SCREENING OF SELECTED TERRESTRIAL PLANTS FOR THE CONTROL OF CYANOBACTERIAL GROWTH

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by

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LIST OF ABBREVIATIONS

AI	Air Itam
ANOVA	Analysis of variance
BLASTn	Basic local alignment search tool nucleotide
CTAB	Cetyltrimethyl Ammonium Bromide
CYN	Cylindrospermopsin
EB	Extraction buffer
EDTA	Ethylenediaminetetraacetic acid
DNA	Deoxyribonucleic acid
NCBI	National Centre for Biotechnology Information
NRPS	Non-ribosomal peptide synthetase
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PKS	Polyketide synthase
T_a	Annealing temperature
TAE	Tris base, acetic acid and EDTA
TB	Teluk Bahang
TE	Tris - EDTA
ITS	Internal transcribe spacer

UJIAN SARINGAN TERHADAP TUMBUHAN DARATAN YANG TERPILIH BAGI MENGGAWAL PERTUMBUHAN SIANOBAKTERIA

ABSTRAK

Kawalan pertumbuhan sianobakteria yang efektif adalah penting kerana sianobakteria boleh menyebabkan masalah seperti bau dan rasa yang tidak menyenangkan, dan yang lebih penting, pengeluaran toksin yang boleh membawa maut kepada manusia dan haiwan. Kajian terdahulu menunjukkan bahawa pelbagai tumbuhan dapat menghalang pertumbuhan sianobakteria. Oleh itu, sekiranya daun dari tumbuhan liar mampu mengawal pertumbuhan sianobakteria sama seperti kajian tersebut, ia boleh mengurangkan kos dan memberi satu alternatif yang mesra alam sekitar dalam pengurusan sianobakteria. Kajian terdahulu juga menunjukkan bahawa keupayaan jerami barli dalam mencegah pertumbuhan sianobakteria melibatkan penguraian mikrob. Oleh itu, kajian dilakukan untuk mengkaji keberkesanan 10 gL^{-1} batang kelapa sawit dan hampas tebu yang diurai oleh kulat bagi mengawal pertumbuhan sianobakteria selama 25 – 30 hari berdasarkan kuantiti klorofil *a*. Hasil kajian menunjukkan bahawa batang kelapa sawit yang diurai oleh kulat dapat mengawal pertumbuhan sianobakteria dengan lebih berkesan, mengukuhkan lagi teori penglibatan penguraian mikrob dalam mencegah pertumbuhan sianobakteria. Manakala bahagian kedua kajian pula melibatkan ujian keberkesanan daun dari 15 jenis tumbuhan liar secara individu dalam menghalang pertumbuhan lapan jenis sianobakteria yang berbeza. Kajian dijalankan dengan menguji 1 – 10 gL^{-1} dedaun terpilih yang kering terhadap pertumbuhan sianobakteria selama 15 – 30 hari. Berdasarkan kuantiti klorofil *a*, kebanyakkan daun dapat mengawal pertumbuhan sianobakteria secara berkesan pada kadar yang berbeza, bergantung kepada spesies sianobakteria dan daun tumbuhan yang digunakan. Oleh itu, kajian ini menunjukkan

bahawa daun dari tumbuhan liar menghasilkan anti-sianobakteria yang berkesan yang boleh menjadi kawalan semulajadi terhadap pertumbuhan sianobakteria. Tumbuhan tersebut kemudiannya dikenalpasti berdasarkan jujukan DNA *rbcL* dan pemerhatian morfologi. Daripada 15 daunan daratan tersebut, hanya 10 daun berjaya dikenalpasti dengan mengunakan primer RbcL tersebut. Sementara itu, kulat yang digunakan dalam kajian in telah dikenalpasti sebagai *Lichtheimia* sp. berdasarkan jujukan DNA ruang tertranskripsi dalaman dan permerhatian secara morfologi.

SCREENING OF SELECTED TERRESTRIAL PLANTS FOR THE CONTROL OF CYANOBACTERIAL GROWTH

ABSTRACT

Efficient cyanobacterial bloom management is important because a bloom in a water body may cause problems such as unpleasant odour and taste, and most importantly, toxins production that are potentially fatal to human and animals. Previous researches have shown that various plants were able to inhibit the cyanobacterial growth. Therefore, if any wild terrestrial leaf can perform the same control, it would provide a low cost and environmental friendly alternative of cyanobacterial management. Previous researches also showed that the ability of barley straw to control cyanobacteria might likely involved complex microbial degradation. Therefore, experiments were set up to test the effect of 10 gL^{-1} fungi-degraded palm oil trunk and sugarcane bagasse on cyanobacterial growth for 25 – 30 days by measuring chlorophyll *a* content. Increased ability of fungi-degraded palm oil trunks in inhibiting cyanobacterial growth was observed, strengthening the theory of involvement of microbial degradation in controlling of cyanobacterial growth. However, no difference was observed in sugarcane study, but it was observed that sugarcane also worked as adsorption treatment. While second part of this project is to test 15 individual wild terrestrial plants leaves leachates individually for their abilities to inhibit the growth of eight isolated cyanobacterial strains. The study was conducted by introducing 1 – 10 gL^{-1} of dried leaf into cyanobacterial culture for 15 – 30 days. Based on chlorophyll *a* content, most leaves effectively controlled all cyanobacterial growth at different efficiency, depending on the species of the cyanobacteria and leaves used. The results suggested that the leaves released effective anti-cyanobacterial substances, which can perform as natural biological

controls of cyanobacterial bloom. The plants selected were then identified based on *rbcL* plastid DNA sequence and morphology observation. Of 15 terrestrial leaves, only 10 terrestrial leaves were successfully identified using RbcL primer. Meanwhile, Fungus isolated and used in the study was also identified as *Lichtheimia* sp. based on internal transcribe spacer gene sequence and morphological approach.

