2693. doi:10.1897/07-213.1

Ahmad-Abas, K., Ahmad, I., & Chay, S. F. (2001). A preliminary study of phytoplankton at Lake Chini, Pahang. *Pakistan Journal of Biological Sciences*, *4*(3), 309–313. Retrieved from http://docsdrive.com/pdfs/ansinet/pjbs/2001/309-313.pdf

Al-Shehri, A. M. (2010). Toxin-producing blooms of the cyanobacterium *Microcystis aeruginosa* in rainwater ponds in Saudi Arabia. *Oceanological and Hydrobiological Studies*, *39*(4), 171–187. doi:10.2478/v10009-010-0056-y

Amano, Y., Sakai, Y., Sekiya, T., Takeya, K., Taki, K., & Machida, M. (2010). Effect of phosphorus fluctuation caused by river water dilution in eutrophic lake on competition between blue-green alga *Microcystis aeruginosa* and diatom *Cyclotella* sp. *Journal of Environmental Sciences*, *22*(11), 1666–1673. doi:10.1016/S1001-0742(09)60304-1

American Public Health Association (APHA). (1998). *Standard methods for the examination of water and wastewater* (20th ed.). Washington: Prepared and published jointly by American Public Health Association, American Water Works Association, and Water Pollution Control Federation.

Andersson, A., Höglander, H., Karlsson, C., & Huseby, S. (2015). Key role of phosphorus and nitrogen in regulating cyanobacterial community composition in the northern Baltic Sea. *Estuarine, Coastal and Shelf Science*, *164*, 161–171. doi:10.1016/j.ecss.2015.07.013

Asai, R., Horiguchi, Y., Yoshida, A., McNiven, S., Tahira, P., Ikebukuro, K., … Karube, I. (2001). Detection of phycobilin pigments and their seasonal change in Lake Kasumigaura using a sensitive in situ fluorometric sensor. *Analytical Letters*, *34*(14), 2521–2533. doi:10.1081/AL-100107533

Avault, J. W. (1996). *Fundamentals of aquaculture: A step by step guide to*

commercial aquaculture. Lousiana, USA: AVA Publishing, Baton Rouge.

Azevedo, S. M. F. O., Carmichael, W. W., Jochimsen, E. M., Rinehart, K. L., Lau, S., Shaw, G. R., & Eaglesham, G. K. (2002). Human intoxication by microcystins during renal dialysis treatment in Caruaru - Brazil. *Toxicology*, *181-182*, 441–446. doi:10.1016/S0300-483X(02)00491-2

Azmi, M. Y. J., Junidah, R., Siti, M. A., Safiah, M. Y., Fatimah, S., Norimah, A. K., … Tahir, A. (2009). Body Mass Index (BMI) of adults: Findings of the Malaysian Adult Nutrition Survey (MANS). *Malaysian Journal of Nutrition*, *15*(2), 97–119. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/22691810

Backer, L. C., Carmichael, W., Kirkpatrick, B., Williams, C., Irvin, M., Zhou, Y., … Cheng, Y. S. (2008). Recreational exposure to low concentrations of microcystins during an algal bloom in a Small Lake. *Marine Drugs*, *6*(2), 389–406. doi:10.3390/md20080018

Baldia, S. F., Conaco, M. C. G., Nishijima, T., Imanishi, S., & Harada, K. I. (2003). Microcystin production during algal bloom occurrence in Laguna de Bay, the Philippines. *Fisheries Science*, *69*, 110–116. doi:10.1046/j.1444-2906.2003.00594.x

Ballot, A., Pflugmacher, S., Wiegand, C., Kotut, K., & Krienitz, L. (2003). Cyanobacterial toxins in Lake Baringo, Kenya. *Limnologica - Ecology and Management of Inland Waters*, *33*(1), 2–9. doi:10.1016/S0075-9511(03)80003-8

Barros, L. S. S., de Souza, F. C., Tavares, L. H. S., & Amaral, L. A. (2010). Microcystin-LR in Brazilian aquaculture production systems. *Water Environment Research : A Research Publication of the Water Environment Federation*, *82*, 240–248. doi:10.2175/106143009X447849

Bartram, J. (Ed.). (2015). *Routledge handbook of water and health*. Retrieved from https://books.google.com.my/books?id=hBHKCgAAQBAJ&pg=PT163&lpg=PT163&dq=morphological+difference+between+planktothrix+an+oscillatoria&source=bl&ots=pd0LFyf8Hx&sig=JUK9jfBM3ctjfxnmUVokPuUjplI&hl=en&sa=X&redir_esc=y#v=onepage&q=morphological difference bet

Bellinger, E. G., & Sigee, D. C. (2010). *Freshwater algae: Identification and use as bioindicators*. United Kingdom: John Wiley & Sons Ltd.

Berry, J. P., Lee, E., & Laenen, A. (2011). Bioaccumulation of microcystins by fish associated with a persistent cyanobacterial bloom in Lago de Patzcuaro (Michoacan, Mexico). *Environmental Toxicology and Chemistry*, *30*(7), 1621–1628. doi:10.1002/etc.548

Bogusz, M. J. (Ed.). (2000). *Handbook of analytical separations*. Elsevier Science & Technology.

Boopathi, T., & Ki, J.-S. (2014). Impact of environmental factors on the regulation of

cyanotoxin production. *Toxins*, *6*(7), 1951–1978. doi:10.3390/toxins6071951

Boyd, C. E. (1998). *Water quality for pond aquaculture*. Alabama, USA: Department of Fisheries and Allied Aquacultures, Auburn University, Alabama.

Boyd, C. E., & Tucker, C. S. (1998). *Pond aquaculture water quality management* (1st ed.). New York: Springer US.

Brock, T. D. (1973). Evolutionary and ecological aspects of the cyanophytes. In N. G. Carr & B. A. Whitton (Eds.), *The Biology of the Blue-Green Algae* (pp. 487–500). Oxford: Blackwell Scientific Publications.

Brookes, J. D., O'Brien, K. R., Burford, M. A., Bruesewitz, D. A., Hodges, B. R., McBride, C., & Hamilton, D. P. (2013). Effects of diurnal vertical mixing and stratification on phytoplankton productivity in geothermal Lake Rotowhero, New Zealand. *Inland Waters*, *3*, 369–376. doi:10.5268/IW-3.3.625

Brutemark, A., Engström-Öst, J., Vehmaa, A., & Gorokhova, E. (2015). Growth, toxicity and oxidative stress of a cultured cyanobacterium (*Dolichospermum* sp.) under different CO2 /pH and temperature conditions. *Phycological Research*, *63*, 56–63. doi:10.1111/pre.12075

Bury, N. R., Eddy, F. B., & Codd, G. A. (1995). The effects of the cyanobacterium *Microcystis aeruginosa*, the cyanobacterial hepatotoxin microcystin-LR, and ammonia on growth rate and ionic regulation of brown trout. *Journal of Fish Biology*, *46*(6), 1042–1054. doi:10.1111/j.1095-8649.1995.tb01408.x

Butler, N., Carlisle, J. C., Linville, R., & Washburn, B. (2009). *Microcystins: A brief overview of their toxicity and effects, with special reference to fish, wildlife and livestock*. Retrieved from http://oehha.ca.gov/ecotox/documents/Microcystin03 1209.pdf

Butterwick, C., Heaney, S. I., & Talling, J. F. (2005). Diversity in the influence of temperature on the growth rates of freshwater algae, and its ecological relevance. *Freshwater Biology*, *50*(2), 291–300. doi:10.1111/j.1365-2427.2004.01317.x

Campbell, D., Hurry, V., Clarke, a K., Gustafsson, P., & Oquist, G. (1998). Chlorophyll fluorescence analysis of cyanobacterial photosynthesis and acclimation. *Microbiology and Molecular Biology Reviews : MMBR*, *62*(3), 667–683. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC98930/

Carneiro, R. L., Beatriz, A., Pacheco, F., Maria, S., & Oliveira, F. De. (2013). Growth and saxitoxin production by *Cylindrospermopsis raciborskii* (cyanobacteria) correlate with water hardness. *Marine Drugs*, *11*(8), 2949–2963. doi:10.33 90/md11082949

Carrasco, D., Moreno, E., Sanchis, D., Wörmer, L., Paniagua, T., Del Cueto, A., & Quesada, A. (2006). Cyanobacterial abundance and microcystin occurrence in Mediterranean water reservoirs in Central Spain: Microcystins in the Madrid

area. *European Journal of Phycology*, *41*(3), 281–291. doi:10.1080/09670260600801724

Castenholz, R. W. (2012). Cyanobacteria. In D. R. Boone & R. W. Castenholz (Eds.), *Bergey's manual of systematic bacteriology: Volume One: The archaea and the deeply branching and phototrophic bacteria* (2nd ed., pp. 473–599). New York: Springer Science & Business Media.

