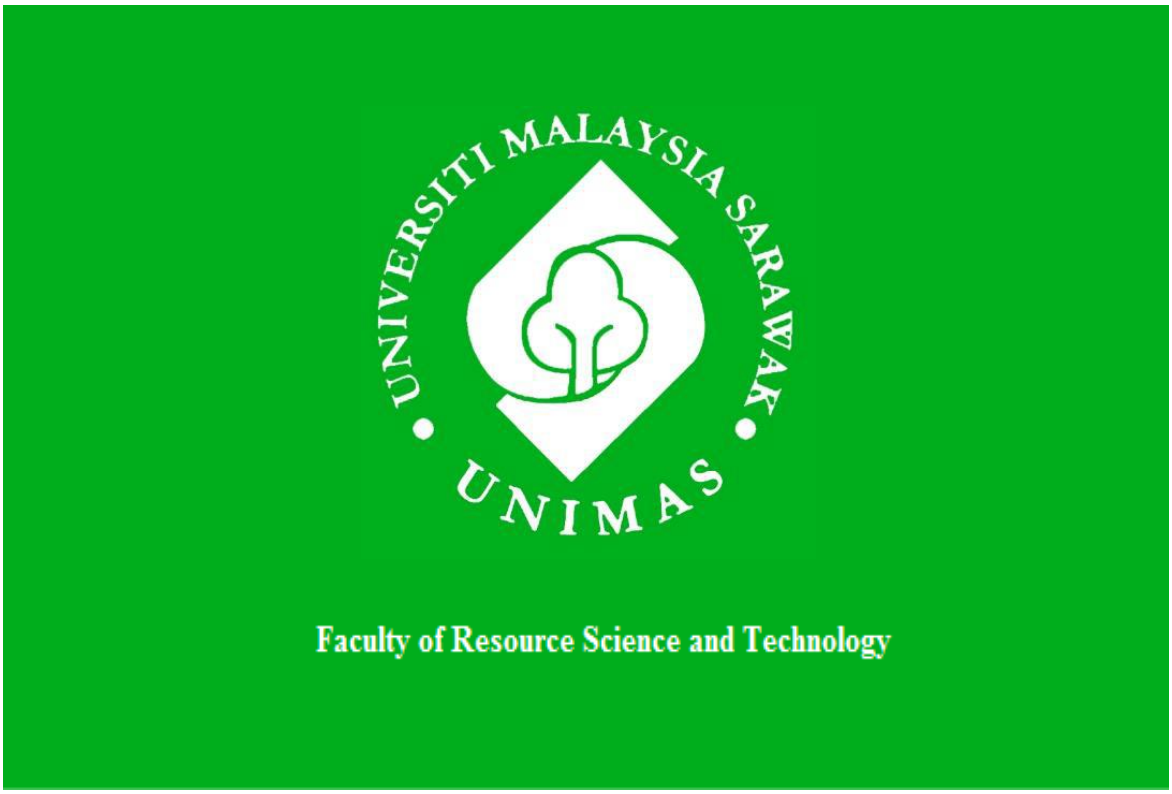

STUDY OF TOXIN PRODUCTION OF *MICROCYSTIS* UNDER LABORATORY CONDITION WITH DIFFERENT NUTRIENT TREATMENT

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Bachelor of Science with Honours

(Aquatic Resource Science and Management)

2015

Study of Toxin Production of *Microcystis* under Laboratory Condition with Different Nutrient Treatment

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This dissertation is submitted in partial fulfillment of requirement for the degree of Bachelor Science with Honours in Aquatic Resource Science and Management

Faculty of Resource Science and Technology

University Malaysia Sarawak

2015

Acknowledgement

First and foremost, I would like express my gratitude and appreciation to Department of Aquatic Science for providing the facility and support throughout this one year of final year project.

Secondly, my express gratitude and sincere appreciation to my supervisor, Dr. Samsur Mohamad for giving the chances to handle and organize the project, and also advise for thesis writing.

