# BIODIVERSITY OF CYANOBACTERIA FROM PRE-TREATED AND POST-TREATED WATER RESOURCES IN SURANAREE UNIVERSITY OF TECHNOLOGY

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# ความหลากหลายทางชีวภาพของไซยาโนแบคทีเรียจากแหล่งน้ำก่อนการบำบัดและหลังการบำบัดในมหาวิทยาลัยเทคโนโลยีสุรนารี

นายฮิโรยูกิ วะคุอิ



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2561

# BIODIVERSITY OF CYANOBACTERIA FROM PRE-TREATED AND POST-TREATED WATER RESOURCES IN SURANAREE UNIVERSITY OF TECHNOLOGY

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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มหาวิทยาลัยเทคโนโลยีสุรนารี จังหวัดนครราชสีมา ประเทศไทย มีอ่างเก็บน้ำอยู่ 2 อ่าง คือ อ่างสุระ 1 และ อ่างสุระ 2 อ่างเก็บน้ำดังกล่าวจัดเป็นแหล่งน้ำหลักในมหาวิทยาลัยเทคโนโลยีสุรนารี แต่ยังไม่มีความชัดเจนเกี่ยวกับชุมชนของแบคทีเรีย เช่น ไซยาโนแบคทีเรีย ที่พบในอ่างเก็บน้ำ ไซยาโนแบคทีเรียเป็นหนึ่งในแบคทีเรียที่ผลิตสารพิษซึ่งพบแพร่กระจายได้ทั่วไปในทะเล แหล่งน้ำจืด และบนบก ดังนั้นการศึกษาชุมชนของไซยาโนแบคทีเรียในอ่างเก็บน้ำอาจช่วยในการแยกแยะคุณภาพของน้ำได้ งานวิจัยนี้ได้ทำการเก็บน้ำจากอ่างเก็บน้ำทั้งสองแห่งที่จัดเป็นน้ำก่อนการบำบัด และน้ำจากห้องน้ำซึ่งจัดเป็นน้ำที่ผ่านการบำบัดแล้ว โดยน้ำก่อนการบำบัดจะใช้ในการศึกษาชุมชนของไซยาโนแบคทีเรียในอ่างเก็บน้ำและน้ำที่ผ่านการบำบัดแล้วจะใช้เพื่อตรวจสอบคุณภาพของน้ำหลังจากผ่านระบบบำบัด

โดยวิธีการทดลองประกอบไปด้วยการแยกจีโนมสิ่งแวดล้อมจากน้ำ และทำการเพิ่มจำนวนยีน 16S rRNA ของไซยาโนแบคทีเรียจากจีโนมที่ได้จากตัวอย่างน้ำด้วยไพรเมอร์เฉพาะของไซยาโนแบคทีเรีย Cya106F และ Cya781Ra โดยชิ้นยีน 16S rRNA ที่ได้จะถูกนำมาเชื่อมต่อกับพลาสมิดเวคเตอร์ pTG19T และถ่ายเข้าสู่เซลล์ของเชื้อ *Escherichia coli* DH5α จากนั้นจะทำการหาลำดับนิวคลีโอไทด์ของยีน 16S rRNA ที่อยู่บนพลาสมิดลูกผสม จากลำดับเบสของยีน 16R rNA ทั้งหมดยี่สิบสองยีนพบว่า สิบสองยีนมีความสัมพันธ์ใกล้เคียงกับไซยาโนแบคทีเรียในจีนัส *Cylindrospermopsis*, *Prochlorothrix*, *Ancylothrix* และ *Synechococcus* โดยประชากรไซยาโนแบคทีเรียที่พบมากที่สุดในการศึกษานี้อยู่ในจีนัส *Cylindrospermopsis* และ *Synechococcus* ซึ่งสายพันธุ์ของเชื้อในสองจีนัสนี้อาจเป็นได้ทั้งสายพันธุ์ที่สร้างสารพิษหรือไม่สร้างสารพิษ ผลจากการวิเคราะห์คุณภาพของน้ำที่ผ่านการบำบัดแล้วพบว่าไม่พบยีน 16S rRNA ของแบคทีเรียรวมถึงไซยาโนแบคทีเรีย

กล่าวโดยสรุปคือ สามารถจำแนกชนิดของไซยาโนแบคทีเรียในอ่างเก็บน้ำของมหาวิทยาลัยเทคโนโลยีสุรนารีได้สำเร็จ และยังตรวจพบจีนัสของไซยาโนแบคทีเรียที่มีความเป็นไปได้ว่าจะผลิตสารพิษ อย่างไรก็ดีน้ำที่ผ่านการบำบัดแล้วไม่พบว่ามีการปนเปื้อนของแบคทีเรียและไซยาโนแบคทีเรีย จึงปลอดภัยที่จะนำมาใช้เพื่อวัตถุประสงค์ในการอุปโภค



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RESERVOIRS/PRE-TREATED/POST-TREATED/CYANOBACTERIA/ *CYLINDROSPERMOPSIS RACIBORSKII*

Suranaree University of Technology (SUT), Nakhon Ratchasima, Thailand, there are two water reservoirs, Ang Sura 1 and Ang Sura 2, on the campus. The reservoirs are the main source of water supply in SUT yet community of Bacteria such as Cyanobacteria is uncertain. Cyanobacteria is one of the toxin-producing bacteria widely spread in marine, freshwater, and terrestrial resources. Thus, the study of Cyanobacterial communities in reservoirs may help to distinguish the quality of water. In this research, water was collected from two reservoirs represented as pretreated water and bathroom water represented as post-treated water. Pre-treated water was used to study the community of Cyanobacteria in reservoirs and post-treated water was used to determine the quality of water after treatment system. The approach consists of isolation of the environmental genome from water and then 16S rRNA gene of Cyanobacteria was amplified with Cyanobacteria specific primers set, Cya106F and Cya781Ra. The amplified 16S rRNA genes were ligated with pTG19T-vectors, transformed into Escherichia coli DH5α, and then 16S rRNA genes on the recombinant plasmids were sequenced.

From a total of twenty-two 16S rRNA gene sequences, twelve were identified as high relative to Cyanobacteria which were *Cylindrospermopsis*, *Prochlorothrix*, *Ancylothrix*, and *Synechococcus*. The most abundant population found in this study was belong to *Cylindrospermopsis* which can be either toxin or non-toxin producer. Quality of post-treated water was analyzed and bacterial 16S rRNA gene includes cyanobacteria were not amplified.

In conclusion, the Cyanobacterial community in SUT reservoirs was successfully identified in which possible toxin producer genus was detected. Since post-treated water was not contaminated with bacteria and Cyanobacteria, the water should be safe to use as non-drinking purposes.



