DETECTION AND IDENTIFICATION OF CYANOBACTERIAL TOXIN ENCODING GENE IN MALAYSIA

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DETECTION AND IDENTIFICATION OF CYANOBACTERIAL TOXIN ENCODING GENE IN MALAYSIA

by

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LIST OF ABBREVIATIONS

ATX	anatoxin-a
BLAST	Basic Local Alignment Search Tool
CYN	cylindrospermopsin
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
HAB	harmful algal bloom
mcy	microcystin
NCBI	National Centre for Biotechnology Information
NPRS	non-ribosomal peptide synthetase
PCR	polymerase chain reaction
PKS	polyketide synthase
PS	peptide synthetase
PSP	paralytic shellfish poison
rRNA	ribosomal RNA
SXT	saxitoxin
TBE	Tris-Borate-EDTA
TES	2-[Tris(hydroxymethyl)-methylamino]-ethanesulfonic acid
WHO	World Health Organization

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MENGESAN DAN MENGENALPASTI GEN PENGEKODAN TOKSIN SIANOBAKTERIA DI MALAYSIA

ABSTRAK

Ledakan sianobakteria boleh menjadi masalah utama kerana sebahagian sianobakteria berupaya menghasilkan toksin yang boleh mendatangkan mudarat kepada haiwan serta manusia. Penghasilan toksin oleh sianobakteria adalah berasaskan kepada gen-spesifik dan bukannya spesies-spesifik. Oleh itu, pengecaman secara morfologi tidak boleh dipercayai dalam menentukan ketoksikan sianobakteria. Tujuan utama kajian ini adalah untuk menentukan taburan sianobakteria bertoksik dalam persekitaran Malaysia yang terpilih. Sebanyak sembilan puluh enam sampel diambil di Pulau Pinang, Kedah, Perak, Kelantan, Sabah serta Sarawak dan kehadiran toksin sianobakteria disiasat menggunakan tindakbalas rantai polimerase (PCR). Sianobakteria dikesan daripada sembilan puluh lima sampel. Gen pengekodan *microcystin* telah dikesan di Empangan Air Itam, Pulau Pinang dan Miri City Fan, Sarawak dan *cylindrospermopsin* telah dikesan di Empangan Air Itam, Pulau Pinang. Kehadiran sianobakteria bertoksik di Malaysia yang dikenalpasti boleh menjadi satu ancaman kerana gen pengekodan *microcystin* dan *cylindrospermopsin* telah berjaya dikesan. Bahagian kedua dalam ujikaji ini adalah mengenalpasti sianobakteria yang berkebolehan menghasilkan toksin. Sebanyak empat puluh dua spesies sianobakteria terpencil telah dikenalpasti melalui teknik molekul 16S rRNA menggunakan tindakbalas rantai polimerase (PCR). Sebahagian daripada strain sianobakteria ini pernah merekodkan kehadiran toksin, dan kerana itu pengesanan bagi gen pengekodan toksin terhadap sianobakteria terpencil ini telah dijalankan. Gen *anatoxin* (ATX) yang terlibat dalam penghasilan toksin *anatoxin* telah dikesan dalam dua sianobakteria

terpencil iaitu *Limnothrix* sp. B15 dan *Leptolyngbya* sp. D1C10. Akhir sekali, ujian terhadap keberkesanan penyimpanan strain sianobakteria secara jangka panjang dikaji menggunakan teknik pembekuan dalam suhu rendah dengan kehadiran bahan pelindung. Tujuh belas strain sianobakteria digunakan dalam ujikaji ini di mana metanol digunakan sebagai bahan pelindung dan disimpan dalam suhu -20 darjah bagi jangka masa tertentu. Sembilan strain sianobakteria berupaya untuk hidup kembali selepas penyimpanan selama tiga bulan dalam suhu rendah dengan menggunakan metanol sebagai bahan pelindung. Setiap sianobakteria menunjukkan tindak balas yang berbeza apabila disimpan dalam suhu rendah bergantung kepada kumpulan sianobakteria. Hasil daripada kajian ini, pemantauan berterusan perlu dijalankan kerana toksin sianobakteria telah dikesan dalam persekitaran Malaysia dan teknik pembekuan menunjukkan keberkesanan bagi tujuan penyimpanan untuk jangka masa panjang.