CHAPTER 1: INTRODUCTION

1.1 Background

Cyanobacteria or blue-green algae are prokaryotes that obtain their nutrients mainly through the photosynthetic process. They are highly adaptable to the environment, where they can be found in soils, rocks and most water bodies, from hot springs to the cold water of Antarctic lakes and even in the low nutrient freshwater environment. As part of the aquatic system, cyanobacteria plays an important role in the ecosystem maintenance (WHO, 1999). Photosynthesis of the bacteria provides oxygen, while nitrogen-fixing cyanobacteria made atmospheric nitrogen utilizable to other organisms. Cyanobacteria is also important as a potential source for renewable energy, biofertilizers, and in facilitating degradation of complex organic compounds such as oil and herbicides (Abed et al, 2009). However, excessive growth of cyanobacteria may forms blooms in the water. The blooms may cause several problems such as unpleasant odours and taste, and, most importantly, toxin production (WHO, 1999).

Presence of toxic bloom was recorded earliest by Francis (1878). The cyanobacterial toxins are generally categorized into four major groups based on its toxicology effects, namely hepatotoxin (microcystin and nodularin), neurotoxin (anatoxin-a, anatoxin-a(s), and saxitoxin), cytotoxin (cylindrospermopsin) and dermatotoxin (aplysiatoxins, lyngbyatoxin-A) (Merel et al, 2013). Consumption or direct contact with these toxins has caused severe health consequences. For examples, microcystin leads to the death of 60 dialysis patients in Brazil (Codd et al, 1999), hospitalized of 148 children in the Palm Island, Australia due to cylindrospermopsin toxication (Mihali et al, 2008), and lead to several cases of animal death such as death of two

dogs in La Loue River in South France (Gugger et al, 2005), infecting cattle in Swiss alpine pastures (Mez et al, 1997) and cause neurotoxicity to 37 dogs in the Tarn River valley in South France (Cadel-Six et al, 2007). Currently, more than 65 countries worldwide including Thailand (Mahakhant et al., 1998), the Philippine (Cuvin-Aralar et al, 2002), Vietnam (Hummert et al, 2001) and Singapore (Te & Gin, 2011) have recorded the detection of toxic cyanobacteria in the water environment. Malaysia was reported to have the presence of toxic cyanobacteria in 2015 (Sinang et al, 2015) where toxin-producing *Microcystis* sp. was successfully isolated from Ayer Itam reservoir, Penang by Sim (2015).

Increasing concern on harmful and unpleasant cyanobacterial blooming in the freshwater environment leads to extensive researches on cyanobacterial growth control. Currently, the most widely used chemical for water treatment, copper (II) sulphate ($CuSO_4$) has harmed a wide spectrum of species, risking a secondary pollution in the water environment (Shao et al, 2013). While physical treatments such as sedimentation has lower secondary pollution risk, the treatments can injure other organisms and are usually energy consuming and expensive (Shao et al, 2013). Hence, more scientists are in search of biological-derived treatments as an alternative.

Many researches focus on an anti-cyanobacterial compound derived from waste and plant biomass, and so far, the most effective and researched cyanobacterial bio-control is barley straw. Other researchers observed inhibition of cyanobacterial growth by different terrestrial plant and herbs, such as sugarcane bagasse, palm oil trunk (Sim, 2015), *Gingko biloba* (Zhang et al, 2013a) and oak trees (Park et al, 2006), aquatic plant such as *Myriophyllum spicatum* (Nakai et al, 2005) and *Hydrilla verticillata* (Zhang et al, 2012).

Several active compounds released from the plants have been successfully isolated and characterized in previous researches, which include polyphenol (Ni et al, 2013), terpenoid (Ni et al, 2011) and fatty acid (Nakai et al, 2005). These compounds inhibit growth via different pathways, such as inhibition of photosynthesis, disruption of cellular structure, and inactivation of enzymatic and non-enzymatic functions (Ni et al, 2013).

1.2 Problem Statements

Although many of the studies showed biological-derived compounds to be effective, several studies indicate that effectiveness depended on cyanobacterial species. For instance, palm oil trunk is able to inhibit *Microcystis* sp. effectively, but unable to inhibit the growth of *Synechocystis minuscula* (Sim, 2015). Similarly, various studies on barley straw also indicated the dependency of cyanobacterial species (Lalung, 2012). Types of barley straw also showed different effectiveness in inhibiting cyanobacteria (Xiao et al, 2014).

Researchers have also hypothesized that the composition and complexity of microbial in degrading lignin in barley straw influenced its capability to control algae growth, such as a study conducted by Murray et al (2010) using barley straw pre-treated with fungus. However, currently, information on if in fact, similar to barley straw, fungus also able to assist palm oil trunk and sugarcane bagasse in inhibition of cyanobacteria is not known.

As previous researches showed that plant leaves released anti-cyanobacterial compounds, we could hypothesize that in natural environment, leaf litter of wild terrestrial plants around lakes or reservoirs leaches compounds that are able to inhibit

growth of cyanobacteria. Consequently, it would provide a low cost and environmental friendly alternative for bloom management.

However, many of the researches mainly focused on *Microcystis* sp. and limited researches on other bloom-forming cyanobacteria. In addition, most researches were conducted on plants grow outside the tropical regions and other countries. Therefore, abilities of terrestrial plant leaves from Malaysia in inhibiting cyanobacterial growth are not yet fully known.

1.3 Objectives of Study

As such, experiments were set up to achieve the following objectives:

1. To compare the effect between fungi-degraded palm oil trunk and sugarcane bagasse with fresh palm oil trunk and sugarcane bagasse on cyanobacterial growth.
2. To examine the potential of selected terrestrial leaves in Malaysia to inhibit cyanobacterial bloom formation.
3. To identify the species of plants and fungus using morphological and molecular approaches.