Throughout the whole project, there are several people I would like to express my gratitude and appreciation for their time and effort. First, million thanks are extended to officer from Indigenous Fisheries Research and Production Center (IFRPC), Mrs. Josephine and her field assistant Mr. Albert for their warm welcome and guidance during my sampling trip to IFRPC in Tarat.

Next, I would like to thank the science officer and laboratory staff: Mr. Benedict, Mr. Nazri Latip, Mr. Zaidi Nin Haji Ibrahim and other Aquatic Department staffs for their help on the instrument and companionship throughout this project.

Last but not least, my devoted appreciation for all my labmates: Er Huey Hui, Jovina Chang Pei Fong, Muhd Zaid B Nasir, Wan Nurain Farahan Bt Wan Basri, Nur Afifah Hanun Bt Ismail, and Nurin Syahidah Syasya for their companionship and cheerful time we have together throughout the project. I would thanks my other coursemates: Tan Kian Leong, Ching Kai Siang, Tan Xinh Guan and Goh Hao Chin for their help during the fieldtrip sampling and on statistical analysis. Lastly, special thanks to Mr. Tan Toh Hii for his counsel and advice on cell culturing technique in this experiment.

Declaration

No portion of the work referred to this dissertation has been submitted in support of an application for another degree or qualification of this or any other university or institution of higher learning.

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List of Abbreviations

HABs- Harmful Algal Bloom

HPLC- High Performance Liquid Chromatography

LPS- Lipopolysaccharides

MCYST- microcystins

N-Nitrogen

P- Phosphorus

RT- retention time

LC/MS- Liquid chromatography-mass spectrometry

ELISA- Enzyme-linked immunosorbent assay

PPIA- Protein phosphatase inhibition assay

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Study of Toxin Production of *Microcystis* under Laboratory Condition with Different Nutrient Condition

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ABSTRACT

Aquaculture is an important industries in Malaysia, however, cyanobacterial blooms with potential toxins are problematic in pond water management in aquaculture. In this study, potential toxin-producing cyanobacteria, *Microcystis* sp. were isolated from selected aquaculture ponds in Indigenous Fisheries Production and Research Center, Tarat, Sarawak. The isolated *Microcystis* sp. were successfully maintained and mass culture using BG11 medium under laboratory condition. *In-situ* and *ex-situ* water parameter of selected aquaculture pond were recorded and microcystins concentration were detected in bloom-forming ponds using HPLC analysis. There are no significant relation found between chl-*a* concentration and microcystins concentration of pond samples and culture samples. Meanwhile, in nutrient experiment using different N:P (nitrogen:phosphorus) ratio, NP15(15:1 ratio) give the best growth performance of *Microcystis* sp. with highest optical density and overall specific growth rate. Highest microcystins concentration was detected in sample with N:P ratio 15:1 at 8.510 μg mg^{-1}. In this experiment, a relation between microcystins production and growth of *Microcystis* in term of growth density and growth rate was observed but no relation was observed between nitrate concentration and microcystins production. Nevertheless, N:P ratio = 15 was found to be the optimum nutrient ratio for growth of *Microcystis* sp. and highest microcystins content was detected.