Cazenave, J., Wunderlin, D. A., Bistoni, M. de los Á., Amé, M. V., Krause, E., Pflugmacher, S., & Wiegand, C. (2005). Uptake, tissue distribution and accumulation of microcystin-RR in *Corydoras paleatus*, *Jenynsia multidentata* and *Odontesthes bonariensis*: A field and laboratory study. *Aquatic Toxicology*, *75*(2), 178–190. doi:10.1016/j.aquatox.2005.08.002

Chan, H. Y. (2009). *Kepelbagaian fitoplankton di Tanjung Kuin, Tasek Bera, Pahang* (Final year project, Universiti Kebangsaan Malaysia, Selangor, Malaysia). Retrieved from http://www.ukm.my/ahmad/tesispelajar/ fitoberakuin.htm

Chia, A., Oniye, S., Ladan, Z., Lado, Z., Pila, A., Inekwe, V., & Mmerole, J. (2009). A survey for the presence of microcystins in aquaculture ponds in Zaria, Northern-Nigeria: Possible public health implication. *African Journal of Biotechnology*, *8*(22), 6282–6289. doi:10.5897/AJB09.1263

Chorus, I., & Bartram, J. (Eds.) (1999). *Toxic cyanobacteria in water: A guide to their public health consequences, monitoring and management*. *World Health Organisation*. doi:10.1046/j.1365-2427.2003.01107.x

Codd, G. A., Azevedo, S. M. F. O., Bagchi, S. N., Burch, M. D., Carmichael, W. W., Harding, W. R., … Utkilen, H. C. (2005). *Cyanonet: A global network for cyanobacterial bloom and toxin risk management: initial situation assessment and recommendations*. Paris: United Nations Educational, Scientific and Cultural Organisation (UNESCO).

Codd, G. A., Steffensen, D. A., Burch, M. D., & Baker, P. D. (1994). Toxic blooms of cyanobacteria in Lake Alexandrina, South Australia - Learning from history. *Marine and Freshwater Research*, *45*(5), 731–736. doi:10.1071/MF9940731

Common Symptoms. (n.d.). Retrieved from http://www.fishnet.org/sick-fish-chart.htm

Correll, D. L. (1998). The Role of phosphorus in the eutrophication of receiving waters: A review. *Journal of Environment Quality*, *27*(2), 261. doi:10.2134/jeq1998.00472425002700020004x

Cuiya, R. J. X. (1998). Distribution characteristics of chlorophyll-*a* in principal culture areas in Xinlin and Tongan of Xiamen. *Journal of Fujian Fisheries*, *4*, 000.

Davis, T. W., Berry, D. L., Boyer, G. L., & Gobler, C. J. (2009). The effects of

temperature and nutrients on the growth and dynamics of toxic and non-toxic strains of *Microcystis* during cyanobacteria blooms. *Harmful Algae, 8*, 715–725. doi:10.1016/j.hal.2009.02.004

Davis, T. W., Watson, S. B., Rozmarynowycz, M. J., Ciborowski, J. J. H., McKay, R. M., & Bullerjahn, G. S. (2014). Phylogenies of microcystin-producing cyanobacteria in the lower Laurentian Great Lakes suggest extensive genetic connectivity. *PLoS ONE, 9*(9), e106093. doi:10.1371/journal.pone.0106093

Department of Fisheries Malaysia (DoF Malaysia). (2008). *Annual fisheries statistics 2008*. Retrieved from http://www.dof.gov.my/en/annual-fisheries-statistics20082

Department of Fisheries Malaysia (DoF Malaysia). (2009). *Annual fisheries statistics 2009*. Retrieved from http://www.dof.gov.my/en/list-of-annual-fisheries statistics -20093

Department of Fisheries Malaysia (DoF Malaysia). (2010). *Annual fisheries statistics 2010*. Retrieved from http://www.dof.gov.my/en/list-of-annual-fisheries-statistics-20103

Department of Fisheries Malaysia (DoF Malaysia). (2011). *Annual fisheries statistics 2011*. Retrieved from http://www.dof.gov.my/en/senarai-perangkaan-perikanan-2011

Department of Fisheries Malaysia (DoF Malaysia). (2012a). *Annual fisheries statistics 2012*. Retrieved from http://www.dof.gov.my/en/perangkaan-perikanan-tahunan-2012

Department of Fisheries Malaysia (DOF Malaysia). (2012b). *Newsletter 2012*. Retrieved from http://www.dof.gov.my

Department of Fisheries Malaysia (DoF Malaysia). (2013a). *Annual fisheries statistics 2013*. Retrieved from http://www.dof.gov.my/senarai-perangkaan-perikanantahunan-2013

Department of Fisheries Malaysia (DoF Malaysia). (2013b). *Perangkaan tahunan perikanan 2013*. Retrieved from http://www.dof.gov.my/senarai-perangkaan-perikanan-tahunan-2013

Deusch, O., Landan, G., Roettger, M., Gruenheit, N., Kowallik, K. V., Allen, J. F., … Dagan, T. (2008). Genes of cyanobacterial origin in plant nuclear genomes point to a heterocyst-forming plastid ancestor. *Molecular Biology and Evolution, 25*(4), 748–761. doi:10.1093/molbev/msn022

Dokulil, M. T., & Teubner, K. (2000). Cyanobacterial dominance in lakes. *Hydrobiologia, 438,* 1–12. doi:10.1023/A:1004155810302

Dong, G., Zhu, X., Han, D., Yang, Y., Song, L., & Xie, S. (2009). Effects of dietary cyanobacteria of two different sources on growth and recovery of hybrid tilapia

(*Oreochromis niloticus* x *O. aureus*). *Toxicon*, *54*(3), 208–216. doi:10.1016/j.toxicon.2009.03.031

El Ghazali, I., Saqrane, S., Carvalho, a. P., Ouahid, Y., Del Campo, F. F., Vasconcelos, V., & Oudra, B. (2010). Effects of the microcystin profile of a cyanobacterial bloom on growth and toxin accumulation in Common carp (*Cyprinus carpio*) larvae. *Journal of Fish Biology*, *76*(6), 1415–1430. doi:10.1111/j.1095-8649.2010.02572.x

Elçi, Ş., (2008). Effects of thermal stratification and mixing on reservoir water quality. *Limnology*, *9*, 135–142. doi:10.1007/s10201-008-0240-x

Elliott, J. A. (2010). The seasonal sensitivity of cyanobacteria and other phytoplankton to changes in flushing rate and water temperature. *Global Change Biology*, *16*(2), 864–876. doi:10.1111/j.1365-2486.2009.01998.x

Ernst, B., Hitzfeld, B., & Dietrich, D. (2001). Presence of *Planktothrix* sp. and cyanobacterial toxins in Lake Ammersee, Germany and their impact on Whitefish (*Coregonus lavaretus L.*). *Environmental Toxicology*, *16*(6), 483–488. doi:10.1002/tox.10006

Fatimah, M. Y., Mohd. Mohsin, A. K., & Mustafa, K. (1984). Phytoplankton composition and productivity of a shallow tropical lake. *Pertanika*, *7*(3), 101–113.

Ferrão-Filho, A. D. S., & Kozlowsky-Suzuki, B. (2011). Cyanotoxins: Bioaccumulation and effects on aquatic animals. *Marine Drugs*, *9*, 2729–2772. doi:10.3390/md9122729

Food and Agriculture Organisation (FAO). (2014). *The state of world fisheries and aquaculture: Opportunities and challenges*. Rome: Food and Agriculture Organisation of the United Nations. doi:92-5-105177-1

Food and Agriculture Organisation (FAO). (2015). *Tilapia - April 2015*. Retrieved from http://www.fao.org/in-action/globefish/market-reports/resource-detail/en/c/336942/

Francis, G. (1878). Poisonous Australian Lake. *Nature*, *18*(444), 11–12.doi:10.1038/018011d0

Fujiki, H., & Suganuma, M. (2011). Tumor promoters - Microcystin-LR, nodularin and TNF-α and human cancer development. *Anti-Cancer Agents in Medicinal Chemistry*, *11*(1), 4–18. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/21269254

Fujimoto, N., Sudo, R., Sugiura, N., & Inamori, Y. (1997). Nutrient-limited growth of *Microcystis aeruginosa* and *Phormidium tenue* and competition under various N : P supply ratios and temperatures. *Limnology and Oceanography*, *42*(2), 250–256. doi:10.4319/lo.1997.42.2.0250

Ghazali, I. El, Saqrane, S., Carvalho, A. P., Ouahid, Y., Oudra, B., Campo, F. F. Del, & Vasconcelos, V. (2009). Compensatory growth induced in zebrafish larvae after pre-exposure to a *microcystis aeruginosa* natural bloom extract containing microcystins. *International Journal of Molecular Sciences*, *10*, 133–146. doi:10.3390/ijms10010133

Goettsch, B., & Hernández, H. M. (2006). Beta diversity and similarity among cactus assemblages in the Chihuahuan Desert. *Journal of Arid Environments*, *65*(4), 513–528. doi:10.1016/j.jaridenv.2005.08.008

Gregor, J., & Maršálek, B. (2005). A simple in vivo fluorescence method for the selective detection and quantification of freshwater cyanobacteria and eukaryotic algae. *Acta Hydrochimica et Hydrobiologica*, *33*(2), 142–148. doi:10.1002/aheh.200400558