CHAPTER 2: LITERATURE REVIEW

2.1 Cyanobacterial Taxonomy and Morphology

Cyanobacteria phylum consists of bacteria classified as greenish-blue due to its chlorophyll pigment, and possesses vastly different forms and structures. The bacteria are either in unicellular or filamentous which are determined by the mode of reproduction. Unicellular forms are often seen as unicellular cocci bacteria whilst the filamentous forms are observed with a filamentous-like shape or rod shape. Many filamentous cyanobacteria are capable of forming heterocyst or akinetes in a specific environment. Heterocyst is formed in the absence of nitrogen, especially in a clean water environment, allows the cyanobacteria to fix nitrogen from the atmosphere and survive, although certain non-heterocystous cyanobacterial species such as filamentous *Trichodesmium* sp., *Lyngbya* sp. and *Oscillatoria* sp. and unicellular *Gloeothece* sp. and *Cyanothece* sp. are also capable of fixing nitrogen under aerobic conditions (Bandyopadhyay et al, 2013).

Akinete is a resting-state form, which acts as a survival strategy similar to bacterial endospores. Akinete is formed in harsh conditions such as low temperature, drought, high level of salt, and iron depletion (Olsson-Francis et al, 2009). Both heterocyst and akinete are observed as a thick cell wall, a trait to distinguish cyanobacterial species using microscopic observation. Besides heterocyst and akinete characteristics, the presence or absence of sheath, true or false branching, and cell size are also used for cyanobacterial identification (Komárek, 2010).

Previously, cyanobacterial taxonomy mainly depended on the morphological characteristics described. However, some of the morphological data conflict with the molecular results; that is when cyanobacterial species have similar morphology but distinctive 16S ribosomal RNA (rRNA) DNA sequence (Komárek, 2010). In addition, relying only on morphological observation in cyanobacterial classification would lead to misidentification, as cyanobacteria are capable of changing their taxonomical characteristics. Researchers recorded that about 50% of the cyanobacterial cultures contradicted their taxonomical descriptions (Lyra et al, 2001).

Therefore, in recent change of taxonomy, a combination data of molecular, biochemical, and ulrastructural patterns of thylakoids and ecology are required for cyanobacterial speciation (Komárek, 2010). Molecular data for cyanobacterial taxonomy uses 16S rRNA as the marker for identification and classification of cyanobacterial genus and higher. 16S rRNA can also distinguish different ecological habitats of cyanobacteria that have similar morphological appearances (Komárek, 2010). After the re-evaluation of taxonomy, eight orders have been established: Gloeobacterales, Synechococcales, Spirulinales, Chroococcales, Pleurocapsales, Oscillatoriales, Chroococcidiopsidales and Nostocales (Komarek et al, 2014).

2.2 Cyanotoxins

Cyanotoxins are generally categorized in four major groups based on its toxicology effects. They are hepatotoxins, neurotoxins, cytotoxin and dermatotoxins as shown in Plate 2.1. Hepatotoxins include microcystin and nodularia, that affect liver cells, neurotoxins such as saxitoxin and anatoxin-a(s) that affect nerve system, cytotoxin affecting cells includes cylindrospermopsin while dermatotoxins such as aplysiatoxins and lyngbyatoxin-A are toxins affecting skin cells (Merel et al, 2013).