Keywords:, *Microcystis*, aquaculture, microcystins, HPLC, N:P ratio

ABSTRAK

Akuakultur merupakan industri yang penting di Malaysia. Padahal, pertumbuhan sianobakteria menjadi isu kritikal dalam pengurusan sumber air akuakultur kerana sianobakteria mempunyai potensi untuk mengeluarkan toksin mycrocystins. Dalam kajian ini, sianobakteria yang berpotensi untuk menghasilkan toksin, ialah Microcystis *sp. Sianobakteria ini diasingkan dari kolam akuakultur yang terpilih iaitu, IFRPC, Tarat, Sarawak.* Microcystis *sp. yang diasing telah berjaya dikultur and dikekalkan dengan menggunakan BG11 media dalam makmal. Parameter air kolam akuakultur yang dipilih telah direkodkan.Kolam dengan ledakan alga telah dikesan memgandungi mikrosistin selepas dianalisis dengan KCPT. Tiada hubungan signifikan didapati antara konsentrasi klorofil dan kandungan mikrosistin kolam sampel dan sampel kultur. Selain itu, dalam eksperimen nutrien yang menggunakan nisbah berbeza N:P (nitrogen: fosforus), NP15 (15:1 nisbah) menunjukan prestasi pertumbuhan yang optimum untuk* Microcystis *sp. dalam kepadatan optik dan kadar pertumbuhan yang tertinggi. Konsentrasi mikrosistin tertinggi dikesan dalam sampel dengan nisbah N:P 15:1 iaitu 8.510 μg mg^{-1}. Dalam eksperimen ini, hubungan antara pengeluaran mikrosistin dan pertumbuhan* Microcystis *dari segi kepadatan pertumbuhan dan kadar pertumbuhan telah didapati tidak berkaitan antara konsentrasi nitrat dan pengeluaran mikrosistin. Walau bagaimanapun, nisbah N:P = 15 didapati nisbah nutrien yang optimum bagi pertumbuhan* Microcystis *sp. dan kandungan mikrosistin yang tertinggi telah dikesan.*

***Kata kunci**: Microcystis, akuakultur, mikrosistin, KCPT, N:P nisbah*

1.0 Introduction

Aquaculture has been the most progressive and gradually become the main solution in world food security problem. Aquaculture industries been expanding over the year, world aquaculture production has increase exponentially and contribute significantly to global fish production for human consumption (Costa *et al*., 2014). However, intensive aquaculture industries also give rise to ecological risk such eutrophication which cause by large input of nutrient for boosting the growth and fattening of products (Paerl & Tucker, 1995). In freshwater system such as aquaculture pond, eutrophication often lead to algal blooms. Generally these blooms are harmless, but if the blooms produce toxins which lead to fishkill and poisoning of human through consumption, they are identified as harmful algal blooms (HABs) (Paerl & Otten, 2013). The main culprit of harmful algal blooms in freshwater system likely to be cyanobacteria species which produce cyanotoxins.

Cyanobacteria are widely known capable of forming blue-green scum on the water surface accompany by a foul smell. The cyanobacterial bloom often associate with a condition of high availability of nutrient in the water known as eutrophication (Merel *et al*., 2013). However, the main problem of cyanobacterial blooms are that certain species of cyanobacteria can produce a variety of secondary metabolites, some of which exhibit toxic properties and are referred to as cyanotoxins. The present known toxigenic cyanophyceans constitute about 40 species (Skulberg *et al*., 1993).

Cyanotoxins are group of most powerful natural toxins known produced by cyanobacteria. Cyanotoxins vary widely in their chemical structures and the structural diversity reflects on the different mechanisms of toxicity (Carmichael, 1992). One of the most extensively study of cyanotoxins is microcystins. Microcystins are categorized under hepatotoxins family produced by some freshwater species cyanobacteria, primarily *Microcystis* species such as *Microcystis aeruginosa* and other genera, namely *Anabaena,*

Planktothrix and *Nostoc* (Codd *et al*., 2010). Microcystins are potent hepatotoxins, which have been linked to many cases of livestock and wildlife poisoning incidents, aquatic organism as well as human fatalities (Falconer, 2005).

Aquaculture ponds are most likely to experience cyanobacterial blooms as it contain high nutrient availability due to large influx of fertilizer which mostly contains nitrogen and phosphorus (Rodgers, 2008). This will lead to eutrophic and hyper-eutrophic condition in aquaculture ponds which favor blooms and toxin production (Neilan *et al*., 2013). Besides, the fish product in cyanobacteria blooming water often carry a musty smell and off-flavour. The musty smell is caused by a compound produced by cyanobacteria known as geosmin (Merel *et al*., 2013). Fish mortality in aquaculture pond has been reported in various places (Chia *et al*., 2009; Jewel *et al*., 2003; Padmavathi & Prasad, 2007; Padmavathi & Veeraiah, 2009).