School of Preclinic Student's Signature_____

Academic Year 2018 Advisor's Signature_____

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# LIST OF ABBREVIATIONS

µl = Microliter

ml = Milliliter

mg/ml = Milligram per milliliter

µm = Micrometer

min = minutes

h = hour

PCR = Polymerase Chain Reaction

rpm = Round per minutes

rRNA = Ribosomal ribonucleic acid

TBE = Tris-borate EDTA

# CHAPTER I

# INTRODUCTION

## 1.1 Background/ Problem

The relationship between water resource and health is significant since number of infections and toxic agents are caused by consumption of water. Drinking water contamination in public water system may affect human health. Disease occur from the consumption of contaminated water are often referred as waterborne.

Cyanobacteria are photosynthetic prokaryotes which are found in poor treated water and possess a hazard to human and animals by producing cyanotoxins. The first acute cyanotoxin poisoning of human and cattle was reported in Lake Alexandrina, Australia in 1878 (Hitzfeld, Höger, and Dietrich, 2000). People who consumed contaminated water had high incidence of primary liver cancer. The presence of Cyanobacteria in drinking water causes serious problems in water treatment facilities.

The water related disease are classified into four groups; waterborne disease, water washed disease, water based disease, and water related disease (Table 1) (Bartram and Hunter, 2015). The waterborne diseases are caused by bacteria, viruses and parasites derived mainly from feces and contaminated water. These pathogens cause cholera, typhoid, hepatitis A, cryptosporidiosis and giardiasis. Toxic chemicals are also included in this group. The water washed diseases are caused by personal and domestic hygiene. Water hygiene diseases invade skin and eye infections such as tinea, scabies, pediculosis and trachoma. These infections can be controlled by managing water

hygiene quality. The water based diseases are mainly caused by parasites through skin contact. The main example is schistosomiasis which transmit from snail intermediate host to human. This group also include non-infectious agents that can cause allergies and skin irritation. The water related diseases are diseases that transmitted by insect vectors such as malaria (Fewtrell and Bartram, 2001). The cyanotoxins may cause waterborne disease when ingested, and water washed disease through recreational exposure (Chorus and Bartram, 1999).

**Table 1** A classification of infective diseases related to water (Bartram and Hunter, 2015).

| **Category** | | **Example** |
|---|---|---|
| I. | Water borne | Typhoid, Infectious hepatitis |
| II. | Water-washed | Trachoma, Scabies, Shigella Dysentery |
| III. | Water-based | Bilharziasis, Guinea worm |
| IV. | Water-related insect vector | Gambian sleeping sickness, Onchocerciasis |



In Suranaree University of Technology, there are two major reservoirs that are used as water supply for non-drinking purposes. However, the chance to consume these water via recreational and daily use may happen. Therefore, the study of Cyanobacteria from pre-treatment and post-treatment water in Suranaree University of Technology (SUT) campus may help to determine water quality.

## 1.2 Research objectives

To study the diversity of toxin and non-toxin producing Cyanobacteria in water reservoir in Suranaree University of Technology.

## 1.3 Research hypothesis

The results of 16S rRNA sequencing and phylogenetic tree from water reservoir of Suranaree University of Technology shows non-toxin producing Cyanobacteria and possible toxin producing Cyanobacteria species.

## 1.4 Scope and limitations of study

This work involves the identification of uncultured Cyanobacteria from water reservoir collected in Suranaree University of Technology, Nakhon Ratchasima, Thailand. The environmental sample genome was extracted by FavoPrep™ Soil DNA Isolation Mini Kit (Favorgen, Taiwan). The 16S rRNA gene of Cyanobacteria was amplified from environmental genome by using PCR technique. The sequence of amplified 16S rRNA gene was analyzed and used for the construction of phylogenetic tree.

# CHAPTER II

# LITERATURE REVIEWS

## 2.1 Cyanobacteria

Cyanobacteria are known as oldest photosynthetic prokaryotes which are widely spread in marine, freshwater, and terrestrial resources (Fristachi et al., 2008). The life of Cyanobacteria are simple, they only require water, carbon dioxide, inorganic substances and light (Lau, Matsui, and Abdullah, 2015). They are able to grow in a wide range of environments, ranging from marine, freshwater, terrestrial, to extreme environments. All Cyanobacteria are capable of oxygenic photosynthesis and perform fermentation under dark or anoxic conditions. Some Cyanobacteria can tolerate huge changes in salinity and temperature, and low light intensity (Zanchett and Oliveira-Filho, 2013).

The basic cell morphology of Cyanobacteria are unicellular, colonial or multicellular filamentous forms (Figure 1). Unicellular forms of Cyanobacteria may vary from spherical, ovoid to cylindrical shape. Filamentous morphology is the result of repeated perpendicular cell division at right angle to the main axis of the filament. This multicellular cell arrangement of a chain is called trichome. The trichome can be straight or coiled which has variety of sizes and shapes. Some filamentous Cyanobacteria have two specialized cells which are heterocysts and akinetes. The heterocysts develop from vegetative cells and produce thick cell walls. Heterocysts produce nitrogenase which enables nitrogen fixation. The akinetes also produce thick

cell wall to the end of vegetative growth period. Akinetes function to store the photosynthetic products and surviv9e harsh conditions (Komárek and Johansen, 2015).