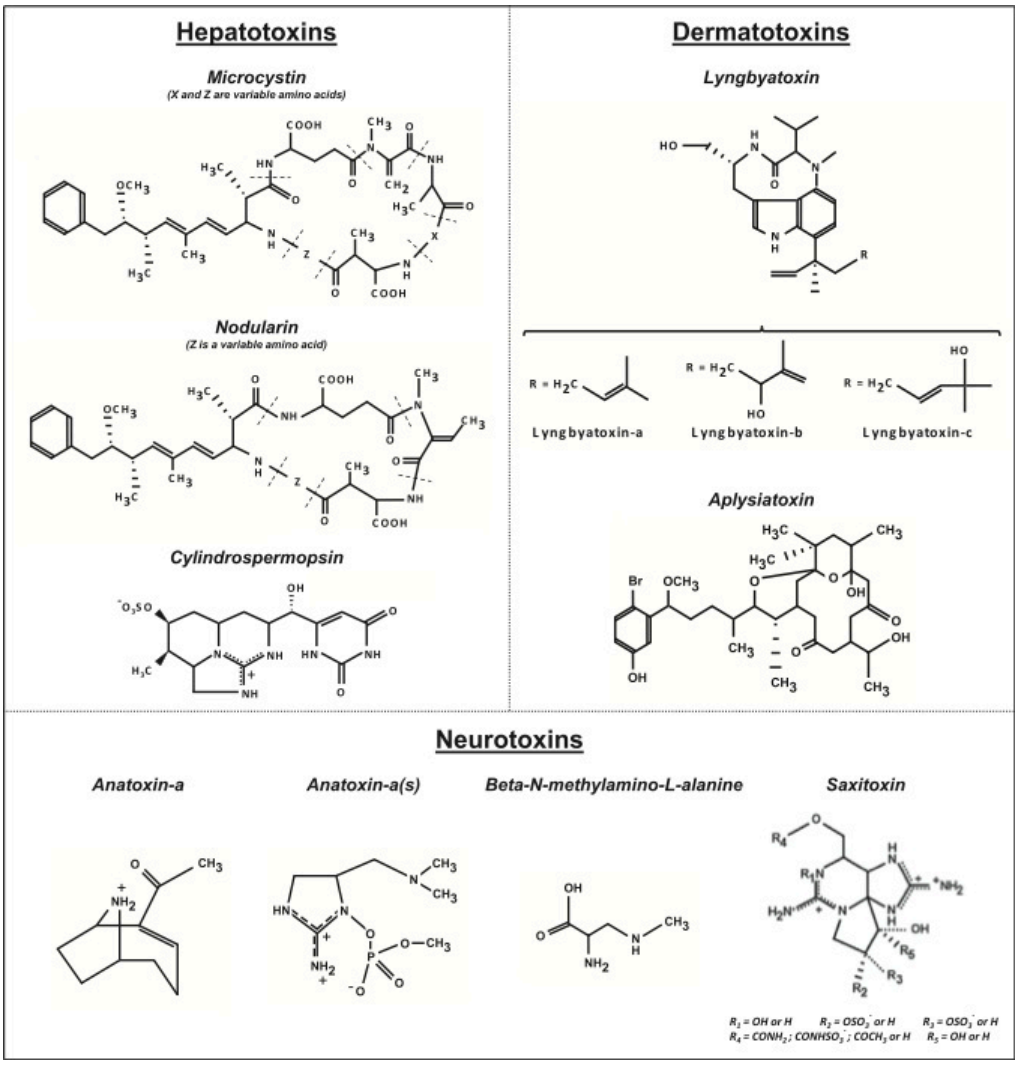

Plate 2.1 Cyanobacterial toxin structures and their effect on tissues [Adopted from Merel et al (2013)].

2.2.1 Hepatotoxins

Hepatotoxins that are capable of destroying liver cells include microcystin and nodularin. The toxins have a unique cyclic peptide structure that enables the inhibition of eukaryote proteins phosphatase type 1 and 2A, which are important in the dephosphorylating of amino acid serine or threonine of the liver cells. Inhibition of the toxins to the proteins will lead to excessive phosphorylation of structural filament (Pearson et al, 2010) and subsequently lead to cytoskeleton instability, which in turn causes cell death.

Microcystin is the most prevalent and routinely monitored cyanotoxin and is more intensely studied compared to other toxins. This is due to the fact the toxin is the most commonly found in cyanobacterial blooms. Unlike other toxins, microcystin has caused human fatality in Brazil (Merel et al, 2013). Researchers also relate high incidences of liver cancer in China from the consumption of microcystin-contaminated water (Blaha et al, 2009). Therefore, there is possibility of the toxin causing liver cancer. The earliest cyanobacterium species detected as a microcystin producer is *Microcystis* sp.. Later, researchers identified *Planktothrix* sp.*, Nostoc* sp.*, Anabaena* sp.*, Nodularia* sp.*, Phormidium* sp. and *Chroococcus* sp. to also capable of producing microcystin (Pearson et al, 2008).

Microcystin is structurally the most varied among the cyanotoxins, consisting of about 90 different isoforms. In the environment, the microcystin is stable in chemical hydrolysis and extremely high temperatures (>300℃) (WHO, 1999), thus, it may accumulate in the water body for several days to years (Gągała & Mankiewicz-Boczek, 2012). However, microcystin is also easily degraded by strong oxidation molecules such as ozone (WHO, 1999) and a breakdown by aquatic bacteria such as

Sphingomonas sp. and *Pseudomonas aeruginosa* (Gągała & Mankiewicz-Boczek, 2012).

Tillett and colleagues (2000) were the first to characterise microcystin gene biosynthesis using gene cloning and sequencing. The toxin is encoded in 55 kb microcystin synthetase (*mcyS*) gene cluster. The gene cluster consists of two operons and encoded 10 genes.

Nodularin is produced specifically by planktonic *Nodularia* sp. such as *N. spumigena* (Moffitt & Neilan, 2004) and has a similar structure as the microcystin (Pearson et al, 2010) due to its cyclic structure, which exists in seven different structures. Two of the structure isoforms comprised of a variation of 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid (ADDA) residues, which directly affect the toxicity level of the toxins (Moffitt & Neilan, 2004). In 1997, it was observed that the pure nodularin remained stable in sunlight and dark conditions after nine days (Twist & Codd, 1997).

The nodularin gene cluster, *nda*, was characterised by sequencing toxic *N. spumigena* strain to detect potential toxic *Nodularia* sp. (Moffitt & Neilan, 2004). *nda* gene cluster, which is 48-kb gene long, consists of nine open frames (ORFs), encoding for gene *ndaA* to *ndaI* genes.

2.2.2 Cytotoxin

Cytotoxin has various effects on human and animal cells, potentially caused hepatotoxic, neurotoxic and even tumour development. The main cytotoxin produced by the cyanobacteria is the cylindrospermopsin (CYN) toxin. The toxin is a polyketide-derived alkaloid containing guanidiono and sulfate groups (Neilan et al, 2013). The toxicity of CYN relies on the inhibition of cytochrome P450 and

glutathione molecule as well as the inhibition of protein synthesis (Mihali et al, 2008). The toxin has been documented in all continents; therefore, it is a threat to public health (Mihali et al, 2008). *Cylindrospermopsis raciborskii* is the first cyanobacteria identified as a CYN producer. Other cyanobacteria species then identified as CYN producers are *Aphanizomenon ovalisporum*, *Anabaena bergii*, *Raphidiopsis curvata*, *Aphanizomenon flos-aquae*, *Anabaena lapponica, Lyngbya wollei* and *Oscillatoria* sp. (Bormans et al, 2014).

A comparison between non-toxic *C. raciborskii* and toxic *C. raciborskii* genome sequence show several genome differences. The most important difference between the toxic and non-toxic *C. raciborskii* genome is the *cyr* gene clusters, which encode important molecules for cylindrospermopsin (CYN) production. The *cyr* gene cluster encodes 15 open reading frames (ORFs) which encode for 15 different genes (Sinha et al, 2014). The release of toxin to the extracellular environment occurred mainly during bloom decline. The extracellular toxin is extremely susceptible to the heat and sunlight, and can be degraded easily, with 90% of the toxin broken down after two to three days when exposed to light (WHO, 1999).