DETECTION AND IDENTIFICATION OF CYANOBACTERIAL TOXIN ENCODING GENE IN MALAYSIA

ABSTRACT

Cyanobacterial blooms could become a major concern since certain cyanobacteria are able to produce toxins which can be harmful to animals and human. The production of the toxins are gene-specific, and not species-specific. Thus morphological identifications are not reliable to determine the toxicity of the cyanobacteria. The main aim of this study was to detect the presence of toxic cyanobacteria in selected water bodies in Malaysia. A total of ninety-six samples were collected from Penang, Kedah, Perak, Kelantan, Sabah and Sarawak and investigated for the presence of cyanobacterial toxins using PCR amplification of toxin-encoding genes. Cyanobacteria were detected in ninety-five samples. Microcystin-producing gene was detected in Air Itam Dam, Penang and Miri City Fan, Sarawak, while cylindrospermopsin-producing gene was detected in Air Itam Dam, Penang. Through this study, the presence of cyanobacterial toxin in Malaysia can now be determined as potential threat because microcystin and cylindrospermopsin-encoding genes were detected in samples from some locations. The second part of the study was to isolate and identify toxin and non-toxin producing strains from the selected study sites. Forty-two cyanobacterial strains were identified using 16S rRNA gene sequence analysis. Some of the isolated strains have recorded some histories of cyanotoxin production, thus detection of cyanobacterial toxins were carried out to detect the toxin-encoding genes in the isolated strains. Anatoxin-a-encoding gene, which is involved in the biosynthesis of anatoxin-a was detected in two isolated strains namely *Limnothrix* sp. B15 and *Leptolyngbya* sp. D1C10. The last part of this study was to evaluate the

effectiveness of long-term preservation of cyanobacterial strains using deep freezing technique with the presence of cryopreservative agents. Seventeen isolated cyanobacterial strains were used to study the effects of methanol as cryopreservation agents in deep freezing (-20^{o}C) for certain periods of times. A total of nine strains exhibited high post-thaw viability after three months of preservation with the presence of methanol as cryopreservative agent. Cyanobacterial strains responses differently to the cryopreservation methods according to the cyanobacterial groups. From the results, continuous monitoring should be conducted due to the presence of cyanobacterial toxin in water bodies in Malaysia and cryopreservation technique has demonstrated to be effective for the long terms of storage.

CHAPTER ONE

INTRODUCTION

1.1 Background

Over the past few years, greater attention has been focused on algal that affect the quality of drinking water perhaps due to demand of consumers for a safe source of drinking water (Hunter et al., 2010). Water pollution can affects the quality of drinking water sources as well as recreational waters and caused a negative impact on the sustainability of water resources, human health and economy of the countries (Salih et al., 2013). Besides pH, temperature, TSS, COD, BOD, turbidity and *E. coli*, algal density becomes one of the parameters in drinking water and recreational waters quality standard. A wide variety of algal types have been involved in development of unfavorable tastes and odors in water supplies and blue green algae are one of those algal that most often encountered in blooms formation (Yoo et al., 1995).

Cyanobacteria also known as blue-green algae is a photosynthetic bacteria and can be found in most of the water column (Waterbury, 2006). Cyanobacteria can multiply rapidly in water surface and form blooms when in large population (Azevedo et al., 1994). Cyanobacterial blooms can be found in eutrophic water bodies of freshwater, estuarine as well as marine ecosystem. As an example, cyanobacterial blooms in freshwater ecosystem mainly consisted of *Microcystis* spp., *Anabaena* spp. or *Cylindrospermopsis* spp., while estuarine ecosystems mainly consisted of *Nodularia* spp. and *Aphanizomenon* spp. Cyanobacterial blooms in marine ecosystem consisted of *Lyngbya* spp., *Synechococcus* spp. and *Trichodesmium* spp. (O'Neil et al., 2012).

Cyanobacterial blooms can degrade water quality as their presence will cause foul odors and tastes, deoxygenation of bottom waters, toxicity, aquatics kills and disruption of

food web (WHO, 1999). They also can produce toxins known as cyanotoxins that severely affect animals as well as human health (Paerl et al., 2001). A wide range of toxins produced by cyanobacteria can be classified into few distinct categories in terms of their structures and mode of actions. The toxins can be divided into hepatotoxins, cytotoxins, neurotoxins, and dermatotoxins (Dittmann et al., 2013). The problem with the cyanobacterial toxins is that the water is unsuitable for multi-purposes even for irrigation because the toxin persists in water even after the disappearance or death of the cyanobacterial cells (Sarma, 2012).

Fifty-two haemodialysis patients in Brazil died due to intoxication of microcystin in the dialysis fluid in 1996. This incident known as 'Caruaru Syndrome' might be the worst case that was ever recorded (Azevedo et al., 2002). Following the incident, World Health Organization (WHO) had considered cyanobacteria as 'contaminants' and increased the awareness of cyanobacteria toxicity in the year 2000 (WHO, 1999). Provisional guidelines value of 1 μg L^{-1} for microcystin, one of the cyanobacterial toxins was set up by the WHO for drinking water (WHO, 2003, WHO, 1998). Up to 2012, some countries including European Union, Australia, Brazil, Canada, Cuba, New Zealand, Singapore and some states in the USA has implemented the regulation for cyanobacterial toxins in their water-bodies used for drinking and recreational purposes (Chorus, 2005).