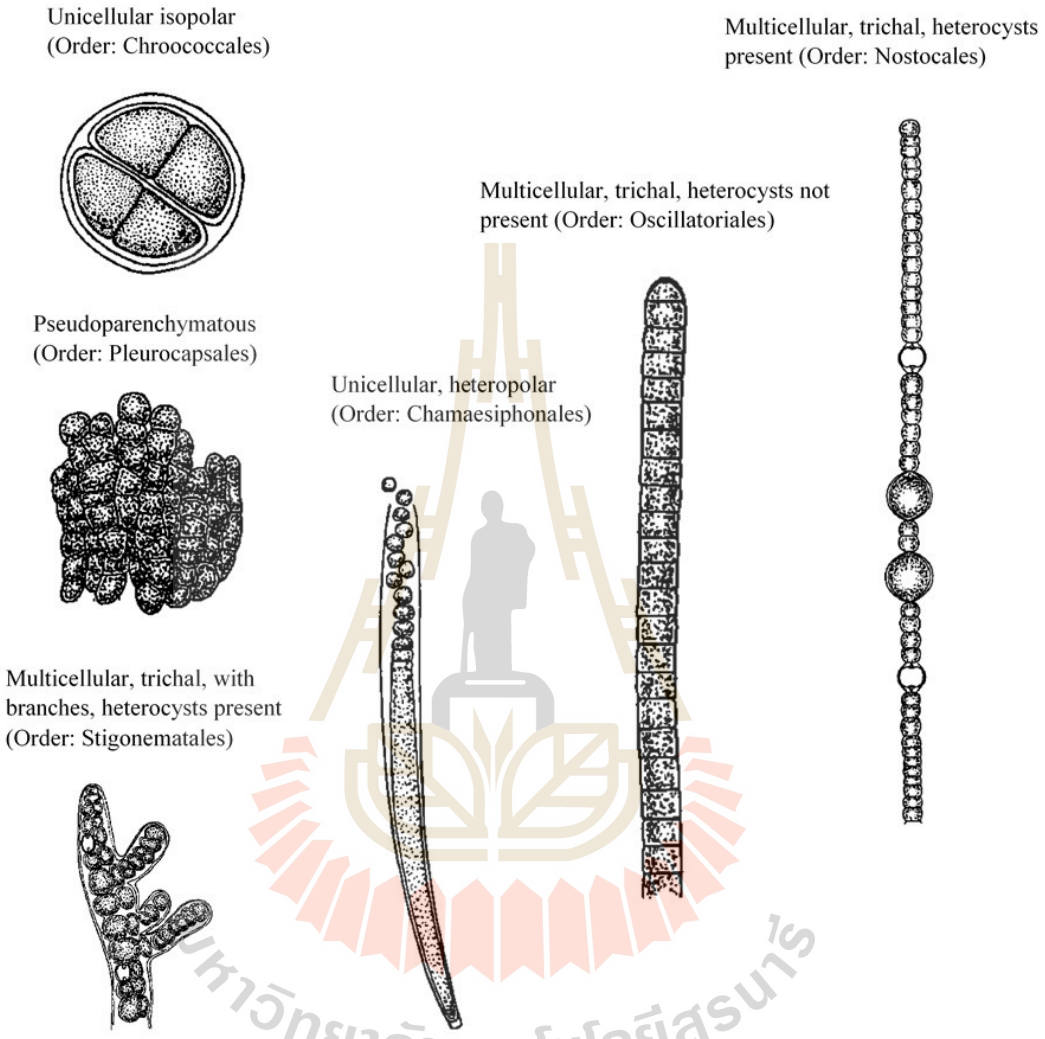



**Figure 1** Basic morphology of Cyanobacteria. *Chroococcales*: unicellular form separate by binary fission. *Chamaesiphonales*: exospores are budded off from the upper ends of cells. *Pleurocapsales*: The binary fission converting a single mother cell into many daughter cells. *Oscillatoriales*: with unseriated and unbranched trichomes, are composed of essentially identical cells. *Nostocales* and *Stigonematales*: Characterized with trichomes having a heterogeneous cellular composition (Chorus and Bartram, 1999).

Cyanobacteria are oxygenic phototrophs possess Photosystem I (PSI) and Photosystem II (PSII). The photosynthetic pigments found in Cyanobacteria are chlorophyll *a* and phycocyanin. The photosynthetic pigments of Cyanobacteria are located in thylakoids membrane that lie in the cytoplasm near cell periphery. PSII releases an excited electron, it returns to its ground state by accepting an electron from the oxidation of $H_2O$. PSI transfer electrons to ferredoxin and finally to reduce $NADP^+$ to generate NADPH (Figure 2). The NADPH is further used for $CO_2$ fixation to produce carbohydrate. Since Cyanobacteria have an ability to store essential nutrients and metabolites within their cytoplasm, excess supply of synthesized glycogen and lipids will be accumulated (Chorus and Bartram, 1999).

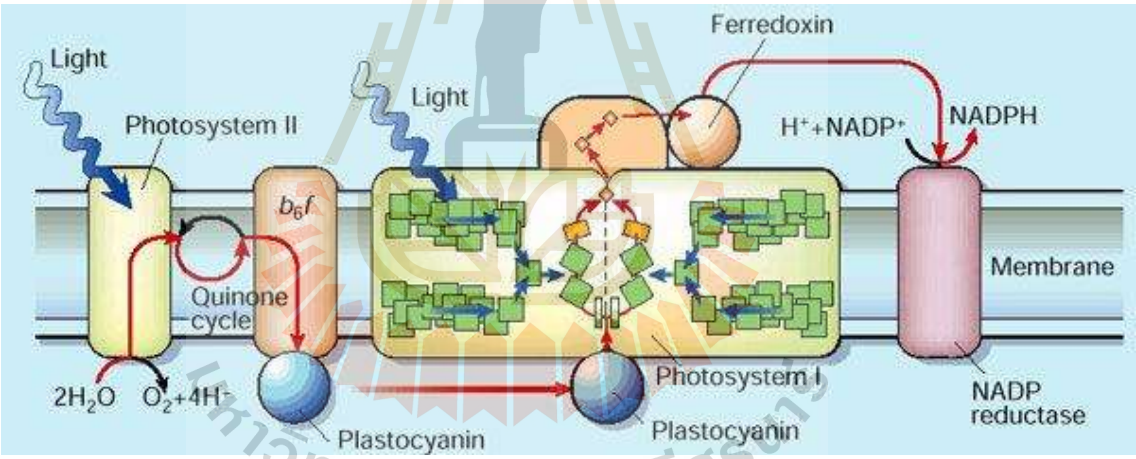



**Figure 2** Photosynthetic electron transport pathways in thylakoid membranes of the Cyanobacteria (Kühlbrandt, 2001) $b_6f$, cytochrome $b_6f$ complex.

Dinitrogen fixation is a fundamental metabolic process of Cyanobacteria that produce simplest nutrition to all living organisms. Cyanobacteria use nitrogenase enzyme to convert $N_2$ directly into ammonia ($NH_3$). Nitrogenase is a complex of dinitrogenase reductase and dinitrogenase (Figure 3). The dinitrogenase reductase consists of a dimeric Fe-protein function as an electron carrier to the dinitrogenase. The dinitrogenase consist of tetrameric MoFe-protein which reduces molecular nitrogen to ammonia (Issa, Abd-Alla, and Ohyama, 2014). Since both enzymes are highly sensitive to oxygen, anaerobic condition is required for dinitrogen fixation. However, some Cyanobacteria which contain heterocysts are able to fix nitrogen in anaerobic condition. Heterocysts consist of thick multilayered wall which the outer and inner layers of the heterocyst envelope contain polysaccharides and glycolipids. The multilayer wall of heterocysts prevents the entry of oxygen, thus creating anaerobic environment. As a result, filamentous heterocystous Cyanobacteria are able to reduce nitrogen in aerobic environment by using heterocysts (Issa et al., 2014). *Anabaena* and *Nostoc* spp. are example of nitrogen fixing Cyanobacteria (Bothe, Schmitz, Yates, and Newton, 2010).