2.2.3 Neurotoxins

Cyanotoxins such as saxitoxin, anatoxin-a, and anatoxin-a(s) are neurotoxins that affect mainly human and animal nervous systems through different mechanisms. Saxitoxin or paralytic shellfish toxin (PSTs) is a trialkyl tetrahydropurine toxin, consisting of 30 different structures isoform (Pearson et al, 2010). Saxitoxin affects the neuron system by blocking the voltage gated sodium channels of neuron cells. It also affects heart cells by blocking calcium channels and lengthens the gating of potassium channels of the cells (Pearson et al, 2010). The *sxt* gene cluster encodes proteins that are important for biosynthesis of saxitoxin. Different from other

cyanotoxins, the intoxication of saxitoxin occurred mainly through seafood consumption such as seashells as the toxin accumulates in the food chain (Pearson et al, 2010). In the environment, saxitoxin is stable and able to accumulate in the fresh water environment for nine to 28 days depending on its variant (Hardy, 2011). *Lyngbya wollei* (Pearson et al, 2010) and *C. raciborskii* (Mihali et al, 2009) are two cyanobacterial species that are capable to produce saxitoxin.

Another cyanobacterial neurotoxin, Anatoxin-a is a potent nicotinic acetylcholine receptor agonist, a receptor in nervous cells that is important for muscle contraction signal. Binding of anatoxin-a to the receptor leads to muscle fasciculation, gasping, seizures and possibly death due to respiratory arrest in human and animals. Anatoxin-a that is encoded in *ana* gene cluster is highly similar between different species however with different gene arrangement (Rantala-Ylinen et al, 2011). The toxin are produced by cyanobacteria such as *Aphanizomenon* sp. (Ballot et al, 2010), *Anabaena* sp. (Rantala-Ylinen et al, 2011) and *Oscillatoria* sp. (Méjean et al, 2009).

Anatoxin-a(s) has a similar toxicity mechanism with anatoxin-a. However, unlike anatoxin-a, the anatoxin-a(s) structure consists of unique phosphate ester of a cyclic N-hydroxyguanidine (Neilan et al, 2013). Other neurotoxin, jamaicamides produced by cyanobacteria *Lyngbya majuscula*, is also found to have a sodium channel blocking activity and fish toxicity. Jamaicamides structure consists of alkynyl bromide, vinyl chloride, β-methoxy eneone system and pyrrolinone ring (Edwards et al, 2004).

2.2.4 Dermatotoxins

Dermatotoxins including aplysiatoxin, debromoaplysiatoxin and lyngbyatoxin-A are cyanotoxins that mainly affect the skin. Aplysiatoxin and debromoaplysiatoxin have a phenolic bislactones structure, synthesized by cyanobacterium *Lyngbya majuscule*. The toxins are strong skin irritants, causing skin rashes and blistering, while lyngbyatoxin-A is an indole alkaloid produced by benthic marine cyanobacteria, *L. majuscule* and freshwater *L. wollei w*hich can cause dermatitis and inflammation of oral and gastrointestinal tissues (Rzymski & Poniedziałek, 2012). Among the cyanotoxins, dermatotoxins are the least researched toxins, accounting for less than 2% of all cyanotoxins papers available in 2013 and remain poorly researched (Merel et al, 2013), thus information on the toxins are limited that further studies are required.

2.3 Cyanobacterial Dynamics and Toxin Biosynthesis in The Environment

Even though cyanobacteria are highly adaptable to various environments, different ecologies may be inhabited by different species of cyanobacteria. Cyanobacteria commonly live in the freshwater environment such as lakes, ponds, rivers and reservoirs are the main concern as humans are highly exposed to these resources through drinking water and recreational activities. Cyanobacteria from the order of Oscillatoriales, Nostocales and Chroococcales are the main cyanobacteria found in the freshwater environment, meanwhile, potentially toxic marine cyanobacteria include *L. majuscule* (Rzymski & Poniedziałek, 2012).

Cyanobacterial growth, species variation and concentration in the environment are influenced by both abiotic and biotic environmental factors. Abiotic factors such as wind, and the characteristics of water bodies, such as depth, stream flow and tides

affect cyanobacterial accumulation and concentration whilst light intensities, nutrient and temperature, as well as biotic factors, have influenced toxin biosynthesis and the cyanobacterial population, species and strain variation.

2.3.1 Characteristic of water bodies and wind direction

The freshwater environment is made up of two different habitats: benthic and planktonic. Benthic is the lowest region of the freshwater environment whereas planktonic is characterized as the upper region of the habitat. The benthic habitat is commonly inhabited by cyanobacteria that lacked of gas vacuole, the non-toxic *Nodularia* sp. such as *N. sphaerocarpa*, and *N. harveyana* (Lyra et al, 2005) and benthic toxic, *Phormidium favosum* (Gugger et al, 2005). Meanwhile, planktonic is inhabited by cyanobacteria consisting of gas vesicle organelle, enabling them to float. Planktonic cyanobacteria include *Planktothrix* sp. (Walsby et al, 2004), toxic *Nodularia* sp. (Lyra et al, 2005), *Anabaena* sp., *Microcystis* sp., *Aphanizomenon* sp., and *Oscillatoria* sp. (Oliver & Walsby, 1984). In addition, the concentration of cyanobacteria changes within hours depending on wind, water stream flow and tides (Baxa et al, 2010).