Despite of many cases of cyanobacterial toxicities recorded, studies on cyanobacterial toxins in Malaysia are still limited despite of its harmfulness due to the lack of awareness and monitoring. Many countries including Malaysia does not include cyanobacterial toxins into any of its guidelines or standards for the drinking and recreational water quality. Malaysia National Standard for drinking water quality revised in 2004 did not consider any of cyanobacterial toxins as potential threat.

Previous cyanobacterial studies in Malaysia only focused on the cyanobacterial communities in the water bodies. Studies of cyanobacterial communities were carried out in Putrajaya (Yusoff and Seman, 2013, Malek et al., 2012, Mansoor et al., 2011), Sarawak (Harith and Hassan, 2012, Harith and Hassan, 2011, Harith and Hassan, 2007), Penang (Makhlough, 2008) and Johor (Singh et al., 2013) and the water bodies revealed a number of cyanobacterial genera and species. Out of the detected cyanobacteria, some of them were recognized as potential toxin producers.

However, the monitoring was done based on morphological looks, a method which is no longer reliable. The technique is inaccurate and it also cannot predict the ability of the cyanobacterial species to produce toxins because the toxicity in cyanobacteria is gene-specific and not species-specific (do Carmo Bittencourt-Oliveira, 2003). With the recent molecular analysis, it is now possible to identify cyanobacterial species accurately and determine whether a strain is capable of producing toxin. Different species that carry the same gene may produce the same toxin but the same species may or may not produce the toxin (do Carmo Bittencourt-Oliveira, 2003).

Identification of cyanobacterial strains based on morphological characteristics and 16S rRNA gene sequences can be used to detect the presence of cyanobacteria in the samples as well as to identify unknown cyanobacterial strains, but does not recognized whether the strains are toxin-producing or non-toxin-producing cyanobacteria (Nubel et al., 1997). Thus, to identify the potential toxin producers in cyanobacteria, detection of toxin-encoding genes using PCR amplification of specific sequences is likely the best options.

Due to this, potential threats of cyanobacterial toxins in Malaysia can now be determined by the detection of toxin-encoding genes using molecular analysis. Presence

or absence of toxin-encoding genes in the samples can be related to the potential risks of cyanobacterial toxins in Malaysia's water environments. Production of cyanobacterial toxin was determined by the presence of cyanobacterial toxin-encoding genes. For example, *mcyE* gene was responsible for the biosynthesis of microcystin (Rantala et al., 2006, Rantala et al., 2004). Microcystin is one of the cyanobacterial hepatotoxin. Thus, detection of toxin-encoding gene is crucial to determine the potential cyanobacterial toxins present in selected Malaysia's environments.

Presence of microcystin has been reported in freshwater lakes in Selangor (Sinang et al., 2015) and cyanobacterial species producing toxin, microcystin has been successfully isolated from Air Itam Dam, Penang (Sim, 2015). These studies indicated the presence of cyanobacterial toxins in Malaysia's water bodies.

Isolation of cyanobacterial strains can be laborious and time-consuming. Thus, best preservation methods should be applied to maintain cyanobacterial strains in cultured conditions especially for the toxin-producing cyanobacteria. Various methods have been used to preserve cyanobacterial strains such as batch cultures, agar slants and cryopreservation methods (Day, 2007, Romo and Bécares, 1992). Cryopreservation methods include cool storage (temperature ranging from 5 to 8°C), deep freezing (-20°C) and storage at low temperatures (−80°C) using cyroprotectant agents such as glycerol, sucrose, methanol and lyophilization (drying cultures using silica gel) (Prasad et al., 2013, Esteves-Ferreira et al., 2013, Syiem and Bhattacharjee, 2010).

1.2 Problem statements

As a photosynthetic bacteria, the problems caused by cyanobacteria are more apparent in tropical climates such as Malaysia. Due to this tropical climate, cyanobacterial blooms has the possibly to occur all year round due to the abundance of sunlight as it plays a vital role in the formation of cyanobacterial blooms. As a comparison, research on the presence of cyanobacterial toxin has been carried out in Singapore and revealed the presence of microcystin; one of the toxin produced by cyanobacteria, in Keranji Reservoir. They have also discovered that the percentage of the presence of cyanobacterial toxin was higher in subtropical and temperate regions (Te and Gin, 2011). Considering the fact Malaysia shares the same climate as Singapore, the situation is similar in Malaysia. But the potential threat of cyanobacterial toxins remain unknown until an extensive study is conducted. A preliminary study of cyanobacterial diversity and potential toxin in freshwater lakes in Selangor, Malaysia revealed the presence of microcystin in their research (Sinang et al., 2015). However, only microcystin was reported, and the status of other cyanobacterial toxins was still unknown. Thus, there is a need to monitor and detect cyanobacterial toxins in Malaysia's water environment.