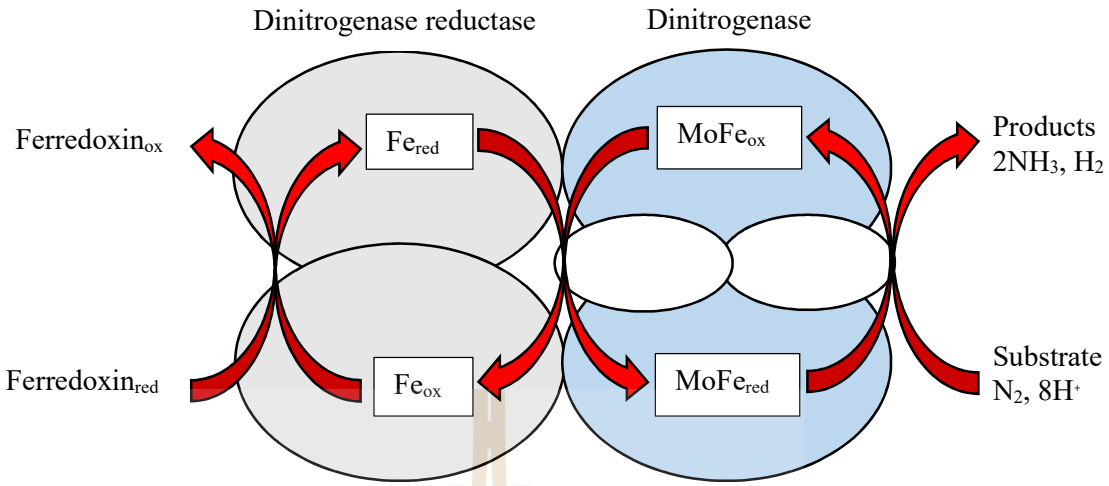



**Figure 3** The structure of nitrogenase (Issa et al., 2014). The nitrogenase is an enzyme that catalyzes the transfer of electrons from ferredoxin to reduce $N_2$. The reduction of $N_2$, thus, generate $NH_3$. The nitrogenase is composed from two subunits which are dinitrogenase reductase and dinitrogenase.

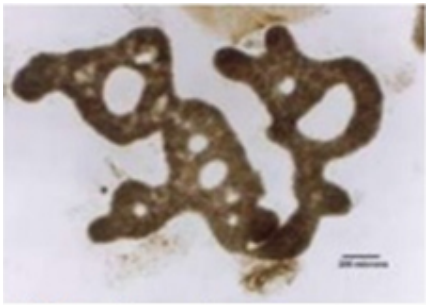
The periodic increase of Cyanobacteria resulting in high biomass known as harmful algal blooms which is commonly distributed in warm climates (Magana-Arachchi, Wanigatunge, and Jeyanandarajah, 2008). The maximum growth rate of most Cyanobacteria are above 25 ˚C thus, Cyanobacteria bloom is generally appeared during summer. There are several factors that can lead to the bloom of Cyanobacteria. These factors are hydrology, nutrients, sunlight, temperature, and ecosystem disturbance (Perovich et al., 2008). When bloomed Cyanobacteria dies, decomposers consumes oxygen decay bloom which create hypoxic conditions that result in plants and animal die-off (Chorus and Bartram, 1999). Some Cyanobacteria can secrete toxic compounds into fresh water which raises a major concern for the production of safe drinking water.

## 2.2 Cyanotoxins

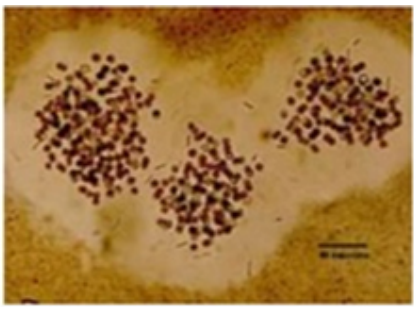
Some species of Cyanobacteria can produce a variety of toxic secondary metabolites known as cyanotoxins (Figure 4). Cyanotoxins are divided into three groups: cyclic peptide (microcystins and nodulatin), alkaloids (anatoxins and saxitoxins), and lipopolysaccharide (LPS) (Table 2) (Hitzfeld et al., 2000). In most cases, toxins are synthesized during bloom intracellularly and release into water when cell dies (Magana-Arachchi et al., 2008). On the other hand, some species can release toxin into water during bloom. Acute exposure to cyanotoxin result in fever, headache, muscle and joint pain, diarrhea, liver failure, respiratory arrest, and may be death.

**Table 2** General features of Cyanotoxins (Chorus and Bartram, 1999).

| | Toxin group | Primary target organ in mammals | Cyanobacterial genera |
|---|---|---|---|
| **Cyclic peptides** | Microcystins | Liver | Microcystis, Anabaena, Planktothrix (Oscillatoria), Nostoc, Hapalosiphon, Anabaenopsis |
| | Nodularin | Liver | Nodularia |
| **Alkaloids** | Anatoxin-a | Nerve synapse | Anabaena, Planktothrix (Oscillatoria), Aphanizomenon |
| | Anatoxin-a(S) | Nerve synapse | Anabaena |
| | Saxitoxins | Nerve axons | Anabaena, Aphanizomenon, Lyngbya, Cylindrospermopsis |
| | Cylindrospermopsins | Liver | Cylindrospermopsis, Aphanizomenon, Umezakia |
| | Aplysiatoxins | Skin | Lyngbya, Schizothrix, Planktothrix (Oscillatoria) |
| | Lyngbyatoxin-a | Skin, gastro-intestinal tract | Lyngbya |
| **Lipopolysaccharides (LPS)** | LPS | Potential irritant; affects any exposed tissue | All |

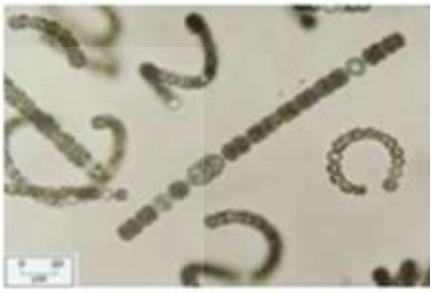


*Microcystis aeruginosa*

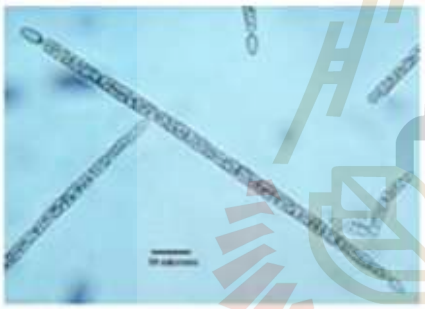


*Microcystis aeruginosa*

*Anabaena circinalis*



*Anabaena planktonica*



*Cylindrospermopsis raciborskii*

*Aphanizomenon ovalisporum*

*Phormidium amoenum*

*Nodularia spumigena*

**Figure 4** Microscope photographs of some of the common toxic and odor producing Cyanobacteria found in Australia (Newcombe, Ho, and Baker, 2010).