2.3.2 Light intensity and temperature

Many of the planktonic cyanobacteria regulate water buoyancy and position themselves for optimum light conditions by regulating the expression of the gas vesicle gene (Halinen et al, 2008). Altering buoyancy leads to the sinking of cyanobacteria during midday and floating of cyanobacteria at night (Walsby et al, 2004). In addition to buoyancy regulation for optimum light, specific cyanobacteria use phototaxis motility via gliding or twitching, as observed in *Anabaena* sp. and

Oscillatoria sp., and unicellular cyanobacteria such as *Synechocystis* sp. respectively (Hoiczyk, 2000).

Light intensity is also involved in toxin release and bio-production rate. By using PCR and RT-PCR, Gobler et al (2007) indicated increase in *mcy* gene cluster expression in *Microcystis* sp. during summer, when light intensity is in abundance but decline during fall, similar to the CYN released into the environment by *A. flos-aquae* (Preußel et al, 2009). Interestingly, light intensity also influences the amount of toxin released by benthic cyanobacteria as shown in benthic *Oscillatoria* sp. (Bormans et al, 2014).

In addition to light intensity, stress induced at specific temperatures also influenced toxin release from the cell. For instance, Preußel et al (2009) indicates that at 25℃ CYN production increases significantly compared to at 20℃, which is in contrast to the anatoxin-a levels produced by *Anabaena* sp. and *Aphanizomenon* sp., that decrease at high temperature (Neilan et al, 2013). In the meantime, Conradie & Barnard (2012) observed different domination of species in different seasons, where the non-toxic *Planktothrix* sp. dominated during the autumn whilst hot summer exhibited presence of both non-toxic and toxic *Microcystis* sp.

As such, Malaysia with continuous favorable environment to cyanobacterial growth will have high risk of cyanobacterial toxin exposure to human and animals. However, currently, only toxic *Microcystis* sp. has been reported in Selangor (Sinang et al, 2015), Penang (Sim, 2015), and Sarawak (Mohamad et al, 2016), whilst status of other toxins in Malaysia have not been reported, possibly due to lack of survey and awareness. Even so, it is important for effective cyanobacterial growth control in Malaysia, as there is a high risk of the toxin presence in the country.

2.3.3 Nutrients

Cyanobacteria obtained energy through the photosynthetic process, with essential nutrients, such as phosphorus, nitrogen and iron required for cell growth (WHO, 1999). However, evidently, these nutrients may not influence bloom formation, as more cyanobacteria are able to utilise phosphate in a phosphate-limited environment using alkaline phosphatase enzyme, and more scientists observed non nitrogen-fixing cyanobacteria in nitrogen-limited and even in nitrogen and phosphorus co-limited environment (Paerl et al, 2011).

Even so, nutrient may affect the cyanobacterial species and strain domination in a population due to different nutrient utilization by different species. For instances, Akcaalan et al (2006) observed the *Planktothrix agardhii* domination in nutrient-rich water bodies whereas *Planktothrix rubescens* was generally found in low nutrient content lakes. Meanwhile, the growth of toxic *Microcystis* sp. showed a positive correlation with phosphorus concentration and a negative correlation with nitrate concentration in the environment (Li et al, 2012).

In addition, nutrient levels may also affect toxin gene expression. As shown by Gobler et al (2007), the *mcy* gene expression level that increases as nitrogen and phosphorus levels increased. In contrast, anatoxins-a concentration increases with phosphorus depletion and the presence of nitrogen increased the level of the toxin (Gobler et al, 2007). Gobler et al (2007) also proposed that this is due to high nitrogen levels, which lead to phosphorus depletion, which in turn increases the toxin biosynthesis. However, the results contradicted previous research, which shows that nitrogen depletion increased the level of anatoxin-a biosynthesis of *Anabaena* sp. and *Aphanizomenon* sp. (Neilan et al, 2013). Thus, transcriptional regulation studies on

the anatoxin-a gene clusters are yet to be further investigated by researchers (Neilan et al, 2013).

Meanwhile, studies indicated that nitrate depletion increases the production of saxitoxin during the initial growth of heterocyst-forming *A. flos-aquae*. However, as the cells grow and are capable of fixing nitrogen from the environment, no significant difference is shown in the production of saxitoxin in nitrate-depleted and nitrate-supplied media. Based on the results, Stucken et al (2014) suggested that nitrogen does not directly affect toxin production in heterocyst-forming cyanobacteria, but instead the growth of cyanobacteria correlates with the biosynthesis of the toxin.

On the other hand, using RT-PCR, Alexova et al (2011) found an increasing expression of the toxin gene in *M. aeruginosa* in iron-depleted culture. They also discovered that the microcystin-producing cyanobacteria were able to uptake iron higher than the non-toxin encoding cyanobacteria, subsequently leads to the importance of microcystin for iron metabolism (Alexova et al, 2011). However, by investigating the rate of iron intake between genetically modified non-microcystin producing *M. aeruginosa* and microcystin producing *M. aeruginosa* of the same strain, Fujii et al (2011) showed that the difference in the iron intake between non-toxic and toxic cyanobacteria was strain specific, rather than due to microcystin production. Therefore, microcystin do not directly involve in iron metabolism of the cyanobacterium.

Although nutrients may not involve in bloom formation, it may affect cyanobacterial species dominations and toxin encoding gene expression. However, as the literatures are often contradictory, more researches with constant experimentations set up should be conducted.

2.3.4 Biotic factors

Biotic factors also play an important role in cyanobacteria population and toxin production. Jang et al (2003) observed that an increasing number of zooplankton (the main predator of cyanobacteria) has increased production of microcystins. They also found that the microcystin-producing cyanobacteria have better survival in combating zooplankton. The research leads to the theory that the expression of microcystin molecule is important to protect the cells from harsh conditions (Jang et al, 2003).