Maintaining cyanobacterial strains especially toxin-producing cyanobacteria using serial transfer or subculturing requires extensive man-power, is time-consuming and higher exposure to contamination (Bui et al., 2013, Romo and Bécares, 1992). Thus, to overcome the circumstances, cryopreservation methods were introduced for better solution in preserving cyanobacterial strains.

1.3 Hypothesis

Throughout this research, we are expected to evaluate the presence of cyanobacteria and cyanobacterial toxin-encoding genes in Malaysia's water bodies using molecular analysis. Upon detection of the presence or absence of the toxin-encoding genes, the current status of toxin-encoding genes in Malaysia can be clarified to prevent any potential disaster such as human fatality.

Cyanobacterial communities are hypothesised to be present in all samples collections, thus cyanobacterial species are expected to be isolated and identified using molecular analysis and presence of cyanotoxin in these cyanobacterial species can be determined. Since the toxin production in cyanobacteria is gene-specific, toxin-producing and non-toxin producing cyanobacterial species isolated from Malaysia's water bodies can also be distinguished using molecular techniques.

Following the isolation and identification of cyanobacteria, preserving the cultures is crucial for maintaining the cultures collection. Thus, cryopreservation methods were expected to be effective in maintaining a large numbers of cultures for a long period. We also hypothesised that cryopreservation technique can be the best options for the long preservation of cyanobacterial strains in the cultured laboratory to replace conventional techniques such as serial transfer. The conventional technique is rather laborious and time-consuming, thus, cryopreservation technique is developed to overcome these problems. Cryopreservation technique may offers new insight in preservation of cyanobacterial strains.

1.4 Research Objectives

The purpose of this study is to determine the current status of toxin-encoding genes in drinking and recreational water bodies in Malaysia. Using the molecular analysis approaches, the current status of cyanobacterial toxins in Malaysia can be assured to meet the following objectives:

1. To evaluate the presence of cyanobacteria and toxins-encoding genes from Malaysia's water bodies using molecular analysis.
2. To isolate and identify cyanobacterial strains from Malaysia for their potential ability to produce toxins.
3. To evaluate the effectiveness of cryopreservation methods for isolated cyanobacterial strains.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction to cyanobacteria

Cyanobacteria are among the earliest organisms that exists on earth, and has been reported in existence for at least 2.7 billion years (Zanchett and Oliveira-Filho, 2013). Cyanobacteria are prokaryotic organisms that has unique characteristics as compared to other prokaryotes. Cyanobacteria are prokaryotes that contain photosynthetic pigment; chlorophyll and among the ancient organisms on Earth that developed an efficient photosynthetic capacity (Zanchett and Oliveira-Filho, 2013). About 20-30% of the total carbon fixed on earth are produced by cyanobacteria and they become the primary biomass producers (Pisciotta et al., 2010). They can obtain their nutrient mainly via photosynthesis process. Some of them can fix nitrogen from the atmosphere through nitrogen fixation mechanism (Akcaalan et al., 2009).

To date, around 2000 species and 150 genera of cyanobacteria with different morphologies has been identified. Out of 150 genera, 40 of them are capable to produce toxins such as *Anabaena, Aphanizomenon, Cylindrospermopsis, Lyngbya, Microcystis, Nostos, Oscillatoria* and other genera (van Apeldoorn et al., 2007).

Based on morphology characteristics, cyanobacteria can be classified into unicellular and filamentous cyanobacteria. Unicellular cyanobacteria exist in both single or colony forms, while filamentous type may exist as thin or thick filaments, single or bundles as well as with or without a sheath (Abed et al., 2009). Some of filamentous cyanobacteria develops specialized cells called heterocyst and akinete. Heterocyst is known as site of aerobic nitrogen fixation in heterocystous cyanobacteria, while akinete is a resting spore-like cells that is capable to withstand some extreme environments and

germination (Adams, 2000). All heterocystous cyanobacteria have the ability to fix nitrogen aerobically, but some non-heterocystous cyanobacteria of unicellular species like *Gloeothece* sp. and *Cyanothece* sp. and filamentous species like *Trichodesmium* sp., *Lyngbya* sp. and *Oscillatoria* sp. can also fix nitrogen (Bandyopadhyay et al., 2013). Some filamentous cyanobacteria are capable to develop branching; in forms of true and false branching by binary fission (Moreira et al., 2013, Waterbury, 2006).

2.2 Cyanobacterial environment and adaptation

Cyanobacteria are microscopic cells that grow widely in most water bodies. These bacteria can grow in shallow, warm, slow moving or still water. Cyanobacteria can tolerate wide ranges of salinity, pH and temperature (Waterbury, 2006). Due to this nature, they can adapt and thrive in many diverse and extreme environments all over the world such as, arid desert soils, thermal springs, rocks, plants, marine, brackish and freshwaters, ice, plants and on vertebrates and invertebrates animals (Choudhary, 2010), but most commonly observed in temperate and tropical freshwaters (Te and Gin, 2011).