### 2.2.1 Cyclic peptides

The cyclic peptides are natural products. The molecular weight of cyclic peptides is vary from (MW) 800-1,100. The cyclic peptide contain either five or seven amino acids with two terminal amino acids of the linear peptide being condensed to form a cyclic compound. (Chorus and Bartram, 1999). The general structure of cyclic heptapeptide (microcystin) is cyclo-(D-alanine1-X2-D-MeAsp3-Z4-Adda5-D-glutamate6-Mdha7) in which X and Z are variable L amino acids, D-MeAsp3 is *D-erythro*-β-methylaspartic acid, and Mdha is *N*-methyldehydroalanine (Figure 5). The mammalian toxicity of microcystins is their strong binding to protein phosphatases.

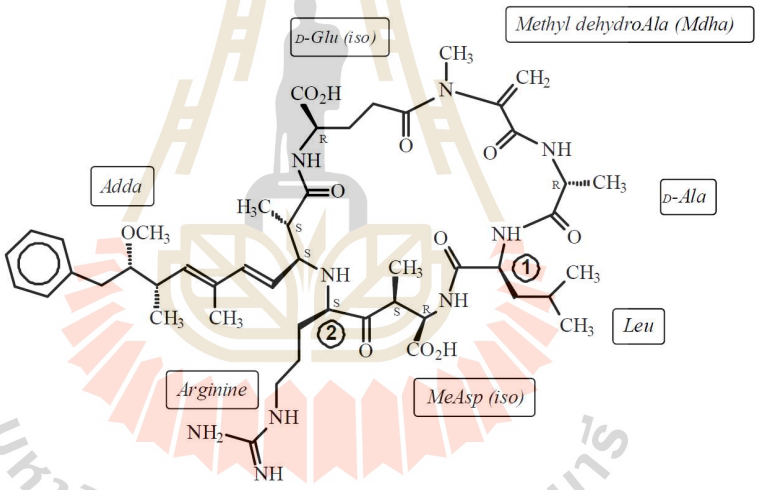



**Figure 5** The generic structure of a microcystin. Variations occur primarily at positions 1 and 2. For example, microcystin–LR contains the amino acids leucine (L) and arginine (R) at positions 1 and 2 respectively; microcystin–RR has arginine at both positions. Nodularins are similar with the five amino acids Adda–γGlu–Mdhb–βMeAsp–Arg making up the core ring system (Fristachi et al., 2008).

Microcystins are the prevalent cyanotoxin contaminated in the environment. The microcystins were first discovered from the Cyanobacterium *Microcystis aeruginosa* (Azevedo, Evans, Carmichael, and Namikoshi, 1994). More than 70 structural variants of microcystins have been identified from *Microcystis* spp. and some species of *Anabaena* (Magana-Arachchi et al., 2008). The microcystins transported across the ileum into the bloodstream through a bile acid type transporter and concentrated in the liver (Chorus and Bartram, 1999). Expose to a lethal dose of microcystin can cause death by liver necrosis within few hours to a few days (Hudnell, 2008). In most cases, microcystins are uptake by oral route from water activities that lead to inhalation of spray and droplets. Accumulation of microcystin toxicity was demonstrated in 1994 (Fitzgeorge, Clark, and Keevil, 1994). A single oral dose of toxin did not increase liver damage, however, daily application of the same dose over seven days increased liver damage by 16 times.

**2.2.2 Alkaloid**

The alkaloid toxins of Cyanobacteria can be grouped into neurotoxic alkaloids, cytotoxic alkaloids, and dermatotoxic alkaloids. Cyanobacterial alkaloid neurotoxins are grouped into three families which have the potential to cause respiratory arrest. The families of neurotoxins are anatoxin-a, anatoxin-a(S), and saxitoxins (Hudnell, 2008). Anatoxin-a is a low molecular weight alkaloid, which is produced by *Anabaena*, *Oscillatoria* and *Aphanizomenon* (Figure 6). Anatoxin-a mimic the effect of acetylcholine which open $Ca^{++}$ and $Na^{+}$ channels to generate action potentials for muscle contraction (Hitzfeld et al., 2000). As a result, high doses of the toxin can cause paralysis (Chorus and Bartram, 1999). Anatoxin-a(S) is phosphate ester of a cyclic N-hydroxyguanine that cause strong salivation, cramps, diarrhea, vomiting, and rapid

death (Figure 6). It can be produced by *Anabaena flos-aquae* strain NRC 525-17 (Chorus and Bartram, 1999). Saxitoxins are group of alkaloid neurotoxins known as paralytic shellfish poisons which block nerve cell sodium channels (Figure 6). Saxitoxins are produced from *Aphanizomenon flos-aquae, Anabaena circinalis, Lyngbya wollei* and *Cylindrospermopsis raciborskii* (Chorus and Bartram, 1999). Compared to microcystins, alkaloid neurotoxins do not occur frequently. Since only small number of blooms were found and no chronic effects have been observed, it is considered as less dangerous than microcystins or cylindrospermopsin, cytotoxic alkaloid, (Hudnell, 2008).

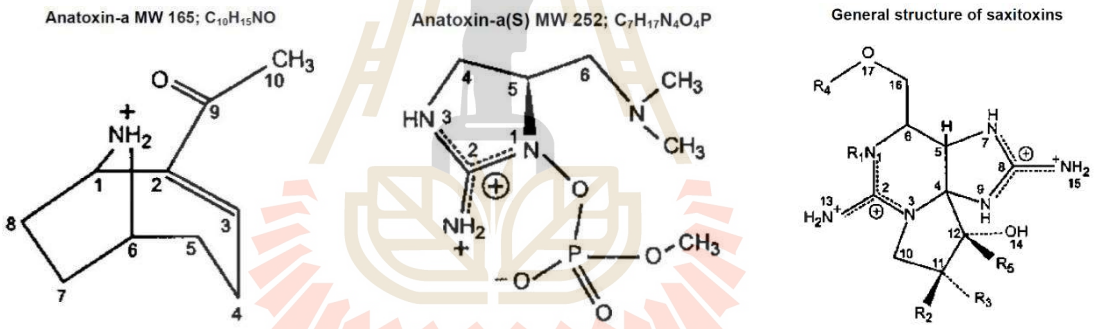



**Figure 6** The chemical structures of Cyanobacterial neurotoxins, anatoxin-a, anatoxin-a(S), and the general structure of saxitoxins (Chorus and Bartram, 1999).