Gregor, J., Maršálek, B., & Šípková, H. (2007). Detection and estimation of potentially toxic cyanobacteria in raw water at the drinking water treatment plant by in vivo fluorescence method. *Water Research*, *41*(1), 228–234. doi:10.1016/j.watres.2006.08.011

Gruber, N. (2008). The marine nitrogen cycle: Overview and challenges. *Nitrogen in the Marine Environment*, 1–50. doi:10.1016/B978-0-12-372522-6.00001-3

Guiry, M. D. (2012). How many species of algae are there? *Journal of Phycology*, *48*(5), 1057–1063. doi:10.1111/j.1529-8817.2012.01222.x

Gupta, M. V., Acosta, B. O., Fish, C., & Fish, M. (2004). A review of global tilapia farming practices. *Aquaculture Asia*, *9*(1), 1–14. Retrieved from http://library.enaca.org/AquacultureAsia/Articles/Jan-March-2004/6global-review-tilapia.pdf

Håkanson, L., Bryhn, A. C., & Hytteborn, J. K. (2007). On the issue of limiting nutrient and predictions of cyanobacteria in aquatic systems. *Science of the Total Environment*, *379*(1), 89–108. doi:10.1016/j.scitotenv.2007.03.009

Hamzah, A., Nguyen, & Ponzoni, R. W. (2008, October). Performance and survival of three Red tilapia strains (*Oreochromis* spp.) in pond environment in Kedah state, Malaysia. In *8th Symposium on Tilapia in Aquaculture*, Egypt.

Hao, L., Xie, P., Fu, J., Li, G., Xiong, Q., & Li, H. (2008). The effect of cyanobacterial crude extract on the transcription of GST mu, GST kappa and GST rho in different organs of goldfish (*Carassius auratus*). *Aquatic Toxicology*, *90*, 1–7. doi:10.1016/j.aquatox.2008.07.006

Harada, K., Tsuji, K., Watanabe, M. F., & Kondo, F. (1996). Stability of microcystins from cyanobacteria - III. Effect of pH and temperature. *Phycologia*, *35*(6S), 83–88. doi:10.2216/i0031-8884-35-6S-83.1

Hargreaves, J. A., & Tucker., C. S. (1996). Evidence for control of water quality in Channel catfish (*Ictalurus punctatus*) ponds by phytoplankton biomass and

sediment oxygenation. *Journal of the World Aquaculture Society*, *27*(1), 21–29.

Hargrove, M., & Hargrove, M. (2011). *Freshwater aquariums for dummies* (2nd ed.). Retrieved from http://www.dummies.com/how-to/content/how-much-food-is enough-for-aquarium-fish.html

Hauser-Davis, R. A., Lavradas, R. T., Lavandier, R. C., Rojas, E. G. A., Guarino, A. W. S., & Ziolli, R. L. (2015). Accumulation and toxic effects of microcystin in tilapia (*Oreochromis niloticus*) from an eutrophic Brazilian lagoon. *Ecotoxicology and Environmental Safety*, *112*, 132–136. doi:10.1016/j.ecoenv.20 14.10.036

Havens, K. E. (2008). Cyanobacteria blooms: Effects on aquatic ecosystems. In H. K. Hudnell (Ed.), *Cyanobacterial harmful algal blooms: State of the science and research needs* (pp. 733–747). New York: Springer Science & Business Media.

Health and ecological effects. (2015, June). Retrieved from http://www2.epa.gov/ nutrient-policy-data/health-and-ecological-effects

Herrero, A., Muro-pastor, A. M., Flores, E., Herrero, A., Muro-pastor, A. M., & Flores, E. (2001). Nitrogen control in cyanobacteria. *Journal of Bacteriology*, *183*(2), 411–425. doi:10.1128/JB.183.2.411

Hotto, A. M., Satchwell, M. F., Berry, D. L., Gobler, C. J., & Boyer, G. L. (2008). Spatial and temporal diversity of microcystins and microcystin-producing genotypes in Oneida Lake, NY. *Harmful Algae*, *7*(5), 671–681. doi:10.1016/ j.hal.2008.02.001

Hudnell, H. K. (Ed.). (2008). *Cyanobacterial harmful algal blooms: State of the science and research needs*. New York: Springer Science & Business Media.

Ibelings, B. W., Bruning, K., De Jonge, J., Wolfstein, K., Dionisio Pires, L. M., Postma, J., & Burger, T. (2005). Distribution of microcystins in a lake foodweb: No evidence for biomagnification. *Microbial Ecology*, *49*(1), 487–500. doi:10.10 07/s00248-004-0014-x

Ibelings, B. W., Vonk, M., Los, H. F. J., van Der Molen, D. T., & Mooij, W. M. (2003). Fuzzy modeling of cyanobacterial surface waterblooms: validation with NOAA-AVHRR satellite images. *Ecological Applications*, *13*(5), 1456–1472. doi:10.1890/01-5345

Ibrahem, M. D., Khairy, H. M., & Ibrahim, M. a. (2012). Laboratory exposure of *Oreochromis niloticus* to crude microcystins (containing microcystin-LR) extracted from Egyptian locally isolated strain (*Microcystis aeruginosa Kützing*): Biological and biochemical studies. *Fish Physiology and Biochemistry*, *38*, 899–908. doi:10.1007/s10695-011-9577-x

Indabawa, I. (2010). Detection of variants of microcystin produced by *Microcystis aeruginosa* in some burrow pits of Kano, Nigeria. *Bayero Journal of Pure and*

Applied Sciences, *2*(1), 189–197. doi:10.4314/bajopas.v2i1.58544

Jackson, P. E. (2000). Ion chromatography in environmental analysis. In R. A. Meyers (Ed.), *Encyclopedia of Analytical Chemistry*. Chichester: John Wiley & Sons Ltd.

Jacoby, J. M., Collier, D. C., Welch, E. B., Hardy, F. J., & Crayton, M. (2000). Environmental factors associated with a toxic bloom of *Microcystis aeruginosa*. *Canadian Journal of Fisheries and Aquatic Sciences*, *57*(1), 231–240. doi: 10.1139/f99-234

Jähnichen, S., Long, B. M., & Petzoldt, T. (2011). Microcystin production by *Microcystis aeruginosa*: Direct regulation by multiple environmental factors. *Harmful Algae*, *12*, 95–104. doi:10.1016/j.hal.2011.09.002

Jasmina, M., Samsur, M., & Ruhana, H. (2010). *Toxicity assessment of cyanobacterial strains using brine shrimp and mouse bioassay*. Paper presented at the Proceedings of the 11th Symposium for the Malaysian Society of Applied Biology 2010, Universiti Malaysia Sarawak, Kota Samarahan, Sarawak. Abstract retrieved from http://ir.unimas.my/1072/1/Toxicity%20Assessment%20of%20 Cyanobacterial%20Strains%20Using%20Brine%20Shrimp%20and%20Mouse% 20Bioassay.pdf

Jayatissa, L. P., Silva, E. I. L., McElhiney, J., & Lawton, L. A. (2006). Occurrence of toxigenic cyanobacterial blooms in freshwaters of Sri Lanka. *Systematic and Applied Microbiology*, *29*(2), 156–164. doi:10.1016/j.syapm.2005.07.007

Jewel, M. A. S., Affan, M. A., & Khan, S. (2003). Fish mortality due to cyanobacterial bloom in an aquaculture pond in Bangladesh. *Pakistan Journal of Biological Sciences*, *6*(12), 1046–1050.

Jiang, Y., Yu, G., Chai, W., Song, G., & Li, R. (2013). Congruence between *mcy* based genetic type and microcystin composition within the populations of toxic *Microcystis* in a plateau lake, China. *Environmental Microbiology Reports*, *5*(5), 637–647. doi:10.1111/1758-2229.12062

Johnson, K. S., Gordon, R. M., & Coale, K. H. (1997). What controls dissolved iron concentrations in the world ocean? - A comment. *Marine Chemistry*, *57*(3-4), 173–179. doi:10.1016/S0304-4203(97)00046-7

Jos, Á., Pichardo, S., Prieto, A. I., Repetto, G., Vázquez, C. M., Moreno, I., & Cameán, A. M. (2005). Toxic cyanobacterial cells containing microcystins induce oxidative stress in exposed tilapia fish (*Oreochromis* sp.) under laboratory conditions. *Aquatic Toxicology*, *72*, 261–271. doi:10.1016/j.aquatox. 2005.01.003

Joung, S. H., Oh, H. M., Ko, S. R., & Ahn, C. Y. (2011). Correlations between environmental factors and toxic and non-toxic *Microcystis* dynamics during bloom in Daechung Reservoir, Korea. *Harmful Algae*, *10*(2), 188–193.

doi:10.1016/j.hal.2010.09.005

Kamjunke, N., Mendonca, R., Hardewig, I., & Mehner, T. (2002). Assimilation of different cyanobacteria as food and the consequences for internal energy stores of juvenile roach. *Journal of Fish Biology*, *60*(3), 731–738. doi:10.1006/jfbi.2002.1894