Increase of eutrophication caused massive development of cyanobacterial population (Komárek et al., 2014). This situation can be seen in Malaysia's water bodies, as they suffered eutrophication due to an increase of human population and urbanization, as well as agricultural and industrialization activities. These factors can lead to the increase of chemical elements in water bodies leading to the formation of algal bloom (Makhlough, 2008).

Cyanobacteria can thrive in wide range of ecological habitats (Choudhary, 2010). As an example, *Planktothrix* spp. can grow under extreme conditions; such as in frozen

water storages and lakes, whereas *Microcystis* spp. and *Cylindrospermopsis* spp. can flourish in tropical reservoirs (Falconer and Humpage, 2006). Some genera and species of cyanobacteria can form blooms in freshwater and brackish water bodies. The colonial species of *Microcystis aeruginosa* is the most common hepatotoxin-producing cyanobacterium in eutrophic freshwater (Azevedo et al., 1994). According to Ye et al. (2009) some cyanobacteria can adapt to low temperature and even survive in the winter. Cyanobacteria can survive in various environments, thus cyanobacteria are expected to be found abundantly in our country because Malaysia is a tropical country that receives sunlight throughout the year (Makhlough, 2008).

2.3 Cyanobacterial blooms

Cyanobacteria are present in most water bodies and sometimes grow to large populations known as blooms (Azevedo et al., 1994). In Malaysia, cyanobacterial blooms occurs throughout the year due to the availability of the sunlight and warm temperature. Due to climate change, more cyanobacterial blooms can be found worldwide (O'Neil et al., 2012). The scums can vary from a small dot at the early formation of a bloom, to thick paint-like accumulations during the peak of the bloom (Azevedo et al., 1994).

Cyanobacterial blooms are a matter of serious concern worldwide as their occurrence is related to problems of the eutrophication of water bodies (O'Neil et al., 2012). Formation of the blooms can affects water turbidity, taste and odor of the drinking water as well as production of toxin. Many blooms were reported to be toxic and had caused significant deaths to animals as well as being implicated with human illness (Carmichael, 1994, Codd et al., 1989). A series of algal blooms were observed in

Seoul's water bodies and had caused major alert to the quality of South Korea's drinking water (Park et al., 2012).

Blue-green algal blooms happen when the favorable conditions are available like high nutrients concentrations (Harith and Hassan, 2007), low water flows and high temperature between 15 to 30^{o}C (Azevedo et al., 1994). Cyanobacterial blooms does not always found floating on water surface, but some of them is suspended at the various depths of water (Yoo et al., 1995). There are various factors that affect the depth at which cyanobacterial blooms float. The most vital factors are light, phosphorus (P) and nitrogen (N) as the availability of these elements are important for cyanobacteria to survive (Makhlough, 2008).

Water flow, turbulence and wind can influence the formation of the cyanobacteria in water bodies. High flow in rivers and streams increase the turbulence and rate of cyanobacteria dispersion. Thus, it prevents the blooms by dispersing the cells further apart and making them harder to retain their buoyancy (Yoo et al., 1995).

Seasonal changes like nutrients availability, light and temperature are believed to influence the succession of toxic cyanobacterial blooms in water columns and also affect the production of the microcystin (Ye et al., 2009). Eutrophication and climate change may as well promote the proliferation and expansions of toxic cyanobacterial blooms. So, the status of nutrients like N and P as well as availability of sunlight can affects the formation of toxic cyanobacterial blooms (Lürling et al., 2017, O'Neil et al., 2012).

Eutrophication is a condition where water bodies contain excessive richness of nutrient especially N and P that can caused the formation of the blooms. Eutrophication can happen naturally by weathering process, or by anthropogenic activities such as

agriculture, urbanization, wastewater treatment plants, and consumption of fossil fuels (Bricker et al., 2008). Climate change often associated with temperature and nutrient input. Regions that receive higher rainfall will have higher nutrient input, resulting in eutrophication (Howarth, 2008). Global warming caused the formation of toxic cyanobacterial blooms due to the increased of water temperature (O'Neil et al., 2012).

2.4 Cyanobacterial taxonomy

Taxonomic classification of cyanobacteria has always been challenging to most taxonomist as the systems had to undergo extensive restructuring and revisions over the years (Komárek et al., 2014). In earlier centuries of 19th and 20th, classification of cyanobacteria were done entirely based on the morphological observation (Hašler et al., 2012). Although identification of cyanobacteria using morphological observation is crucial as preliminary step in a research, but depending solely on morphology characteristics in cyanobacteria detection could lead to misidentification because morphological looks can vary in different environments (Nubel et al., 1997).