Cylindrospermopsin is a cytotoxic alkaloids which is an alkaloid hepatotoxin found in tropical and subtropical water of Australia (Figure 7) (Hitzfeld et al., 2000). It is produced by *Cylindrospermopsis raciborskii*, *Umezakia natans* and *Aphanizomenon ovalisporum (*Chorus and Bartram, 1999*)*. It is a cytotoxin that blocks protein synthesis and cause kidney and liver failure (Hudnell, 2008). It has been shown that crude extract

of Cylindrospermopsin induced pathological symptoms in the kidneys, spleen, thymus, and heart in mice (Chorus and Bartram, 1999).

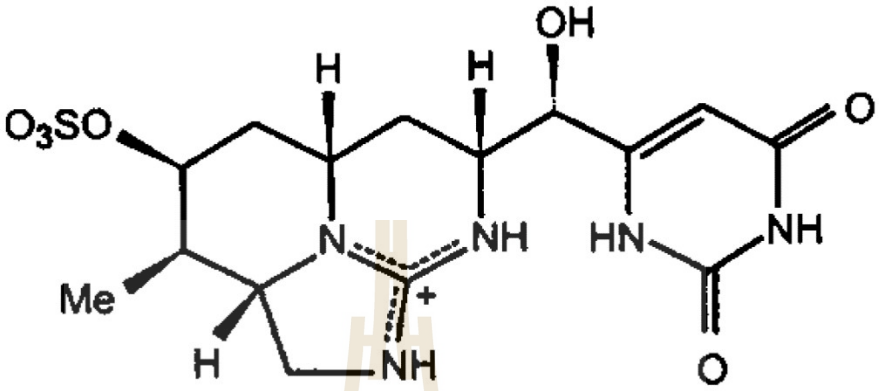



**Figure 7** Structure of Cylindrospermopsin; $C_{15}H_{21}N_5O_7S$ (Chorus and Bartram, 1999).

The dermatotoxic alkaloids can be found from benthic marine Cyanobacteria such as *Lyngbya*, *Oscillatoria* and *Schizothrix* which causes severe dermatitis among swimmers. The inflammatory activity of *Lyngbya* is caused by debromoaplysiatoxin which are strong tumor promoters and protein kinase C activators (Figure 8) (Chorus and Bartram, 1999). Some other species such as *L. majuscule* produce lyngbyatoxin-a that cause dermatitis and severe oral and gastrointestinal inflammation (Figure 8).

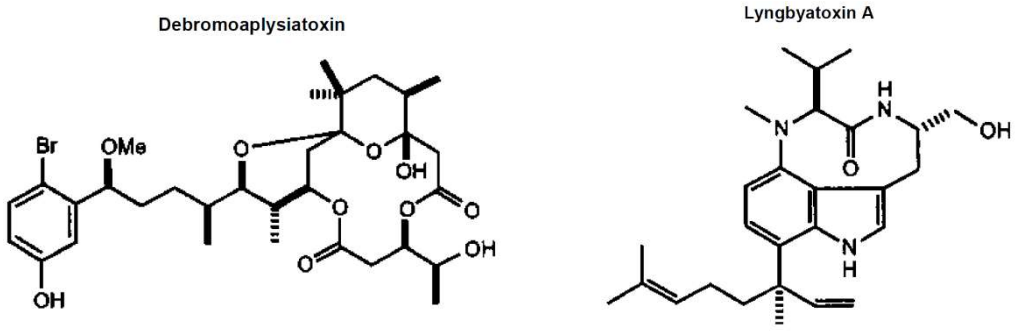



**Figure 8** The chemical structures of debromoaplysiatoxin and lyngbyatoxin-a (Chorus and Bartram, 1999).

### 2.2.3 Lipopolysaccharides

Lipopolysaccharides (LPS) are an integral component of the cell wall of all Cyanobacteria and Gram negative bacteria. The LPS of Cyanobacteria were first isolated from *Anacystis nidulans* (Weise, Drews, Jann, and Jann, 1970). Toxins derived from LPS of Cyanobacteria is pyrogenic, which is fever causing agent. It causes allergic responses in human and animal tissues (Newcombe et al., 2010). However, cyanobacterial LPS are significantly less potent than LPS from other Gram negative bacteria such as *Salmonella* (Chorus and Bartram, 1999).

# CHAPTER III

# MATERIALS AND METHODS

## 3.1 Materials

### 3.1.1 Culture media

- Luria-Bertani (LB) liquid medium contained (per 1 liter) 10 g of peptone, 10 g of NaCl, and 5 g of yeast extract. The final pH adjusted to 7.0±0.2.
- Luria-Bertani agar (LB agar) contained (per 1 liter) 10 g of peptone, 10 g of NaCl, 5 g of yeast extract, and 15 g of agar. The final pH adjusted to 7.0±0.2.
- The sterilization of the medium was performed by autoclaving at 121 ˚C, 15 p.s.i for 15 minutes.

### 3.1.2 Antibiotic

- Ampicillin (Bio-Basic, Canada) stock solution was prepared by dissolving in sterile distilled water to final concentration 100 mg/ml. the antibiotic stock solution was filter sterilized through a 0.22-0.45 µm pore filter (Corning®. Germany) and stored at -20 ˚C until used.

### 3.1.3 Buffer and solutions

- 10X TBE buffer for electrophoresis contained (per 1 liter) 108 g of Tris-HCL, 55 g of boric acid and 40 ml of 0.5 M EDTA pH 8.0.
- MaestroSafe™nucleic acid strain was purchased from MaestroGen, Taiwan.

### 3.1.4 Research kits

- FavorPrep™ Soil DNA Isolation Mini Kit purchased from Favorgen, Taiwan.
- FavorPrep™ GEL/PCR Purification Mini Kit purchased from Favorgen, Taiwan.

### 3.1.5 Primers used for PCR amplification

The name of the oligonucleotides and their sequences are listed in Table 3.

**Table 3** List of primers used in this study.

| Name | Sequences (5′-3′) | Sources |
|---|---|---|
| Cya106F | CGGACGGGTGAGTAACGCGTGA | (Nübel, Garcia-Pichel, and Muyzer, 1997) |
| Cya781Ra | GACTACTGGGGTATCTAATCCCATT | (Nübel, Garcia-Pichel, and Muyzer, 1997) |
| 27F | AGAGTTTGATCMTGGCTCAG | (Wawrik, Kerkhof, Zylstra, and Kukor, 2005) |
| 1525R | AAGGAGGTGATCCAGCC | (Wawrik, Kerkhof, Zylstra, and Kukor, 2005) |

### 3.1.6 Miscellaneous materials

- GoTaq®Green master mix used for PCR amplification was purchased from Promega, USA.
- Just-Add-DNA MasterMix used for PCR amplification was purchased from Cleaver-Scientific, UK.
- One hundred bp DNA Ladder Ready to Load was used as marker. The marker was purchased from Solis Biodyne, Estonia.
- T4 DNA Ligase purchased from Vivantis, Malaysia.
- pTG19-T cloning vector purchased from Vivantis, Malaysia.
- X-Gal purchased from Vivantis, Malaysia.
- X-Gal purchased Bio-Basic, USA.