Many bacteria and viruses also showed anti-cyanobacteria characteristics and are thought to influence the cyanobacteria bloom dynamics. Several researches also indicated that the chemical produces by plants may be the natural inhibitors of cyanobacterial growth (Ni et al, 2011). Subsequently, a same water body may exhibit different toxics or non-toxic strains of cyanobacteria every year (Kaebernick & Neilan, 2001) and different sampling points and depths may have different species and concentration of cyanobacteria. In addition, this also leads to cyanobacteria having distinctive toxicity when placed in different laboratory conditions (Kaebernick & Neilan, 2001) such as various culture media and light intensity.

Even though many researchers suggested that the environmental factors lead to a variation of cyanobacterial genotype domination and cyanobacteria diversity, no research on multiple factors on cyanobacterial growth has been conducted, and how exactly the factors affect the dynamics of cyanobacteria also requires further investigation.

2.4 Controlling of Cyanobacterial Growth

Due to increasing concern on toxic cyanobacteria and unpleasant blooming, researchers take several initiatives to control growth of cyanobacteria. These initiatives are to prevent and treat nuisance cyanobacterial blooming.

2.4.1 Chemical treatments

Chemical treatment is widely used for their easy access, cheap and fast. One of the most successful compounds to control cyanobacteria growth is copper sulphate, which has been used to treat cyanobacteria blooming since 1904 (Hullebusch et al, 2002). However, the chemical has non-specific interaction that reduce diversity of other organism, and could lead to secondary pollution of aquatic environments (Shao et al, 2013). In addition, the treatment reportedly has less affectivity for dense bloom biomass (Hullebusch et al, 2002) and immediate toxic bloom collapse leads to release of highly concentrated cyanotoxin into the water due to cell damage (Hullebusch et al, 2002). Additionally, Wu et al (2007) suggested that effective copper sulfate treatment dependent on the *Microcystis* spp.

In order to cope for non-specificity of copper sulphate treatment, researchers screened for different compounds with toxic selectivity to cyanobacteria. For instances, Schrader & Harries (2001) screened for 39 compounds to selectively control *Oscillatoria perornata* growth. However, only 12 of the compounds were selectively able to kill the species. Matthijs et al (2012) suggest the usage of hydrogen peroxide, a strong oxidizing agent that can kill 99% cyanobacteria without affecting other organisms. The use of potassium as bloom treatment also reduce cyanobacteria level to 50% by disturbing osmotic balance and change the internal pH

from 7 to 9.5 (Shukla & Rai, 2006). However, long-term effect to the environment should be taken into consideration.

2.4.2 Physical treatments

Different physical approaches to reduce cyanobacteria biomass have been conducted, including removal of nutrients or directly removing cyanobacterial cell. The physical treatments are generally expensive and has low efficiency compared to chemical treatment. However, secondary pollution is less likely to occur. Even so, it may also caused injury to non target organism (Shao et al, 2013). Therefore, best methodologies for cyanobacterial growth control should be investigated.

2.4.2(a) Removing nutrient

Physical approaches usually require multiple treatments for an effective water management. For example, phosphorus adsorption-only treatment or dredging-only treatment have lower effectiveness compared to combination of both phosphorus absorption and dredging treatments (Lurling & Faassen, 2012). Additionally, the approach, dredging may cause injury to other organisms, and phosphorus absorption reduced its effectiveness at high pH and humic substances interferences (Lurling & Faassen, 2012).

Furthermore, restriction on nutrient inputs is almost impossible and unavailable for most of the areas across the world due to economical limitation (Jančula & Maršálek, 2011). In addition, as mentioned in Section 2.3.3, evidently, nutrients may not influence bloom formation of cyanobacteria. Therefore, controlling cyanobacterial growth by only removing nutrient from the environment may not be efficient.

2.4.2(b) Removing cyanobacteria

Mixing lake waters using an air compressor, ultrasonic damage to algal cells, and pressure devices to collapse cyanobacterial gas vesicles, are also proposed to control algal blooms (Shao et al, 2013). However, akinetes population could lead reformation of bloom after cyanobacteria treatment, as the akinetes are able to germinate again from the bottom of the water body. While sedimentation drying suggested by Tsujimura (2004) can reduce germination of the cyanobacterial akinetes at low cost, the methodology is limited to akinetes-forming cyanobacteria. Therefore, multiple physical approaches are required for effective reduction of cyanobacterial population in an environment.

2.4.3 Biological treatments

As discussed in Section 2.3.4, biological factors are likely to influence the dynamics and population of cyanobacterial in an environment. Scientists have taken such observations to study potential compounds for further studies as summarized in Table 2.1.

In general, ideal anti-cyanobacterial compounds are characterized by strong inhibition to cyanobacteria, non-toxic to other organisms, readily degraded in the environment, inexpensive and safe to the environment (Shao et al, 2013). Effectiveness of the compounds is also influenced by the hydrophilicity and hydrophobicity (Ni et al, 2011). In addition, ideally, anti-cyanobacterial compounds should be able to inhibit most cyanobacterial species. If the inhibition is species specific, the compounds may enhance the growth of other cyanobacterial species, which is undesirable if the enhanced cyanobacterial species are toxin-producing species (Lalung, 2012).

Bio-control comes with risks such as developing resistance, different efficacy and specificity, and unknown future ecological effect. Bio-control could also be expensive (Shao et al, 2013) and requires complex extraction of anti-cyanobacterial compounds (Xiao et al, 2014). Even so, the approach can be relatively cheaper compared to physical approach, and safer than chemical approach as it readily degraded in the environment (Shao et al, 2013).

2.4.3(a) Viruses and bacteria

Isolation of cyanophage has been conducted since early 1960s. As virus phage infection is strain specific (Yoshida et al, 2006) due to infection mechanism of the phage that requires site recognition on bacterial cell, the use of virus as cyanobacterial biocontrol is safe. However, only specific strain of cyanobacteria is affected, whilst other bloom-forming cyanobacteria may still propagate. In addition, possibility of resistance occurrence may be higher compared to other approach.