In modern taxonomy, cyanobacteria are classified based on phylogenetic tree that reflects the evolutionary relationships. In modern cyanobacterial taxonomy, polyphasic approach which include morphological, ecological and molecular data are used for characterization of cyanobacterial taxa (Komárek et al., 2014, Komárek, 2006). However, thylakoid structures and types of cell division remain as the conservative ultrastructural characters in taxonomy of cyanobacteria. Classification of cyanobacteria based on combined molecular, phenotype, ultrastructural, chemical and ecophysiological criteria were first proposed in 2005 (Hoffmann et al., 2005). Evolution of cyanobacterial taxonomy according to Komárek et al. (2014) is shown in Table 2.1.

Table 2.1: Evolution of classification of cyanobacterial according to Komárek et al. (2014).

Year	Classification
1925	Seven orders: - Chroococcales, Entophysalidales, Pleurocapsales, Dermocarpales, Siphononematales, Nostocales and Stigonematales
1932	Three orders: - Chroococcales, Chamaesiphonales, and Hormogonales
1942	Four orders: - Chroococcales, Dermocarpales, Pleurocapsales and Hormogonales
1979	Five subsections instead of orders based on bacteriological code: - I (= Chroococcales), II (= Pleurocapsales), III (= Oscillatoriales), IV (= Nostocales) and V (= Stigonematales).
2005	Four subclasses: - Gloeobacteriophycidae, Synechococcophycidae, Oscillatoriophycidae and Nostochopycidae
2014	Eight orders: - Gleobacterales, Synechococcales, Oscillatoriales, Chroococcales, Pleurocapsales, Spirulinales, Chroococcidiopsidales and Nostocales

Eight orders are identified in modern taxonomy of cyanobacteria based on phylogenetic analysis and ultrastructural pattern of thylakoids. They are Gleobacterales, Synechococcales, Oscillatoriales, Chroococcales, Pleurocapsales, Spirulinales, Chroococcidiopsidales and Nostocales as presented in Table 2.2.

Table 2.2: Modern classification of cyanobacteria based on preliminary results of phylogenetic analyses and ultrastructural patterns of thylakoids (Komárek et al., 2014).

Order	Characteristics	Families
Gloeobacterales	Lacks of thylakoids; contains only one family and a genus.	Gloeobacteraceae
Synechococcales	Combination of both unicellular and filamentous cyanobacteria; parietal thylakoids; contains eleven families and over seventy genera.	Synechococcaceae, Merismopediaceae, Prochloraceae, Coelosphaeriaceae, Acaryochloridaceae, Chamaesiphonaceae, Romeriaceae, Pseudanabaenaceae, Leptolyngbyaceae, Heteroleibleiniaceae, and Schizotrichaceae
Spirulinales	Filamentous cyanobacteria with parietal thylakoids; screw-like coiled trichomes; absent of sheaths; contains only one family and few genera.	Spirulinaceae
Chroococcales	Coccoids cyanobacteria with more or less irregular thylakoid arrangement; in forms of colonial and not colonial; consists of eight families.	Microcystaceae, Aphanothecaceae, Cyanobacteriaceae, Cyanothrichaceae, Stichosiphonaceae, Chroococcaceae, Gomphosphaeriaceae and Entophysalidaceae
Pleurocapsales	Unicellular aggregates of cells with irregular thylakoids; baeocytes; consists of four families.	Hydrococcaceae, Dermocarpellaceae, Xenococcaceae Pleurocapsaceae
Oscillatoriales	Includes filamentous and coccoid cyanobacteria with radial, or irregular thylakoid arrangement; consists of seven families.	Cyanothecaceae, Borziaceae, Coleofasciculaceae, Microcoleaceae, Homoeotrichaceae, Oscillatoriaceae and Gomontiellaceae
Chroococcidiop-sidales	Unicellular cyanobacteria with irregular thylakoids arrangement; solitary and colonies forms; baeocytes; contains only one family and a genus.	Chroococcidiopsidaceae
Nostocales	Filamentous cyanobacteria with irregular thylakoids arrangement; diversified thallus and special prominent cells (heterocytes, akinetes); contains unbranched and isopolar, and falsely or true branched types; consists of twelve families.	Scytonemataceae, Symphyonemataceae, Rivulariaceae, Tolypothrichaceae, Godleyaceae, Chlorogloeopsidaceae, Hapalosiphonaceae, Capsosiraceae, Stigonemataceae, Gloeotrichiaceae, Aphanizomenonaceae, and Nostocaceae

Up until now, there's no fixed taxonomic classification for cyanobacteria, as the system still undergoes some modification. In 2016, some revision of cyanobacterial groups which contain planktonic freshwater taxa were documented (Komárek, 2016). These revised taxonomy were characterized based on polyphasic evolution as well as ecological adaptation and geographical distribution of different genotypes of cyanobacteria. Although, in Komárek et al. (2014), cyanobacteria was classified into eight orders, but the classification may change in the future with some modification and additional informations of cyanobacterial taxonomic classification.