A variety of studies have shown that environmental factors such as light, temperature, pH, nutrient concentration (nitrogen and phosphorus) and metals stressor such as iron have direct influence on growth and toxin production of toxigenic cyanobacteria under different cultivation conditions (Ame & Wunderlin, 2005; Sivonen, 1990; Smith, 2010; Wiedner *et al*., 2003). Particularly nitrogen and phosphorus content also known as N:P ratio have been widely used to study variation of nutrient composition in medium components towards toxins production (Bortoli *et al*., 2014; Crawford, 2008; Downing *et al*., 2005; Lee *et al*., 2000).

The objectives of this study were: 1) To isolate and established clonal culture of the toxins-producing cyanobacteria (*Microcystis* spp.*)* from aquaculture pond. 2) To study the water quality and microcystins content in selected aquaculture pond 3) To determine the growth and microcystins production of *Microcystis* sp. under different N:P ratio.

2.0 Literature Review

2.1 Cyanophyceae- Cyanobacteria

Cyanophyceae or more commonly known as blue-green algae or cyanobacteria branded as the Earth's pioneer plant. This is because their very existence recorded back at least 2.7 billion years ago and acknowledged to be the main primary producers of organic matter (Falconer, 2005; Zanchett & Oliveira-Filho, 2013). Cyanobacteria is the dominant forms of life on Earth and the most ancient oxygen-producing photosynthesizers (Vincent, 2009); It is believe to be the first to produce chlorophyll-*a* and *b* as well as variety of photosynthetic pigments (Graham & Wilcox, 2000). They have created oxygen in atmosphere 3500 million years ago, long before the emerging of other organism (Adamovsky, 2010). Cyanobacterial colonies appear in fossil record known as stromatolites a layered bio-chemical accretionary structure which dated to approximately 2.09 billion before the present (Falconer, 2005; Graham & Wilcox, 2000)

Cyanobacteria also known as cyanoprokaryotes which possess both characteristics of algae and bacteria. Cyanobacteria are called 'bacteria' as they do not possess nuclei and organelles which are important to eukaryotic cell, instead they possess peptidoglycan cell wall that is distinctive features of gram-negative Eubacteria (Vincent, 2009). However, cyanobacteria contain eukaryotic chlorophyll instead of bacteriochlorophyll. Hence, the photosynthetic production of oxygen by a two-photosystem (PSI and PSII) process bear a resemblance to algae and higher plant (Vincent, 2009). This make them part of bacteria domain and also algae (Vincent, 2009; Graham & Wilcox, 2000). Hence, there are two taxonomic treatment of classification that possible for cyanobacteria; the botanical nomenclature and nomenclature of bacteria. A more recent approach in biological classification of cyanophyceans have cyanobacteria placed within the group of eubacteria in

the phylogenetics taxonomy which is distinct and apart from archaebacterial and eukaryotes (Skulberg *et al*., 1993).

Currently, cyanobacteria are classified under class Cyanophyceae, total of 150 genera and 2000 species have been successfully identified. It has been widely classified into 5 main orders: Chrooccocales, Oscillatoriales, Pleurocapsales, Nostocales and Stigonematales (Falconer, 2005; Mur *et al*., 1999; Vincent, 2009; Waterbury, 2006)

Cyanobacteria can be found throughout wide variety of ecosystem on the surface of the planet as they can tolerate different kind of environmental conditions in terms of temperature, light intensity, salinity and moisture (Falconer, 2005). Hence, beside common limnetic and marine environment, their range of habitat extend to some extreme habitat **s**uch as hot springs, salt lake**s,** desert crusts and even Arctic or Antarctic lakes (Mur *et al*., 1999)

2.2 Morphological features of Order Chroococcales (*Microcystis*)

The basic morphology of cyanobacteria comprises unicellular, colonial and multicellular filamentous forms (Mur *et al*., 1999), and the most advanced type is multiseriate branched filament (Lee, 2008). With this, the Cyanophyceae can be further divided into 5 orders according to their morphological features, organization of cell, mode of reproduction and presence of specialized organelle such as akinetes and heterocysts.