Meanwhile, many bacteria showed anti-cyanobacterial properties as shown in Table 2.1. The bacteria showed no toxicity to cell culture, therefore they are safer options, however, effectiveness may depended on environment, for instance, Nakamura et al (2003) showed *Bacillus cereus* depended on pH for cyanobacterial lysing.

2.4.3(b) Plant biomass

Both aquatic and terrestrial plants are known for producing allelopathy chemicals, a secondary metabolites that affect the surrounding organisms such as microbes, either harmfully or beneficially (Wu et al, 2015). However, plant allelochemical activities also depended on factors such as temperature and plant maturity. In addition, the allelochemicals need to be released into the water, and sufficiently hydrophilic to reach target organisms in effective concentrations (Gross, 2003). Besides that, higher

plants may also release carbon-based organic compounds and dissolved organic nitrogen compounds, that may in turn stimulate growth of cyanobacteria (Gross, 2003). Even so, many researches showed effectiveness of plant biomass as cyanobacterial bloom management.

Several active compounds released from plants have been successfully isolated and characterized in previous researches, which include polyphenol (Ni et al, 2013), terpenoid (Ni et al, 2011) and fatty acid (Nakai et al, 2005). These compounds inhibit growth via different pathways, such as inhibition of photosynthesis, disruption of cellular structure, and inactivation of enzymatic and non-enzymatic functions (Ni et al, 2013). Hence further researches on presence of anti-cyanobacterial compounds in plants should be conducted in future by using compounds separation. In 2014, a research group has successfully isolated and proved that two compounds under the group of flavonolignin, namely salcolin a and salcolin b from barley straw act as algistatic and algicidal toward cyanobacteria respectively (Xiao et al, 2014). As such, there is a possibly that the compounds play similar role in palm oil trunk and leaf leachate.

Previous researches also hypothesized the ability of barley straw to control cyanobacterial growth is due to lignin composition. However, the effectiveness and efficacy varies depending on cyanobacteria species (Lalung, 2012) and type of barley straw (Xiao et al, 2014). Murray et al (2010) had also showed that the barley straw after pre-treated with fungi has enhanced ability as algae bio-control. Additionally, previous research also indicates potential of palm oil trunk as bio-control, but with different effectiveness and outcomes (Sim, 2015).

Table 2.1 Summary of biological approaches for cyanobacterial bloom management.

Biological treatment		Potential bio-derived substances/Mechanisms	Tested Cyanobacterial sp.	Reference
Virus	Cyanophage	Infection	*Microcystis aeruginosa*	(Yoshida et al, 2006)
Bacteria	*Streptomyces neyagawaensis*	Unknown/ anti-algae	*Microcystis aeruginosa* *Anabaena cylindrica*, *Anabaena* Xos-*aquae*, *Oscillatoria sancta*	(Choi et al, 2005)
	Bacillus cereus	Unknown/ allelopathy	*Microcystis* sp.	(Nakamura et al, 2003)
		Cell to cell contact mechanism/Lysis	*Aphanizomenon flos-aquae*	(Shunyu et al, 2006)
	Pseudomonas putida	Unknown/Anti-shock	*Microcystis aeruginosa*	(Zhang et al, 2011)
	Stenotrophomonas F6	Hydroquinone, Cyclo-(Gly-Pro)/ allelopathy	*Microcystis aeruginosa* *Synechococcus*	(Lin et al, 2015)
Waste biomass	Barley straw	Salcolin a, salcolin b/Plasma membrane destruction, inhibition of esterase enzyme	*Microcystis aeruginosa*	(Xiao et al, 2014)
	Banana peel and orange skin	Unknown	*Microcystis aeruginosa*	(Jianzhong et al, 2004)
	Sugarcane bagasse	Unknown	Various	(Sim, 2015)
	Empty fruit bunch	Unknown	Various	(Sim, 2015)
	Palm oil trunk	Unknown	Various	(Sim, 2015)

Table 2.1 (Cont.) Summary of biological approaches for cyanobacterial bloom management.

Biological treatment		Potential bio-derived substances/Mechanisms	Tested Cyanobacterial sp.	Reference
Waste biomass	Haddock peel, Pomegranate	Various allelochemical / allelopathy	*Microcystis aeruginosa*	(Wang et al, 2015)
Aquatic/ wetland plant	*Vallisneria spiralis*	2-Ethyl-3-methylmaleimide, ionone/ allelopathy	*Microcystis aeruginosa*	(Xian et al, 2006)
	Phragmites communis	ethyl 2-methyl acetoacetate/ oxidation	*Microcystis aeruginosa*	(Hong et al, 2008)
	Cyperus alternifolius	Phenolic/ allelopathy	*Microcystis aeruginosa*	(Nakai et al, 2008)
	Myriophyllum verticillatum	Unknown/ allelopathy	*Anabaena variabilis*	(Bauer et al, 2009)
	8 species of aquatic macrophytes	Unknown	*Microcystis aeruginosa*	(Chen et al, 2012)
	Lindernia rotundifolia, Hygrophila stricta, Cryptocoryne crispatula	Removal of nitrogen and phosphorus	Cyanobacteria	(Wang et al, 2012)
	Hydrilla verticillata	Unknown/ oxidation damage	*Anabaena flos-aquae*	(Zhang et al, 2012)
	Pistia stratiotes	Unknown/ oxidative damage	*Microcystis aeruginosa*	(Wu et al, 2015)
	Myriophyllum spicatum	Fatty acids (nonanoic and Octadecanoic acid), polyphenol / allelopathy	*Microcystis aeruginosa*	Various