2.5 Cyanobacterial studies in Malaysia

Studies on cyanobacterial toxin in Malaysia are very limited. Most of cyanobacterial studies in Malaysia does not include cyanobacterial toxins as part of their studies. In 2007, a preliminary study on cyanobacterial composition has been conducted in freshwater fish ponds in Serian, Sarawak. Five genera were detected including *Synechocystis, Oscillatoria, Chroococcus, Nostoc* and *Pleurocapsa* in the water bodies, but does not distinguish between toxic and non-toxic cyanobacteria in the study (Harith and Hassan, 2007). A study on water parameters was conducted in Mengkuang Dam in Penang only focused on phytoplankton community and detected few genera including *Anabaena, Microcystis, Oscillatoria,* and *Nostoc*. Although these genera were known to produce some toxins worldwide, but the study does not examine the toxicity of the detected genera (Makhlough, 2008). So, the status of toxicity of cyanobacteria in Malaysia cannot be determined because identification of cyanobacterial strains does not indicate the cyanobacterial toxicity. Thus, to determine the toxicity of cyanobacteria, toxicity analysis either by chemical or molecular analysis should be carried out.

A study has been conducted in 2011 to investigate the pollution status of the freshwater lake using an image processing techniques and artificial neural network (ANN) algorithms. Five genera of cyanobacteria include *Synechocystis, Oscillatoria, Chroococcus, Nostoc* and *Pleurocapsa* were detected but the technique does not indicate toxicity of the cyanobacteria (Mansoor et al., 2011). Harith and Hassan (2011) has conducted a study to evaluate the diversity and similarity of cyanobacterial populations in selected Sarawak aquatic ecosystems using β- indices. From the study, a total of 43 species of cyanobacteria that belong to 30 genera with the most distributed genera were *Chroococcus, Lyngbya, Nostoc* and *Oscillatoria* were recorded. Although all these genera are known as potential toxin-producer cyanobacteria but cyanobacterial toxins or toxin-encoding genes are not one of the monitored parameters. Thus, the status of cyanobacterial toxins in these water environments cannot be determine because no studies were conducted to determined the toxicity in these genera, even though they are known to produce toxin because the production of cyanobacterial toxin is gene-specific (do Carmo Bittencourt-Oliveira, 2003).

Harith and Hassan (2012) reported the presence of cyanobacteria in Ranchan Pool, Sarawak. Seventeen species of cyanobacteria was recorded from the pool including six genera of potential toxin producing genera; *Cylindrospermopsis, Nostoc, Lyngbya, Oscillatoria, Scytonema* and *Synechococcus*. Nevertheless, there were no reports of the presence of cyanobacterial toxins from the pool (Harith and Hassan, 2012). In Singh et al. (2013), a total of five types of algae were found in Semberong Dam, Johor including cyanophyta, clorophyta, euglenophyta, chrysophyta and pyrrophyta. From the observation using phase-contrast microscope, *Planktothrix* sp. was the dominant species in the dam. *Planktothrix* is known to produce microcystin, but the toxicity was not the observed parameters in this study. Toxicity of cyanobacteria in these studies

cannot be determined because cyanobacterial toxicity analysis was not conducted. Even though some of the cyanobacterial genera found in these studies has the potential to produce toxins, but production of cyanobacterial toxin is not species-specific, but rather gene-specific (do Carmo Bittencourt-Oliveira, 2003). As an example, *Cylindrospermopsis* sp. is known to produce cylindrospermopsin (Fastner et al., 2003), but the production of this toxin is determine by the presence of cylindrospermopsin-encoding gene in the cyanobacterium (Schembri et al., 2001)

According to the research conducted in Putrajaya lakes and wetlands in 2007 to 2012, cyanobacteria always showed the highest number within the phytoplankton population but the species diversity of the phytoplankton begin to decrease in 2012 due to the dominance of cyanobacteria (Yusoff and Seman, 2013). Nine genera including *Microcystis, Oscillatoria* and *Anabaena* and fifteen species were found in the lake. *Oscillatoria* and *Anabaena* were known to pose anatoxin-a and homoanatoxin-a, while *Microcystis* was known as a potential microcystin producer (Stewart et al., 2006). The monitoring was done based on morphological characteristics, which was unable to predict the ability of the species to produce toxin (Yusoff and Seman, 2013). In 2012, Malek et al., evaluated that 28% of total phytoplankton in Putrajaya Lake consisted of cyanobacteria but cyanobacterial toxins or toxin-encoding genes is not one of the monitored parameters in the study (Malek et al., 2012).

Most of cyanobacterial studies in Malaysia focused only on cyanobacterial communities in the water bodies until Sinang et al. (2015) had recorded the presence of microcystin in Selangor freshwater lakes. Furthermore, in 2015, *Microcystis* sp. producing microcystin was successfully isolated from Air Itam Dam, Penang (Sim, 2015). From these studies, it can be summarized that potential risk of cyanobacterial toxins does exist in Malaysia’s water environments.