Member of the order Chroococcales are unicellular forms, the shape can be spherical, ovoid or cylindrical cells. Their mode of reproduction is either binary fission or budding which occurs in one, two, or three planes at right angles to one another (Waterbury, 2006). They commonly amassed in irregular colonies which surrounded by slimy matrix. Cell size range from 0.5-30 µm in diameter. Examples are *Gloeothece, Microcystis* and *Synechococcus* (Mur *et al*., 1999).

The *Microcystis* genus is often characterised by the spherical cells with diameter range from 2 to 8 µm containing gas vesicles and formation of colonies. In natural environment, *Microcystis* always form colonies which size can reach up to a thousand cells. However, in laboratory cell cultures, *Microcystis* are more commonly disaggregate and exist in solitary cells (Marzec *et al*., 2010). In *Microcystis* genera, there are several species that can be distinguished based on cell size, colony shape, sheath characteristics and the number of cell division planes, such as: *M. aeruginosa, M. flos-aquae, M. ichthyoblabe, M. viridis, M. wesenbergii,* and *M. botrys* (Komarek & Komarkova, 2002; Komárek & Hauer, 2013; Marzec *et al*., 2010). The weakness in morphological identification of the species is that the morphological features show great variability and overlapping, this subgeneric classification has turned out to be rather difficult and unreliable (Otsuka *et al*., 2000).

2.3 Toxicology of microcystin

Many bloom-forming species of cyanobacteria are microcystins producer. Microcystins are first asserted as secondary metabolite which define as compounds that are unproductive for organism for its primary metabolism (Carmicheal, 1992). However, the status of microcystins as a secondary metabolite has been redefined as it is widely speculate by researcher that it may have functional role on cellular metabolism or on determining a strain as toxic or non-toxic (Lyck, 2004; Orr & Jones, 1998). Firstly, the known cyanotoxins are currently been classified in accordance to two main criteria:1) Modes of toxicity to multicellular organism cell systems; 2) Chemical composition and structure (Carmichael, 1992; Falconer & Humpage, 2005; Figueiredo *et al*., 2004; Pearson *et al*., 2010; Sivonen & Jones, 1999).

According to Pearson *et al*. (2010), cyanotoxins can be classified into 4 major class from toxicological view: neurotoxins, hepatotoxins, cytotoxic and dermatotoxins (irritant toxins), while according to their chemical structures, it can separate into 3 broad groups; cyclic peptides, alkaloids and lipopolysaccharides (LPS) (Codd *et al*., 2010)

According to Zanchett and Oliveira-Filho (2013), microcystins are classified under hepatotoxins with cyclic peptides chemical structure, the other member of hepatotoxins is nodularins family and cylindrospermopsins as shown in Table 1.0. Both microcystins and nodularins have same mode of toxicity. While target glutathione and protein synthesis as well as cytochrome P450. Microcystins have been characterized from planktonic cyanobacteria such *Anabaena, Microcystis, Oscillatoria (Planktothrix), Nostoc* and *Anabaenopsis* species while nodularins found exclusively in *Nodularia* spp. (Carmichael, 1992; Falconer, 1995; Sivonen & Jones, 1999).

Table 1.0. Main hepatotoxins from its main producer, chemical classification and the mode of action. (Adapted: Zanchett & Oliveira-Filho, 2013)

Hepatotoxins	**Genera of main producers**	**Chemical classification**	**Mode of action**
Microcystins	*Microcystis, Anabaena, Planktothrix, Nostoc, Anabaenopsis*	Cyclic Heptapeptides	Inhibition of protein phosphatases type 1 and 2A
Nodularins	*Nodularia*	Cyclic Pentapeptides	Inhibition of protein phosphatases type 1 and 2A
Cylindrospermopsins	*Cylindrospermopsis raciborskii, Aphanizomenon*	Guanidine alkaloids	Glutathione and protein synthesis as well as cytochrome P450