Kankaanpää, H. T., Holliday, J., Schröder, H., Goddard, T. J., Von Fister, R., & Carmichael, W. W. (2005). Cyanobacteria and prawn farming in northern New South Wales, Australia - A case study on cyanobacteria diversity and hepatotoxin bioaccumulation. *Toxicology and Applied Pharmacology*, *203*, 243–256. doi:10.1016/j.taap.2004.04.012

Kaufman, D. G., & Franz, C. M. (2000). *Biosphere 2000: Protecting our global environment* [DX Reader version]. Retrieved from https://books.google.com.my/books/about/Biosphere_2000.html?id=xBG99KR52mgC&redir_esc=y

Keenum, M. E., & Waldrop., J. E. (1988). *Economic analysis of farm-raised catfish production in Mississippi* [DX Reader version]. https://books.google.com.my/books/about/Economic_Analysis_of_Farm_raised_Catfish.html?id=4UsnAQAAMAAJ&redir_esc=y

Klaassen, C. D., & Watkins, J. B. (1984). Mechanisms of bile formation, hepatic uptake, and biliary excretion. *Pharmacological Reviews*, *36*(1), 1–67. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6144113

Komárek, J. (2005). Phenotype diversity of the heterocytous cyanoprokaryotic genus *Anabaenopsis*. *Czech Phycology*, *5*(1), 1–35. Retrieved from http://fottea.czechphycology.cz/pdfs/fot/2005/01/01.pdf

Komarek, J., & Hauer, T. (2013). *CyanoDB.cz - On-line database of cyanobacterial genera - World-wide electronic publication*. Retrieved http://www.cyano db.cz

Komarek, J., Kastovsky, J., Mares, J., & Johansen, J. R. (2014). Taxonomic classification of cyanoprokaryotes (cyanobacterial genera) 2014, using a polyphasic approach. *Preslia*, *86*, 295–335.

Kotak, B. G., Lam, A. K. Y., Prepas, E. E., & Hrudey, S. E. (2000). Role of chemical and physical variables in regulating microcystin-LR concentration in phytoplankton of eutrophic lakes. *Canadian Journal of Fisheries and Aquatic Sciences*, *57*(8), 1584–1593. doi:10.1139/f00-091

Kranzler, C., Rudolf, M., Keren, N., & Schleiff, E. (2013). Iron in Cyanobacteria. *Advances in Botanical Research*, *65*, 57-105. doi:10.1016/B978-0-12-394313-2.00003-2

Kromkamp, J. (1987). Formation and functional significance of storage products in cyanobacteria. *New Zealand Journal of Marine and Freshwater Research*, *21*(3), 457–465. doi:10.1080/00288330.1987.9516241

Kumar, M., Kulshreshtha, J., & Singh, G. P. (2011). Growth and biopigment accumulation of cyanobacterium *Spirulina platensis* at different light intensities and temperature. *Brazilian Journal of Microbiology*, (42), 1128–1135. doi:10.1590/S1517-83822011000300034

Landsberg, J. H. (2002). The effects of harmful algal blooms on aquatic organisms. *Reviews in Fisheries Science, 10*(2), 113–390. doi:10.1080/20026491051695

Lawton, L. A., Edwards, C., & Codd, G. A. (1994). Extraction and high-performance liquid chromatographic method for the determination of microcystins in raw and treated waters. *Analyst*, *119*, 1525–1530.

Lawton, L. A., Marsalek, B., Padisák, J., & Chorus, I. (1999). Determination of cyanobacteria in the laboratory. In I. Chorus & J. Bartram (Eds.), *Toxic Cyanobacteria in Water: A guide to their public health consequences, monitoring and management. World Health Organisation*. doi:10.1046/j.1365-2427.2003.01107.x

Lee, S. J., Jang, M. H., Kim, H. S., Yoon, B. D., & Oh, H. M. (2000). Variation of microcystin content of *Microcystis aeruginosa* relative to medium N:P ratio and growth stage. *Journal of Applied Microbiology*, *89*, 323–329. doi:10.1046/j.1365-2672.2000.01112.x

Li, X. Y., Chung, I. K., Kim, J. I., & Lee, J. A. (2004). Subchronic oral toxicity of microcystin in Common carp (*Cyprinus carpio L.*) exposed to *Microcystis* under laboratory conditions. *Toxicon*, *44*(8), 821–827. doi:10.1016/j.toxicon.2004.06.010

Li, Y., Sheng, J., Sha, J., & Han, X. (2008). The toxic effects of microcystin-LR on the reproductive system of male rats in vivo and in vitro. *Reproductive Toxicology*, *26*(3-4), 239–245. doi:10.1016/j.reprotox.2008.09.004

Lim, P. T., Gires, U., & Leaw, C. P. (2012). Harmful algal blooms in Malaysian waters. *Sains Malaysiana*, *41*(12), 1509–1515.

Lim, P. T., Leaw, C. P., & Usup, G. (2003). Status of HAB and potential application of remote sensing in detection of HAB events in Malaysian waters. In *Proceedings of the First Red Tide Watcher Symposium*. University of Tokyo, Japan.

Lone, Y., Koiri, R. K., & Bhide, M. (2015). An overview of the toxic effect of potential human carcinogen microcystin-LR on testis. *Toxicology Reports*, *2*, 289–296. doi:10.1016/j.toxrep.2015.01.008

Lorenzen, C. J. (1967). Determination of chlorophyll and pheo-pigments: Spectrophotometric equations. *Limnology and Oceanography*, *12*(2), 343–346. doi:10.4319/lo.1967.12.2.0343

Lürling, M., Eshetu, F., Faassen, E. J., Kosten, S., & Huszar, V. L. M. (2012).

Comparison of cyanobacterial and green algal growth rates at different temperatures. *Freshwater Biology*, *58*, 552–559. doi:10.1111/j.1365-2427.2012.02866.x

Magalhaes, V. F., Soares, R. M., & Azevedo, S. M. F. O. (2001). Microcystin contamination in fish from the Jacarepagua Lagoon (Rio de Janeiro, Brazil): Ecological implication and human health risk. *Toxicon*, *39*, 1077–1085. doi: 10.1016/S0041-0101(00)00251-8

Magalhães, V. F., Marinho, M. M., Domingos, P., Oliveira, A. C., Costa, S. M., Azevedo, L. O., & Azevedo, S. M. F. O. (2003). Microcystins (cyanobacteria hepatotoxins) bioaccumulation in fish and crustaceans from Sepetiba Bay (Brasil, RJ). *Toxicon*, *42*(3), 289–295. doi:10.1016/S0041-0101(03)00144-2

Malaysia's aquaculture production target "to hit 1.76 million metric tonnes in six years." (2014). *Far Eastern Agriculture*. Retrieved from http://www.fareasternagriculture.com/live-stock/aquaculture/malaysia-aquaculture-production-target-to-hit-1-76-million-metric-tonnes-in-six-years

Malaysian Meteorological Department. (2015). *Monthly weather bulletin*. Retrieved from http://www.met.gov.my/index.php?option=com_content&task=view&id=843&Itemid=1586

Malbrouck, C., & Kestemont, P. (2006). Effects of microcystins on fish. *Environmental Toxicology and Chemistry*, *25*(1), 72–86. doi:10.1897/05-029R.1

Malbrouck, C., Trausch, G., Devos, P., & Kestemont, P. (2003). Hepatic accumulation and effects of microcystin-LR on juvenile goldfish *Carassius auratus L. Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*, *135*(1), 39–48. doi:10.1016/S1532-0456(03)00047-4

Mansoor, H., Sorayya, M., Aishah, S., & Mosleh, M. A. A. (2011). Automatic recognition system for some cyanobacteria using image processing techniques and ANN approach. In *2011 International Conference on Environmental and Computer Science*. Singapore.

Martins, J. C., & Vasconcelos, V. M. (2009). Microcystin dynamics in aquatic organisms. *Journal of Toxicology and Environmental Health, Part B*, *12*(1), 65–82. doi:10.1080/10937400802545151

Mazuki, H. (2015, April). *Industry and Market Status of Tilapia in Malaysia*. Paper presented at the 4th International Trade and Technical Conference and Exposition on Tilapia (Tilapia 2015), Kuala Lumpur, Malaysia. Retrieved from http://www.infopesca.org/sites/default/files/complemento/actividadesrecientes/adjuntos/1369/6%20Malaysia-Industry%20and%20Market%20Status%20of%20Tilapia.pdf

Merican, F., Wan-Asmadi, W. A., Wan-Maznah, W. O., & Mashhor, M. (2006). A note on the freshwater algae of gunung stong, Kelantan, Malaysia. *Jurnal*

Biosains, *17*(1), 65–76.

Meriluoto, J. A. O., & Codd, G. A. (2005). *Cyanobacterial monitoring and cyanotoxin analysis*. Finland: ÅBO Akademi University Press.