2.6 Cyanobacterial toxins

Great concerns arised towards cyanobacterial blooms because of their ability to produce toxins or also known as cyanotoxins (Falconer and Humpage, 2006). Cyanobacterial toxins can be divided into few categories; hepatotoxins, neurotoxins, endotoxins and non-other specific toxins. Cyanobacterial toxin types, names and mode of actions are shown in Table 2.3. The neurotoxins, such as anatoxin-a and anatoxin-a (s) are highly toxic nerves poisons that attacks the nervous systems that can lead to muscular paralysis and in severe cases, cause death due to respiratory failure (Yoo et al., 1995). Hepatotoxins, such as microcystin and nodularin inhibit specific enzymes systems and cause damage to gut and liver (Carmichael, 2001). Endotoxins have been involved in gastrointestinal problems and some cause irritations to the skin. General cytotoxins like cylindrospermopsin can inhibit protein synthesis and modify the DNA or RNA structures (Sivonen and Jones, 1999). The toxins attack cells regardless of the organs location and caused damage to kidney and liver (Yoo et al., 1995).

It's estimated that 90% of cyanobacteria researchers originated from only ten countries (Merel et al., 2013) in the past few years. Currently, many countries worldwide including Thailand (Mahakhant et al., 1998), Philippine (Cuvin-Aralar et al., 2002), Vietnam (Hummert et al., 2001) and Singapore (Te and Gin, 2011) have recorded the detection of different cyanobacterial toxins in their water environments and the detailed data are presented in Table 2.4. A study by Sinang et al. (2015) has confirmed the presence of toxic cyanobacteria in Malaysia.

Table 2.3: Types, names and mode of actions of toxins (Stewart et al., 2006, Falconer and Humpage, 2005).

Types of toxin	**Name of toxin**	**Toxin producers genera**	**Effect**
Hepatotoxins	Microcystins,	*Anabaena, Anabaenopsis, Aphanocapsa, Arthrospira, Hapalosiphon, Microcystis, Nostoc, Oscillatoria, Planktothrix, Snowella, Woronichinia*	Inhibit specific enzymes systems and cause damage to gut and liver.
	Nodularins	*Nodularia*	
Neurotoxins	Anatoxins-a	*Anabaena, Aphanizomenon, Arthrospira, Cylindrospermum, Microcystis, Oscillatoria, Phormidium, Planktothrix, Raphidiopsis*	Acts as neuromuscular blocking agents leads to muscular paralysis and in severe cases, death due to respiratory arrest.
	Anatoxins-a(s),	*Anabaena*	Affects nerves and interferes with the smooth transition of stimuli to the muscles.
	Saxitoxin	*Anabaena, Aphanizomenon, Lyngbya, Planktothrix, Cylindrospermopsis*	Paralytic shellfish poisons
Endotoxins	Aplysiatoxin, debromoaplysiatoxin, lyngbyatoxin A	*Lyngbya*	Implicated in gastrointestinal disturbs and cause irritation to skin.
Cytotoxins	Cylindrospermopsin	*Anabaena, Aphanizomenon, Cylindrospermopsis, Raphidiopsis, Umezakia*	Inhibits protein synthesis

Table 2.4: Detection of cyanobacterial toxins in different countries.

Country/region	Toxins	Year of first detection/sample	Citation
Australia	Toxic cyanobacteria	1878	(Francis, 1878)
Great Britain	Microcystin	1982	(Codd and Carmichael, 1982)
Hungary	Toxic cyanobacteria	1983	(Reskóné and Törökné, 2000)
China	Microcystin	1984	(Carmichael et al., 1988) in (Zhang et al., 1991)
Norway	Microcystin	1984	(Utkilen et al., 1996)
Japan	Microcystin	1985	(Ohtake et al., 1989)
America	Toxic cyanobacteria	1987	(Repavich et al., 1990) in (Pelaez et al., 2010)
Argentina	Toxic *M. aeruginosa*	1989	(Scarafia et al., 1995)
Finland	Microcystin	1992	(Namikoshi et al., 1992)
German	Microcystin	1992	(Fastner, 1992)
Russia	Microcystin	1992	(Sivonen et al., 1992)
Canada	Microcystin	1993	(Craig et al., 1993)
Portugal	Microcystin	1993	(Vasconcelos et al., 1993)
Brazil	Microcystin	1994	(Azevedo et al., 1994)
France	Microcystin	1994	(Vezie et al., 1998)
Chile	Microcystin	1995	(Campos et al., 1999)
Denmark	Microcystin	1996	(Henriksen, 1996)
Philippine	Microcystin	1996	(Cuvin-Aralar et al., 2002)