The chemical structure of microcystins are monocyclic heptapeptides which have cyclic structure, the arrangement of the structure are shown in figure below where in the order of: D-Ala^{1}-X^{2}-D-MeAsp^{3}-Z^{4}-Adda^{5}-D-Glu^{6}-Mdha^{7}, whereas the position X and Z are the variable L-amino acids (Figure 1.0) (Rai & Kumar, 2012). The amino acid Adda is a unique β-amino acid that largely responsible for the hepatotoxicity of microcystins (Carmicheal, 1992). There are over 65 different or more variant structure of microcystins exist owing to variable L-amino acids in the two variable residues and some modification in other amino acids (Codd *et al.*, 2010; Harada, 2004). Most studies found that the non-demethylated microcystins (microcystin-LR) form are mostly detected in *Microcystis* blooms (Parrish, 2014). The other non-demethylated microcystins include microcystin-RR and microcystin-YR (Sivonen & Jones, 1999). The most toxic and common variant of microcystins is microcystin-LR, where the – LR indicate the variable residues are leucine (L) and arginine (R) at position X and Z (Rai & Kumar, 2012).

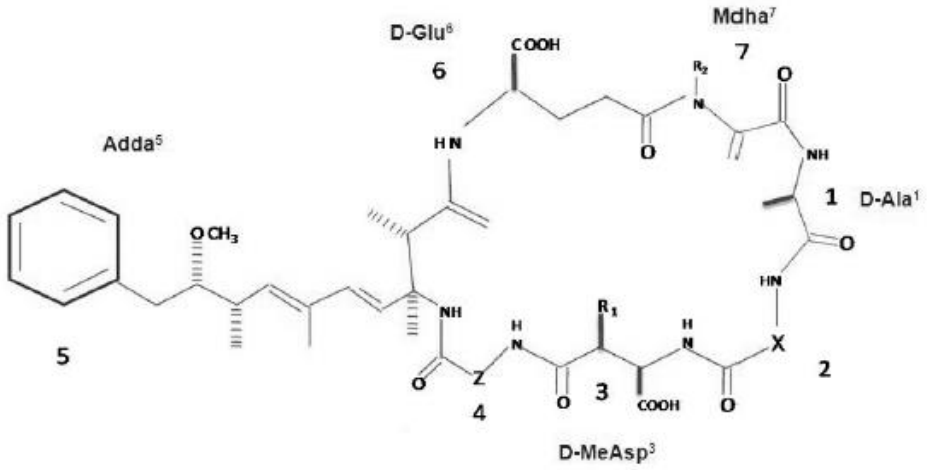

Figure 1.0a. The typical chemical structure of microcystins. Variable L-amino acids residues are found at position X and Z. (taken from Rai & Kumar, 2012)

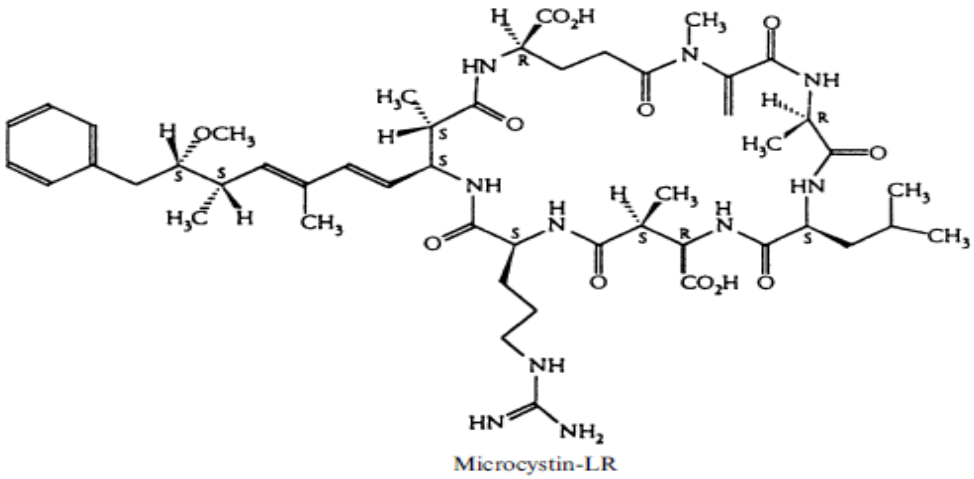

Figure 1.0b. The chemical structure of microcystin-LR. (taken from Dawson, 1998)