Meriluoto, J. A. O., & Spoof, L. E. M. (2008). Cyanotoxins: Sampling, sample processing and toxin uptake. In H. K. Hudnell (Ed.), *Cyanobacterial harmful algal blooms: State of science and research needs* (pp. 483–499). New York: Springer Science & Business Media.

Metcalf, J. S., & Codd, G. A. (2004). Cyanobacterial toxins in the water environment: a review of current knowledge. *Foundation for Water Research.* Retrieved from http://www.fwr.org/cyanotox.pdf

Mioni, C., Kudela, R., Baxa, D., Sullivan, M., Hayashi, K., Smythe, T., & White, C. (2011). Harmful cyanobacteria blooms and their toxins in Clear Lake and the Sacramento-San Joaquin Delta (California). *Delta (California)*, *10*, 58–150.

Mitsoura, A., Kagalou, I., Papaioannou, N., Berillis, P., Mente, E., & Papadimitriou, T. (2013). The presence of microcystins in fish *Cyprinus carpio* tissues : A histopathological study. *International Aquatic Research*, *5*(1), 1–16. doi:10.1186 /2008-6970-5-8

Miyairi, S. (1995). CO_2 assimilation in a thermophilic cyanobacterium. *Energy Conversion and Management*, *36*(6), 763–766. doi:10.1016/0196-8904(95)00 116-U

Mjoun, K., Rosentrater, K. A., & Brown, M. L. (2010). *Tilapia : Environmental biology and nutritional requirements* [DX Reader version]. Retrieved from http://pubstorage. sdstate.edu/AgBio_Publications/articles/FS963-02.pdf

Mohamed, Z. A., Carmichael, W. W., & Hussein, A. A. (2003). Estimation of microcystins in the freshwater fish *Oreochromis niloticus* in an Egyptian fish farm containing a *Microcystis* bloom. *Environmental Toxicology*, *18*(2), 137–141. doi:10.1002/tox.10111

Mohamed, Z. A., & Hussein, A. A. (2006). Depuration of microcystins in tilapia fish exposed to natural populations of toxic cyanobacteria: A laboratory study. *Ecotoxicology and Environmental Safety*, *63*, 424–429. doi:10.1016/j.ecoenv. 2005.02.006

Mohd. Nasarudin, H., & Ruhana, H. (2007). Preliminary study on cyanobacteria composition and selected water quality parameters from freshwater fish (*Tor tambroides*) ponds in Serian, Sarawak. In *Proceedings of NREM & ESH 2007*. Kuching, Sarawak.

Mohd. Nasarudin, H., & Ruhana, H. (2011a). Blue-green algae and nutrient concentrations in two *Tor tambroides* aquaculture ponds differing in construction. *Journal of Tropical Biology and Conservation*, *8*, 51–61. Retrieved

from http://www.ums.edu.my/ibtpv2/images/publication/JTBC/JTBC-VOL-8/part%206%20ruhana.pdf

Mohd. Nasarudin, H., & Ruhana, H. (2011b). Diversity and similarity among cyanobacteria assemblages from selected aquatic ecosystems in Sarawak using *B* – Indices. *Borneo Journal of Resource Science and* Technology, *1*, 28–37. Retrieved from http://www.bjrst.unimas.my/online-issue/item/4-diversity-and-similarity-among-cyanobacteria-assemblages-from-selected-aquatic-ecosystems-in-sarawak-using-%CE%B2-indices.html

Nabout, J. C., da Silva Rocha, B., Carneiro, F. M., & Sant'Anna, C. L. (2013). How many species of cyanobacteria are there? Using a discovery curve to predict the species number. *Biodiversity and Conservation*, *22*(12), 2907–2918. doi:10.1007/s10531-013-0561-x

Nagarkar, S., & Williams, G. A. (1997). Comparative techniques to quantify cyanobacteria dominated epilithic biofilms on tropical rocky shores. *Marine Ecology Progress Series*, *154*, 281–291. doi:10.3354/meps154281

Najiah, M., Aqilah, N. I., Lee, K. L., Khairulbanyyah, Z., Mithun, S., Jalal, K. C. a, … Nadirah, M. (2012). Massive mortality associated with *Streptococcus agalactiae* infection in cage-cultured Red hybrid tilapia *Oreochromis niloticus* in Como River, Kenyir Lake, Malaysia. *Journal of Biological Sciences*, *12*(8), 438–442. doi:10.3923/jobs.2012.438.442

Nasri, A., Bouaïcha, N., & Fastner, J. (2004). First report of a microcystin-containing bloom of the cyanobacteria *Microcystis* spp. in Lake Oubeira, Eastern Algeria. *Archives of Environmental Contamination and Toxicology*, *46*(2), 197–202. doi:10.1007/s00244-003-2283-7

National Hydraulic Research Institute of Malaysia (NAHRIM). (2005). *A Desktop Study on the Status of Lake Eutrophication in Malaysia - Final Report*. Retrieved from http://www.nahrim.gov.my/

Niedzwiadek, B., Scott, P. M., & Lau, B. P. Y. (2012). Monitoring of shrimp and farmed fish sold in Canada for cyanobacterial toxins. *Journal of Food Protection*, *75*(1), 160–163. doi:10.4315/0362-028X.JFP-11-324

Nor Azman, K. (2006). *Kualiti air sungai berdasarkan analisis kimia dan kepelbagaian alga* (Master's thesis, Universiti Teknologi Malaysia, Johor, Malaysia). Retrieved from http://ent.library.utm.my/client/en_AU/main/search/detailnonmodal/ent:$002f$002fSD_ILS$002f619$002fSD_ILS:619088/ada;jsessionid=76421D34E2604BF28ECCAD46A31340B7?qu=Water+quality+--+Analysis&ic=true&ps=300

Nyakairu, G. W. A, Nagawa, C. B., & Mbabazi, J. (2010). Assessment of cyanobacteria toxins in freshwater fish: A case study of Murchison Bay (Lake Victoria) and Lake Mburo, Uganda. *Toxicon*, *55*(5), 939–946. doi:10. 1016/j. toxicon.2009.07.024

Odum, H. T. (1956). Primary production in flowing waters. *Limnology and Oceanography*, *1*(2), 102–117. doi:10.4319/lo.1956.1.2.0102

Offal. (n.d.). In *Wikipedia: The Free Encyclopedia*. Retrieved from https://en.wikipedia.org/wiki/Offal

Oh, H. M., Lee, S. J., Jang, M. H., & Yoon, B. D. (2000). Microcystin production by *Microcystis aeruginosa* in a phosphorus-limited chemostat. *Applied and Environmental Microbiology*, *66*(1), 176–179. doi:10.1128/AEM.66.1.176-179.2000

Okello, W., Portmann, C., Erhard, M., Gademann, K., & Kurmayer, R. (2009). Occurrence of microcystin-producing cyanobacteria in Ugandan freshwater habitats. *Environmental Toxicology*, *24*(3), 296–303. doi:10.1002/tox

Okello, W., Portmann, C., Erhard, M., Gademann, K., & Kurmayer, R. (2010). Occurrence of microcystin-producing cyanobacteria in Ugandan freshwater habitats. *Environmental Toxicology*, *25*(4), 367–380. doi:10.1002/tox.20522

Okogwu, O. I., & Ugwumba, A. O. (2009). Cyanobacteria abundance and its relationship to water quality in the Mid-Cross River floodplain, Nigeria. *Revista de Biología Tropical*, *57*(1/2), 33–43. Retrieved from http://www.ots.ac.cr/rbt/attachments/volumes/vol57-1-2/04-Okechukwu-Cyanobacteria%20abundance%20.pdf

Oliveira, T. A., Koakoski, G., Kreutz, L. C., Ferreira, D., da Rosa, J. G. S., de Abreu, M. S., … Barcellos, L. J. G. (2013). Alcohol impairs predation risk response and communication in Zebrafish. *PLoS ONE*, *8*(10), 1–8. doi:10.1371/journal.pone.0075780

Padisák, J. (1997). Cylindrospermopsis raciborskii (Woloszynska) Seenayya et Subba Raju, an expanding, highly adaptive cyanobacterium: Worldwide distribution and review of its ecology. *Archiv Für Hydrobiologie Supplementband Monographische Beitrage*, *107*(4), 563–593. Retrieved from http://real.mtak.hu/3229/

Paerl, H. W., & Huisman, J. (2008). Climate. Blooms like it hot. *Science*, *320*(5872), 57–58. doi:10.1126/science.1155398

Paerl, H. W., & Huisman, J. (2009). Climate change: A catalyst for global expansion of harmful cyanobacterial blooms. *Environmental Microbiology Reports*, *1*(1), 27–37. doi:10.1111/j.1758-2229.2008.00004.x

Paerl, H. W., & Otten, T. G. (2013). Harmful cyanobacterial blooms: causes, consequences, and controls. *Microbial Ecology*, *65*(4), 995–1010. doi:10.1007/s00248-012-0159-y

Paerl, H. W., & Paul, V. J. (2012). Climate change: Links to global expansion of harmful cyanobacteria. *Water Research*, *46*(5), 1349–1363. doi:10.1016/j.wat

res.2011.08.002

Paerl, H. W., & Tucker, C. S. (1995). Ecology of blue-green algae in aquaculture ponds. *Journal of the World Aquaculture Society*, *26*(2), 109–131. doi:10.1111/j.1749-7345.1995.tb00235.x