### 3.1.7 Equipment

All the instruments used in this study are listed in Table 4.

**Table 4** List of instruments used in this study.

| Name | Source |
|---|---|
| Autoclave | Tomy, USA |
| Hot Air Oven | Memmert, Schwabach |
| Incubator shaker | Appendorf, Germany |
| Spectrophotometer | PG Instrument, UK |
| Centrifuge machine | Hettich, Germany |
| Microcentrifuge | Denville, Canada |
| pH meter | METTLER-TOLEDO, USA |
| Larminar flow | Esco, Switzerland |
| Vortex mixer | FINEPCR, Korea |
| Mini Dry bath incubator | Hercuvan, Malaysia |
| Submairne Agarose Gel Unit | Hoefer, USA |
| UltraBright LED Transilluminator | Maestrogen, Taiwan |
| Dissolved Oxygen Meter | HANNA, USA |

## 3.2 Methods

### 3.2.1 Study sites and sample collection

The study sites were located at the research and residential buildings, and reservoirs in the Suranaree University of Technology (SUT), Nakhon Ratchasima, Thailand. The water samples from residential area were directly collected from faucet in bathroom, and reservoir water was collected from the surface water using a sterile container. Water was collected from surface of the reservoirs since Cyanobacteria contain gas vacuole in cells and float on surface to perform photosynthesis. The water was transferred to sterile polyethylene bag and immediately transferred to laboratory building. The sampling sites of water samples were shown in Figure 9.

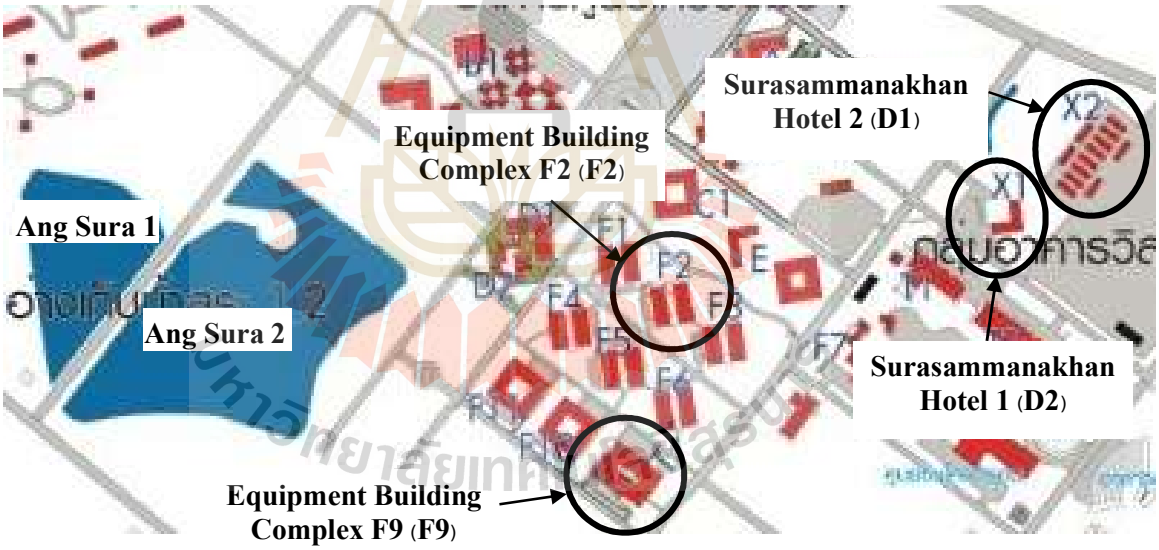



**Figure 9** Map of sampling site in SUT. Ang Sura 1 and Ang Sura 2 represented sampling sites of pre-treated water samples. Equipment Building Complex F2 (F2), Equipment Building Complex F9 (F9), Surasammanakhan Hotel 1 (D1), and Surasammanakhan Hotel 2 (D2) represented the sampling sites of post-treated water.

### 3.2.2 Identification of physical parameters

The physical parameters of dissolved oxygen (DO) concentration, and temperature were determined at the site using a HI 9147 Portable Water-resistant Dissolved Oxygen meter (HANNA, USA). The pH was measured with a pH meter (METTLER TOLEDO S220 model, USA).

### 3.2.3 DNA extraction from water resources

Each sample was used for DNA extraction by using FavoPrep$^{TM}$ Soil DNA Isolation Mini Kit (Favorgen, Taiwan). The water samples were filtrated by using 0.45 µm filter membrane. The filter membrane containing bacteria was cut into pieces, transferred into 50 ml sterile conical centrifuge tube with 200 mg of glass beads and 600 µl of lysis buffer (SDE1 buffer). The suspension was incubated at 70 ˚C for 10 min, followed by the addition of 200 µl of SDE2 buffer. The suspension was incubated on ice for 5 min and centrifuged for 5 min at full speed. The supernatant was transferred into a 1.5 ml microcentrifuge tube, and then mixed well with an equal volume of isopropanol. The mixture was centrifuged at full speed for 10 min. The aqueous layer was removed and extracted with 200 µl of elution buffer, mixed with 100 µl of SDE3 buffer. The mixture was incubated at room temperature for 3 min and centrifuged at full speed for 2 min. The supernatant was transferred into a 1.5 ml microcentrifuge tube, and then mixed well with an equal volume of SDE4 buffer and ethanol. The suspension was transferred into SDE column and centrifuged at full speed for 1 min. The column was washed twice with 750 µl of wash buffer. The column was dried by centrifugation at full speed for 3 min. The DNA was eluted with 50 µl of elution buffer, centrifuged at full speed for 1 min and stored at -20 ˚C.

### 3.2.4 Preparation of competent cells

Competent cells were prepared from *Escherichia coli* DH5α by $CaCl_2$ solution method. First, picked a single bacterial colony from a plate that has been incubated at 37 ˚C for overnight and transferred the colony into 5 ml of LB medium for overnight at 37 ˚C with vigorous shaking in incubator shaker (200 rpm). 1 ml of the culture was transferred into 100 ml of LB medium for 1.5 to 3 hrs at 37 ˚C with vigorous shaking in incubator shaker (200 rpm). $OD_{600}$ of the culture was measured every 10 to 15 min after shaking 1 hr and stop shaking when the $OD_{600}$ of the culture meets 0.3 to 0.4. The bacterial cells were transferred to sterile ice-cold 50 ml conical tubes and cooled the cultures to 0 ˚C by storing the tubes on ice. Recovered the cells by centrifugation at 3000 rpm for 5 min at 4 ˚C, then, decanted the medium from the cell pellets. The pellets were resuspended gently in 5 ml of ice-cold solution containing 0.1 M of $MgCl_2$ and stored it in ice for 30 min. Recovered the cells by centrifugation at 3000 rpm for 5 min at 4 ˚C, then, discarded the solution. Pellets were resuspended gently in 5 ml of ice-cold solution containing 0.1 M of $CaCl_2$ and incubated on ice for 1 hr. Recovered the cells by centrifugation at 3000 rpm for 5 min at 4 ˚C, then, discarded the solution. Pellets were resuspended gently in 300 µl of ice-cold solution containing 0.1 M of $CaCl_2$ with 15 % glycerol and incubated on ice for 1 hr. At last, 100 µl of suspensions were transferred into each centrifuge tube. The suspension will be stored at -80 ˚C for further use.