Microcystins in general are liver toxins where cause liver damage. The toxic mechanism of microcystins come from the strong inhibition towards protein phosphatases types 1 and 2A as the interactions are extremely strong, due to the stoichiometric nature of binding (Adamovsky, 2010). Besides, presence of the unusual amino acid Adda is another reason behind the potency of microcystins toxicity (Rai & Kumar, 2012). As stated by Dawson (1998), microcystins facilitated the uptake by transporting into bloodstream by way of a bile-acid transport mechanism that exists in hepatocytes. The microcystins will form

irreversible inhibition on serine/threonine protein phosphatases 1 and 2A (Figueiredo *et al*., 2004). This resulted in protein phosphorylation imbalance which lead to disintegration of hepatocyte structure and liver necrosis resulted in massive hepatic haemorrhage (Dawson, 1998). Common sign of microcystins intoxication are anorexia, diarrhea, vomiting, piloerection, lethargic, pallor and necrosis of liver (Rai & Kumar, 2012). In serious cases, victim will suffer haemorrhagic shock preceded by disruption of liver structure and function result in death, the onset of the effect can be within few hours after oral ingestion (Dawson, 1998). Previous study suggested that microcystins might act as tumor promoters, an agent that do not cause cancer but stimulate the proliferation of cancer cells (Butler *et al*., 2009; Dawson, 1998).

2.4 Environmental trigger (nutrient) on growth of cyanobacteria and microcystins production

According to Neilan *et al*. (2013), although the genes responsible for microcystins production and the biosynthetic pathway have been elucidated, factor regulating microcystins production are still largely unknown. A review have been done by Pearson *et al*. (2010) on the biosynthetic of microcystins and their toxic nature, in general, microcystins are small nonribosomal peptides that are synthesized by an enzyme complexes called microcystin synthetase which include non-ribosomal peptide synthetase (NRPS) and polyketide synthethase (PKS). The gene cluster that responsible for encoding of these biosynthetic enzyme are known as *mcy* (*mcyABCDEFGHIJ)* gene cluster (55kb) (Neilan *et al*., 2013). At present, the *mcy* gene cluster has been successfully sequenced and characterized in several cyanobacterial genera. A review by Boopathi and Ki (2014) showed that regulation of *mcy* gene cluster play an important part in biosynthetic of microcystins.

The gene cluster is regulated by a complex network system which then influence the biosynthesis process of microcystins. The complex network system may include environmental stress and availability of nutrients (Pearson *et al*., 2010). Therefore, understanding of environmental factors such as temperature, light intensity, pH, nutrients available and micronutrient such as trace metals on inducing succession of toxic *Microcystis* sp. in natural environment and their microcystins production are important for management practices (Boopathi & Ki, 2014).

Most studies on relating microcystins production and environmental factors have been culture-based. The studies mainly manipulating environmental factors which typically associated with bloom formation such as temperature (Sivonen, 1990), light intensity (Wiedner *et al*., 2003), phosphorus and nitrogen (Dolman *et al*., 2012; Downing *et al*., 2005; Vezie *et al*., 2002; Pimentel & Giani, 2014) and iron concentrations (Smith, 2010) in laboratory condition. Environmental factors such as irradiation, temperature, concentration of nutrients and water dynamics can affect the toxicity of a bloom by modifying the population structure and, to a lesser extent, by changing the rate of microcystins biosynthesis (Marzec *et al*., 2010).