Palíková, M., Krejčí, R., Hilscherová, K., Babica, P., Navrátil, S., Kopp, R., & Bláha, L. (2007). Effect of different cyanobacterial biomasses and their fractions with variable microcystin content on embryonal development of carp (*Cyprinus carpio L.*). *Aquatic Toxicology*, *81*, 312–318. doi:10.1016/j.aquatox.2007.01.001

Palikova, M., Mares, J., Kopp, R., Hlavkova, J., Navratil, S., Adamovsky, O., … Blaha, L. (2011). Accumulation of microcystins in Nile tilapia, *Oreochromis niloticus L.*, and effects of a complex cyanobacterial bloom on the dietetic quality of muscles. *Bulletin of Environmental Contamination and Toxicology*, *87*(1), 26–30. doi:10.1007/s00128-011-0279-y

Palinska, K. A., & Surosz, W. (2014). Taxonomy of cyanobacteria: A contribution to consensus approach. *Hydrobiologia*, *740*(1), 1–11. doi:10.1007/s10750-014-1971-9

Palmeri, L., Barausse, A., & Erik, S. J. (2013). *Ecological processes handbook* [DX Reader version]. Retrieved from https://books.google.com.my/books?id=0Xg0AAAAQBAJ&pg=PR11&lpg=PR11&dq=Ecological+Processes+Handbook&source=bl&ots=wJIDJSZ3jI&sig=tzTdeMgQpJ4OiL0JhadCZT1lMAo&hl=en&sa=X&redir_esc=y#v=onepage&q=Ecological%20Processes%20Handbook&f=false

Pandey, G. (2013). Feed formulation and feeding technology for fishes. *International Research Journal of Pharmacy*, *4*(3), 23–30. doi:10.7897/2230-8407.04306

Papadimitriou, T., Kagalou, I., Bacopoulos, V., & Leonardos, I. D. (2009). Accumulation of microcystins in water and fish tissues: An estimation of risks associated with microcystins in most of the Greek lakes. *Environmental Toxicology*, *25*(4), 418–427. doi:10.1002/tox.20513

Paul, V. J. (2008). Global warming and cyanobacterial harmful algal blooms. In H. K. Hudnell (Ed.), *Cyanobacterial harmful algal blooms: State of science and research needs* (pp. 239–257). New York: Springer Science & Business Media.

Pavlova, V., Babica, P., Todorova, D., Bratanova, Z., & Maršálek, B. (2006). Contamination of some reservoirs and lakes in Republic of Bulgaria by microcystins. *Acta Hydrochimica et Hydrobiologica*, *34*(5), 437–441. doi:10.1002/aheh.200600641

Peng, L., Liu, Y., Chen, W., Liu, L., Kent, M., & Song, L. (2010). Health risks associated with consumption of microcystin-contaminated fish and shellfish in three Chinese lakes: Significance for freshwater aquacultures. *Ecotoxicology and Environmental Safety*, *73*(7), 1804–1811. doi:10.1016/j.ecoenv.2010.07.043

Pimentel, J. S. M., & Giani, A. (2014). Microcystin production and regulation under nutrient stress conditions in toxic *Microcystis* strains. *Applied and Environmental Microbiology*, *80*, 5836–5843. doi:10.1128/AEM.01009-14

Poste, A. E., Hecky, R. E., & Guildford, S. J. (2011). Evaluating microcystin exposure risk through fish consumption. *Environmental Science and Technology*, *45*, 5806–5811. doi:10.1021/es200285c

Postlethwaite, M. (2010). *Introduction to Freshwater Fishkeeping* [DX Reader version]. Retrieved from https://books.google.com.my/books?id=35otAgAAQBAJ&pg=PA100&lpg=PA100&dq=fish+stress+sign+loss+of+appetite&source=bl&ots=v1AlBY7ig9&sig=blf1GURoZz9AQcyQHYWjBkNnCRA&hl=en&sa=X&redir_esc=y#v=onepage&q=fish stress sign loss of appetite&f=false

Purdie, E. L., Young, F. M., Menzel, D., & Codd, G. a. (2009). A method for acetonitrile-free microcystin analysis and purification by high-performance liquid chromatography, using methanol as mobile phase. *Toxicon*, *54*(6), 887–890. doi:10.1016/j.toxicon.2009.06.016

Rahman, S., & Jewel, A. S. (2008). Cyanobacterial blooms and water quality in two urban fish ponds. *University Journal of Zoology, Rajshahi University*, *27*, 79–84. doi: 10.3329/ujzru.v27i0.1960

Ramlah, R. (2005). *Diversity of cyanobacteria in Sarawak Lentic Zones.* (Unpublished final year project). Universiti Malaysia Sarawak, Kota Samarahan, Sarawak.

Rapala, J., & Sivonen, K. (1998). Assessment of environmental conditions that favor hepatotoxic and neurotoxic *Anabaena* spp. strains cultured under light limitation at different temperatures. *Microbial Ecology*, *36*(2), 181–192. doi:10.1007/s002489900105

Rapala, J., Sivonen, K., Lyra, C., & Niemelä, S. I. (1997). Variation of microcystins, cyanobacterial hepatotoxins, in *Anabaena* spp. as a function of growth stimuli. *Applied and Environmental Microbiology*, *63*(6), 2206–2212. Retrieved from http://aem.asm.org/content/63/6/2206.full.pdf+html

Ren, Y., Pei, H., Hu, W., Tian, C., Hao, D., Wei, J., & Feng, Y. (2014). Spatiotemporal distribution pattern of cyanobacteria community and its relationship with the environmental factors in Hongze Lake, China. *Environmental Monitoring and Assessment*, *186*(10), 6919–6933. doi:10.1007/s10661-014-3899-y

Reynolds, C. S. (1984). *The ecology of freshwater phytoplankton*. Retrieved from https://books.google.com.my/books?id=0nyrasgaTwMC&pg=PP1&lpg=PP1&dq=The+ecology+of+freshwater+phytoplankton&source=bl&ots=2jyFgoxXpB&sig=IdoCs-gITF8h_PWBL7yP6Cohf7Y&hl=en&sa=X&redir_esc=y#v=onepage&q=The%20ecology%20of%20freshwater%20phytoplankton&f=false

Reynolds, C. S. (2006). *The ecology of phytoplankton*. United States: Cambridge University Press.

Rinta-Kanto, J. M., Konopko, E. A., DeBruyn, J. M., Bourbonniere, R. a., Boyer, G. L., & Wilhelm, S. W. (2009). Lake Erie *Microcystis*: Relationship between microcystin production, dynamics of genotypes and environmental parameters in a large lake. *Harmful Algae*, *8*(5), 665–673. doi:10.1016/j.hal.2008.12.004

Rita, D. P., Valeria, V., Silvia, B. M., Pasquale, G., & Milena, B. (2014). Microcystin contamination in Sea mussel farms from the Italian Southern Adriatic Coast following cyanobacterial blooms in an artificial reservoir. *Journal of Ecosystems*, *2014*, 1–11. doi:10.1155/2014/374027

Robarts, R. D., & Zohary, T. (1987). Temperature effects on photosynthetic capacity, respiration, and growth rates of bloom-forming cyanobacteria. *New Zealand Journal of Marine and Freshwater Research*, *21*(3), 391–399. doi:10.1080/00288330.1987.9516235

Rodgers, J. H. (2008). *Algal toxins in pond aquaculture* (SRAC Publication No. 4605). Retrieved from http://www2.ca.uky.edu/wkrec/AlgalToxinsPond.pdf

Ruangrit, K., Peerapornpisal, Y., Pekkoh, J., & Whangchai, N. (2013). Microcystin accumulation in Nile tilapia, *Oreochromis niloticus*, and Giant freshwater prawns, *Macrobrachium rosenbergii*, in green water system cultivation, *4*, 60–63. doi: 10.4236/ijg.2013.45B010

Rückert, G. Von, & Giani, A. (2004). Effect of nitrate and ammonium on the growth and protein concentration of *Microcystis viridis Lemmermann* (cyanobacteria). *Revista Brasileira de Botânica*, *27*(2), 325–331. doi:10.1590/S0100-84042004000200011

Sahin, A., Tencalla, F. G., Dietrich, D. R., & Naegeli, H. (1996). Biliary excretion of biochemically active cyanobacteria (blue-green algae) hepatotoxins in fish. *Toxicology*, *106*(1-3), 123–130. doi:10.1016/0300-483X(95)03173-D

Schindler, D. W. (2012). The dilemma of controlling cultural eutrophication of lakes. *Proceedings of the Royal Society B: Biological Sciences*, *279*, 4322–4333. doi:10.1098/rspb.2012.1032

Schliewen, U. (2008). *My Aquarium*. Hauppauge, New York: Barron's Educational Series, Inc.

Schmidt, J. R., Shaskus, M., Estenik, J. F., Oesch, C., Khidekel, R., & Boyer, G. L. (2013). Variations in the microcystin content of different fish species collected from a eutrophic lake. *Toxins*, *5*, 992–1009. doi:10.3390/toxins5050992

Schrader, K. K., & Dennis, M. E. (2005). Cyanobacteria and earthy/musty compounds found in commercial catfish (*Ictalurus punctatus*) ponds in the Mississippi Delta and Mississippi-Alabama Blackland Prairie. *Water Research*, *39*(13), 2807–

2814. doi:10.1016/j.watres.2005.04.044

Schreurs, H. (1992). *Cyanobacterial Dominance - Relations to Eutrophication and Lake Morphology* [IDX Reader version]. Retrieved from https://books.google.com.my/books/about/Cyanobacterial_Dominance.html?id=lulnnQEACAAJ&redir_esc=y

Sell, R., & Aakre, D. (2004, March). Farming tilapia. *The Fish Site*. Retrieved from http://www. thefishsite.com/articles/13/farming-tilapia/

Selman, M., & Greenhalgh, S. (2009). *Eutrophication: Sources and drivers of nutrient pollution*. Retrieved from Water Resources Institute website: http://www.wri.org/publication/eutrophication-sources-and-drivers-nutrient-pollution

Shapiro, J. (1990). Current beliefs regarding dominance by blue-greens: the case for the importance of CO_2 and pH. *Verh Int Verein (theor Angrew) Limnol.*, *24*, 38–54.

Sharma, N. K., Rai, A. K., & Stal, L. J. (2013). *Cyanobacteria: An Economic Perspective* [DX Reader version]. Retrieved from https://books.google.com.my/books?id=tncqAgAAQBAJ&dq=how+dissolved+oxygen+promote+cyanobacteria&source=gbs_navlinks_s

Shaw, B., Mechenich, C., & Klessig, L. (1993). *Understanding lake data*. Retrieved from University of Wisconsin System website: http://www3.uwsp.edu/cnr-ap/weal/Documents/G3582.pdf

Sieroslawska, A., Rymuszka, A., Velisek, J., Pawlik-Skowrońska, B., Svobodova, Z., & Skowroński, T. (2012). Effects of microcystin-containing cyanobacterial extract on hematological and biochemical parameters of Common carp (*Cyprinus carpio L.*). *Fish Physiology and Biochemistry*, *38*, 1159–1167. doi:10.1007/ s10695-011-9601-1

Sigman, D. M., & Boyle, E. A. (2000). Glacial/Interglacial variations in atmospheric carbon dioxide. *Nature*, *407*(6806), 859–869. doi:10.1038/35038000

Sin Chew Daily. (2005, January 23). *Sin Chew Daily*. Retrieved from http://sinchew-i.com

Sinang, S. C. (2012a). *Spatial and temporal dynamics of cyanobacteria and microcystins in freshwater systems: Implications for the management of water resources* (Doctoral thesis, The University of Western Australia, Perth, Australia). Retrieved from https://repository.uwa.edu.au/R/-?func=dbin-jump-full&object_id=32911&local_base=GEN01-INS01

Sinang, S. C. (2012b). Toxic cyanobacterial bloom: What should Malaysian know? In *Proceedings of Internal Conference on Water Resources (ICWR)*. Johor.

Sinang, S. C., Keong, B. P., Shamsudin, S., & Sinden, A. (2015). Preliminary assessment of cyanobacteria diversity and toxic potential in ten freshwater lakes in Selangor, Malaysia. *Bulletin of Environmental Contamination and Toxicology*, *95*(4), 542-547. doi:10.1007/s00128-015-1620-7

Sinang, S. C., Reichwaldt, E. S., & Ghadouani, A. (2013). Spatial and temporal variability in the relationship between cyanobacterial biomass and microcystins. *Environmental Monitoring and Assessment*, *25*(4), 367–380. doi:10.1007/s10661-012-3031-0

Singh, S., & Asthana, R. K. (2014). Assessment of microcystin concentration in carp and catfish: A case study from Lakshmikund Pond, Varanasi, India. *Bulletin of Environmental Contamination and Toxicology*, *92*, 687–692. doi:10.1007/s00128-014-1277-7

Singh, S., Rai, P. K., Chau, R., Ravi, A. K., Neilan, B. a., & Asthana, R. K. (2015). Temporal variations in microcystin-producing cells and microcystin concentrations in two fresh water ponds. *Water Research*, *69*, 131–142. doi:10.1016/j.watres.2014.11.015

Sivonen, K., & Jones, G. (1999). Cyanobacterial toxins. In I. Chorus & J. Bartram (Eds.), *Toxic cyanobacteria in water: A guide to their public health consequences monitoring and management* (pp. 41–111). doi:10.1046/j.1365-2427.2003.01107.x

Smith, J. L., Boyer, G. L., & Zimba, P. V. (2008). A review of cyanobacterial odorous and bioactive metabolites: Impacts and management alternatives in aquaculture. *Aquaculture*, *280*, 5–20. doi:10.1016/j.aquaculture.2008.05.007

Snyder, G. S., Goodwin, A. E., & Freeman, D. W. (2002). Evidence that Channel catfish, *Ictalurus punctatus* (Rafinesque), mortality is not linked to ingestion of the hepatotoxin microcystin-LR. *Journal of Fish Diseases*, *25*(5), 275–285. doi:10.1046/j.1365-2761.2002.00374.x

Soares, R. M., Magalhães, V. F., & Azevedo, S. M. F. O. (2004). Accumulation and depuration of microcystins (cyanobacteria hepatotoxins) in Tilapia rendalli (Cichlidae) under laboratory conditions. *Aquatic Toxicology*, *70*(1), 1–10. doi:10.1016/j.aquatox.2004.06.013

Stone, D., & Bress, W. (2007). Addressing public health risks for cyanobacteria in recreational freshwaters: The Oregon and Vermont framework. *Integrated Environmental Assessment and Management*, *3*(1), 137–143. doi:10.1897/1551-3793(2007)3[137:APHRFC]2.0.CO;2

Stone, N., & Daniels, M. (2006). Algal blooms, scums and mats in ponds. Retrieved from http://www.uaex.edu/publications/pdf/fsa-9094.pdf

Su, X., Xue, Q., Steinman, A., Zhao, Y., & Xie, L. (2015). Spatiotemporal dynamics of microcystin variants and relationships with environmental parameters in Lake

Taihu, China. *Toxins*, *7*(8), 3224–3244. doi:10.3390/toxins7083224

Sunda, W. G., & Huntsman, S. A. (2015). High iron requirement for growth, photosynthesis, and low-light acclimation in the coastal cyanobacterium *Synechococcus bacillaris*. *Frontiers in Microbiology*, *6*, 1–13. doi:10.3389/fmicb.2015.00561

Suneerat, R., Wichien, Y., Paveena, T., & Monthon, G. (2014). Cyanobacterial composition and microcystin accumulation in catfish pond. *Chiang Mai Journal of Science*, *41*(1), 27–38. Retrieved from http://it.science.cmu.ac.th/ejournal/journalDetail.php?journal_id=4577

Te, S. H., & Gin, K. Y. H. (2011). The dynamics of cyanobacteria and microcystin production in a tropical reservoir of Singapore. *Harmful Algae*, *10*(3), 319–329. doi:10.1016/j.hal.2010.11.006

The World Bank. (2015). *Population, total*. Retrieved from http://data.worldbank.org/indicator/SP.POP.TOTL

Tsuji, K., Watanuki, T., Kondo, F., Watanabe, M. F., Suzuki, S., Nakazawa, H., … Harada, K.-I. (1995). Stability of microcystins from cyanobacteria - II. Effect of UV light on decomposition and isomerization. *Toxicon*, *33*(12), 1619–1631. doi:10.1016/0041-0101(95)00101-8

Tucker, C. S. (1996). The ecology of Channel catfish culture ponds in Northwest Mississippi. *Reviews in Fisheries Science*, *4*(1), 1–55.doi:10.1080/10641269609388577

Tucker, C. S. (2000). Off-flavor problems in aquaculture. *Reviews in Fisheries Science*, *8*(1), 45–88. doi:10.1080/10641260091129170

Tucker, C. S., & Hargreaves, J. A. (Eds.). (2004). *Biology and culture of Channel catfish*. doi: 10.1111/j.1365-2109.2005.01310.x

Utkilen, H., & Gjølme, N. (1995). Iron-stimulated toxin production in *Microcystis aeruginosa*. *Applied and Environmental Microbiology*, *61*(2), 797–800. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=167340&tool=pmcentrez&rendertype=abstract

Valério, E., Faria, N., Paulino, S., & Pereira, P. (2008). Seasonal variation of phytoplankton and cyanobacteria composition and associated microcystins in six Portuguese freshwater reservoirs. *Annales de Limnologie - International Journal of Limnology*, *44*(3), 189–196. doi:10.1051/limn:2008003

Van de Waal, D. B., Verspagen, J. M., Finke, J. F., Vournazou, V., Immers, A. K., Kardinaal, W. E. a, … Huisman, J. (2011). Reversal in competitive dominance of a toxic versus non-toxic cyanobacterium in response to rising CO_2. *The ISME Journal*, *5*(9), 1438–1450. doi:10.1038/ismej.2011.28

Vasconcelos, J. F., Barbosa, J. E. L., Lira, W., & Azevedo, S. M. F. O. (2013). Microcystin bioaccumulation can cause potential mutagenic effects in farm fish. *The Egyptian Journal of Aquatic Research*, *39*, 185–192. doi:10.1016/j.ejar.2013.11.002

Vasconcelos, V. M. (1999). Cyanobacterial toxins in Portugal: Effects on aquatic animals and risk for human health. *Brazilian Journal of Medical and Biological Research*, *32*(3), 249–254. doi:10.1590/S0100-879X1999000300001

Verma, P. (2004). *Cooling water treatment handbook* (1st ed.). New Delhi, India: Albatross Fine Chem, Ltd.