One of the most studied environmental factors in triggering microcystins production is the effects of nitrogen and phosphorus concentrations on microcystins production. In lentic systems such as lake and ponds, nitrogen:phosphorus (N:P) ratio has been frequently used as indicator in predicting algal biomass, compositions and its seasonal succession (Kim *et al*., 2007). The atomic ratio, 16:1 (N:P) first mentioned by Alfred C. Redfield also known as the Redfield ratio or Redfield stoichiometry are now widely used as an indicator of N and P limitation condition (Liu *et al*., 2011). Numerous of studies were focused on the effect of nitrogen, phosphorus and its respective ratio on growth and dominance of *Microcystis* sp.

and its production of microcystins. This is principally important as bloom-forming species are mostly toxigenic *Microcystis* sp.. Kim *et al*. (2007) suggested that bloom-forming cyanobacteria are likely to be dominant when the N:P ratio is not more than 27 in weight. However, research of the influence of N, P and their concentration ratio on the growth of *Microcystis* sp. strains and microcystins synthesis has shown contradictory results. Lee *et al*. (2000) reported that N:P ratios of 10 to 16 as optima for bloom development. On the other hand, a highest microcystins concentration was determined when at N:P is equal 31:1 (Downing *et al*., 2005). Vezie and co-workers determined this ratio to be much higher, 237 < N:P < 753 (Vezie *et al*., 2002). Nevertheless, a rapid growth rate of *Microcystis* sp. in a hypertrophic reservoir was observed when N:P ratios of < 30 (Kim *et al*., 2007).

2.5 Occurrence of microcystins and its consequences on aquaculture pond

Cyanobacteria can colonize and bloom to great mass in aquatic enviroment. Harmful cyanobacteria that have frequently been reported in freshwater are *Microcystis* and *Planktothirx*. These species mainly produce the peptide hepatotoxins microcystins (MCYST) which have structural variants or congeners that differ in toxicity (Ibelings & Chorus, 2007). The main reason for their bloom are nutrient status, salinity, light conditions, turburlence and mixing, temperature and herbivory (Rodgers, 2008). Occurrence become more frequent as scientist suspect global warming and associated hydrological changes do have implication on metabolism, growth rates and bloom formation of toxic cyanobacteria (Paerl & Paul, 2012). Microcystins affect aquatic biota or other higher trophic level consumers through three mechanisms:1)direct exposure, 2)bioaccumulation in aquatic food chain, 3)alterations to aquatic food chain structure (Paerl *et al*., 2001). Release of cyanotoxins particularly microcystins during bloom will have deleterious effect on phytoplankton and zooplankton

population which ultimately affect the aquatic food chain strucutre, as phytoplankton is the primary producer and zooplankton as primary grazer (Vasconcelos, 2001). Bioaccumulations of microcystins has been observed in both freshwater and marine invertebrates (mollusk, crustacean), vertebrates(fishes) and water fowls as well (Adamovsky, 2010) In more recent study, cases of microcystin intoxication in marine mammals has been reported (Parrish, 2014). Microcystin toxicity to human are mainly through consumption or utilization of microcystin contaminated water supply. The first and notorious case of fatal microcystin poisoning in humans happened in Carauru, Brazil, in 1996. A local renal dialysis treatment center was found using untreated water from dam reservoir which contain microcystin to treat 126 renal disease patients and later 100 patients developed acute liver failure and 52 had died (Azevedo *et al*., 2002).

In the case of aquaculture pond, as reported by Rodgers (2008), normally eukaryotic algae such as green algae or diatoms are more easily proliferate than cyanobacteria. However, cyanobacteria can out-compete algae for nutrient in aquaculture situation because they can thrive with low dissolved oxygen, and efficient photosysthesis in low-light conditions. Besides that, cyanobacteria are less likely affected by turbitidy, high concentration of ammonia and warm temperatures (Rodgers, 2008). Furthermore, cyanobacteria able seize the advantages in eutrophic aquaculture situations by produce allelochemicals that can inhibits competing algae and invertebrate grazers (Rodgers, 2008). Paerl and Tucker (1995) noted that cyanobacteria even though is part of phytoplankton community where its contribute primary productivity in aquaculture ponds, but bloom-forming cyanobacteria are consider undesirable. This is because 1) Cyanobacteria are relatively poor base for aquatic food chain; 2) Cyanobacteria are poor oxygenator of water and its bloom create an oxygen depleted water in aquaculture pond which lead to fish mortality. 3) Cyanobacteria often impart off-flavour and objectionable odor in cultered fish by producing odorues metabolites