Vézie, C., Rapala, J., Vaitomaa, J., Seitsonen, J., & Sivonen, K. (2002). Effect of nitrogen and phosphorus on growth of toxic and nontoxic *Microcystis* strains and on intracellular microcystin concentrations. *Microbial Ecology*, *43*(4), 443–454. doi:10.1007/s00248-001-0041-9

Vincent, W. F. (2009). Cyanobacteria. *Encyclopedia of Inland Waters*, *3*, 226–232

Von Westernhagen, H. (1988). Sublethal effects of pollutants on fish eggs and larvae. *Fish Physiology*, *11*, 253–346. doi: 10.1016/S1546-5098(08)60201-0

Walsby, A. E., Hayes, P. K., Boje, R., & Stal, L. J. (1997). The selective advantage of buoyancy provided by gas vesicles for planktonic cyanobacteria in the Baltic Sea. *New Phytologist*, *136*(3), 407–417. doi:10.1046/j.1469-8137.1997.00754.x

Wannemacher, R. W. (1989). Chemical stability and laboratory safety of naturally occurring toxins. *Fort Detrick, Frederick, MD: US Army Medical Research Institute of Infectious Disease*.

Whitton, B. A. (Ed.). (2012). *Ecology of cyanobacteria II: Their diversity in space and time*. doi:10.1007/978-94-007-3855-3

Wilmotte, A. (1994). Molecular evolution and taxonomy of the cyanobacteria. In D. A. Bryant (Ed.), *The molecular biology of cyanobacteria* (pp. 1–25). doi: 10.1007/0-306-48205-3_1

Wilson, A. E., Gossiaux, D. C., Höök, T. O., Berry, J. P., Landrum, P. F., Dyble, J., & Guildford, S. J. (2008). Evaluation of the human health threat associated with the hepatotoxin microcystin in the muscle and liver tissues of Yellow perch (*Perca flavescens*). *Canadian Journal of Fisheries and Aquatic Sciences*, *65*(7), 1487–1497. doi:10.1139/F08-067

WorldFish. (2015). *Genetically improved farmed tilapia* (*GIFT*). Retrieved from WorldFish website: http://www.worldfishcenter.org/content/genetically-improved-farmed-tilapia-gift

Wu, S., Wang, S., Yang, H., Xie, P., Ni, L., & Xu, J. (2008). Field studies on the environmental factors in controlling microcystin production in the subtropical

shallow lakes of the Yangtze River. *Bulletin of Environmental Contamination and Toxicology*, *80*(4), 329–334. doi:10.1007/s00128-008-9378-9

Wurts, W. a, & Durborow, R. M. (1992). *Interactions of pH, carbon dioxide, alkalinity, and hardness in fish ponds* (SRAC Publication No. 464). Retrieved from http://www2.ca.uky.edu/wkrec/InteractionspHEtc.PDF

Xie, L., Xie, P., Guo, L., Li, L., Miyabara, Y., & Park, H. D. (2005). Organ distribution and bioaccumulation of microcystins in freshwater fish at different trophic levels from the eutrophic Lake Chaohu, China. *Environmental Toxicology*, *20*(3), 293–300. doi:10.1002/tox.20120

Yang, H., Xie, P., Xu, J., Zheng, L., Deng, D., Zhou, Q., & Wu, S. (2006). Seasonal variation of microcystin concentration in Lake Chaohu, a shallow subtropical lake in the People's Republic of China. *Bulletin of Environmental Contamination and Toxicology*, *77*(3), 367–374. doi:10.1007/s00128-006-1075-y

Yang, X., Wu, X., Hao, H., & He, Z. (2008). Mechanisms and assessment of water eutrophication. *Journal of Zhejiang University. Science. B*, *9*(705824), 197–209. doi:10.1631/jzus.B0710626

You, L., Cui, L. F., Liu, Z. W., Yang, B., & Huang, Z. F. (2007). Correlation analysis of parameters in algal growth. *Environmental Science & Technology*, *30*(9), 42–44.

Zang, C., Huang, S., Wu, M., Du, S., Scholz, M., Gao, F., … Dong, Y. (2011). Comparison of relationships between pH, dissolved oxygen and chlorophyll-*a* for aquaculture and non-aquaculture waters. *Water, Air and Soil Pollution*, *219*(1-4), 157–174. doi: 10.1007/s11270-010-0695-3

Zati, S., & Salmah, Z. (2008). Lakes and reservoir in Malaysia : Management and research challenges. In M. Sengupta, M. & R. Dalwani, R. (Eds.), *Proceedings of Taal2007: The 12th World Lake Conference, India*, 1349–1355. Retrieved from http://www.moef.nic.in/sites/default/files/nlcp/P%20-%20World%20Case%20Studies/P-3.pdf

Zhang, D., Xie, P., & Chen, J. (2010). Effects of temperature on the stability of microcystins in muscle of fish and its consequences for food safety. *Bulletin of Environmental Contamination and Toxicology*, *84*(2), 202–207. doi:10.1007/s00128-009-9910-6

Zhang, D., Xie, P., Liu, Y., & Qiu, T. (2009). Transfer, distribution and bioaccumulation of microcystins in the aquatic food web in Lake Taihu, China, with potential risks to human health. *Science of the Total Environment*, *407*(7), 2191–2199. doi:10.1016/j.scitotenv.2008.12.039

Zhao, M., Xie, S., Zhu, X., Yang, Y., Gan, N., & Song, L. (2006a). Effect of dietary cyanobacteria on growth and accumulation of microcystins in Nile tilapia (*Oreochromis niloticus*). *Aquaculture*, *261*(3), 960–966. doi:10.1016/j.aquacul

ture.2006.08.019

Zhao, M., Xie, S., Zhu, X., Yang, Y., Gan, N., & Song, L. (2006b). Effect of inclusion of blue-green algae meal on growth and accumulation of microcystins in Gibel carp (*Carassius auratus gibelio*). *Journal of Applied Ichthyology*, *22*(1), 72–78. doi:10.1111/j.1439-0426.2006.00706.x

Zhong, F., Gao, Y., Yu, T., Zhang, Y., Xu, D., Xiao, E., … Wu, Z. (2011). The management of undesirable cyanobacteria blooms in Channel catfish ponds using a constructed wetland: Contribution to the control of off-flavor occurrences. *Water Research, 45*(19), 6479–6488. doi:10.1016/j.watres.2011.09.044

Ziková, A., Trubiroha, A., Wiegand, C., Wuertz, S., Rennert, B., Pflugmacher, S., … Kloas, W. (2010). Impact of microcystin containing diets on physiological performance of Nile tilapia (*Oreochromis niloticus*) concerning stress and growth. *Environmental Toxicology and Chemistry*, *29*(3), 561–568. doi:10.1002/etc.76

Zilliges, Y., Kehr, J.-C., Meissner, S., Ishida, K., Mikkat, S., Hagemann, M., … Dittmann, E. (2011). The cyanobacterial hepatotoxin microcystin binds to proteins and increases the fitness of *Microcystis* under oxidative stress conditions. *PLoS ONE*, *6*(3), e17615. doi:10.1371/journal.pone.0017615

Zimba, P. V., Camus, A., Allen, E. H., & Burkholder, J. M. (2006). Co-occurrence of White shrimp, *Litopenaeus vannamei*, mortalities and microcystin toxin in a southeastern USA shrimp facility. *Aquaculture*, *261*(3), 1048–1055. doi:10.1016/j.aquaculture.2006.08.037

Zimba, P. V., Khoo, L., Gaunt, P. S., Brittain, S., & Carmichael, W. W. (2001). Confirmation of catfish, *Ictalurus punctatus* (Rafinesque), mortality from *Microcystis* toxins. *Journal of Fish Diseases*, *24*(1), 41–47. doi:10.1046/j.1365-2761.2001.00273.x

Znachor, P., Jurczak, T., Komarkova, J., Jezberova, J., Mankiewicz, J., Kastovska, K., & Zapomelova, E. (2006). Summer changes in cyanobacterial bloom composition and microcystin concentration in eutrophic Czech reservoirs. *Environmental Toxicology*, *21*, 236–243. doi:10.1002/tox.20176