### 3.2.5 16S rRNA gene amplification

The genomic DNA extracted from water resources were used as DNA template for PCR amplification of 16S rRNA gene. PCR amplification of the 16S rRNA gene of samples was performed by using specific universal primers, Cya106F, Cya781Ra, 27F, and 1525R (Nübel, 1997; Wawrik, 2005). The thermal cycling conditions were as follows: initial denaturation at 95 ˚C for 5 min, followed by 25 cycles of denaturation at 95 ˚C for 60 s, annealing at 60 ˚C for 60 s, extension at 72 ˚C for 30 s, and a final elongation at 72 ˚C for 7 min. The PCR products were verified on 0.7 to 1.5 % (w/v) agarose gel in 1X Tris/borate/EDTA (TBE) buffer. Amplified fragment length between 600 to 700 bp were cut from agarose gel and the band was purified by using FavorPrep$^{TM}$ GEL/PCR Purification Kit (Favorgen, Taiwan).

The purified 16S rRNA fragment obtained from PCR was ligated to the pTG 19-T cloning vector (Vivantis, Malaysia) (Figure 10). The recombinant plasmid was mixed with 100 µl of the chemically competent *Escherichia coli* DH5α cells. The suspension kept on ice for 1 h. The mixture was incubated at 42 ˚C for 60 sec and immediately put on ice and incubated for 2 min. A volume of 900 µl of LB added into the mixture and incubated at 37 ˚C for 1 h. The transformed cells were spread onto LB plate supplemented with 0.1 mg/ml of ampicillin and 40 µl/ml of X-gal. The plate was incubated overnight at 37 ˚C.

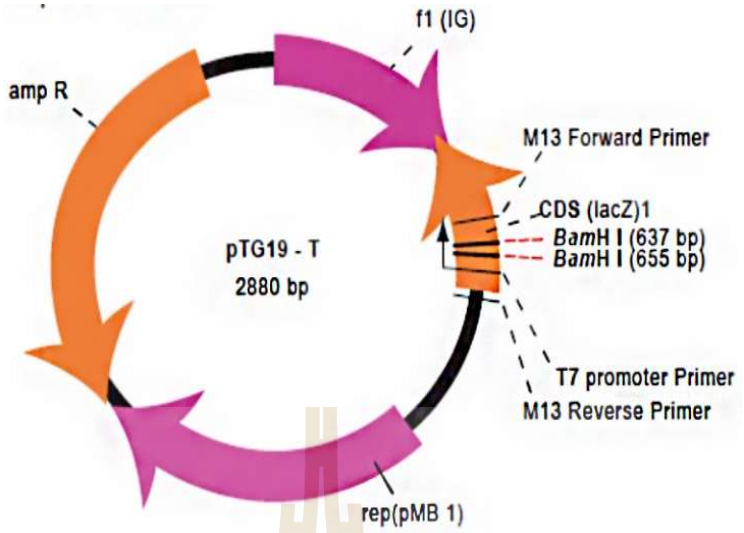



**Figure 10** Map of pTG19-T vector (Vivantis, Malaysia). The PCR product was inserted between *Bam*HI recognition regions.

### 3.2.6 16S rRNA gene sequencing

The recombinant plasmid containing 16s rRNA gene were submitted for sequencing at Macrogen, Korea. The similarities of obtained sequences were detected between a query sequence and database sequences in the GenBank using Blastn (Altschul et al., 1997).

### 3.2.7 Construction of phylogenetic tree

The sequences of 16S rRNA gene were compared with known sequence from NCBI database. The sequences were aligned with closest related strains by using ClustalW. The phylogenetic tree was constructed by the neighbor-joining method using the Molecular Evolutionary Genetics Analysis software version 7.0 (MEGA 7.0). The tree topologies were evaluated by using bootstrap analysis (500 replications).

# CHAPTER IV

# RESULTS

## 4.1 Sample collection

Nineteen samples were collected from SUT, Thailand, on May 2018. Thirteen samples were collected from Ang Sura 1 and Ang Sura 2 water reservoirs (Figure 9). Six post-treated water samples were collected from Equipment Building Complex F2, Equipment Building Complex F9, Surasammanakhan Hotel 1, and Surasammanakhan Hotel 2 (Figure 9). Since water from the reservoirs (Pre-treated) are purified and mostly used in bathrooms, post-treated water was collected from bathroom in the buildings. Water samples were collected into polyethylene bags and transferred to the laboratory. The sampling location of reservoirs were named with alphabet from A to M (Figure 11). The post-treated water samples from Equipment Building Complex F2 and F9 were named as F2-1, F2-2, F9-1, F9-4, and from Surasammanakhan Hotel 1 and 2 were named as D1 and D2 (Figure 9).

## 4.2 Physical parameters of water

### 4.2.1 Physical parameters of water from reservoirs

Thirteen samples were collected from Ang Sura 1 and Ang Sura 2 water reservoirs. The physical parameters of the water reservoirs (Pre-treated) are shown in Table 5. According to the results, temperature of the reservoirs were ranged from 28 to 31.0 ˚C, dissolved oxygen (DO) were from 7.4 to 7.8 mg/L, pH were from 8.27 to 8.76.


**Figure 11** Sample collecting site of reservoirs from Ang Sura 1 and Ang Sura 2 water reservoirs. The sample collecting position were named from A to M.

**Table 5**. Physical parameters from Ang Sura 1 (A-F) and Ang Sura 2 (G-M) water reservoirs.

| Samples name | pH | Dissolved Oxygen (mg/L) | Temperature (˚C) |
|---|---|---|---|
| A | 8.60 | 7.7 | 28.6 |
| B | 8.76 | 7.7 | 29.0 |
| C | 8.56 | 7.7 | 28.6 |
| D | 8.55 | 7.7 | 29.0 |
| E | 8.90 | 7.6 | 29.8 |
| F | 8.55 | 7.6 | 30.0 |
| G | 8.49 | 7.5 | 30.0 |
| H | 8.49 | 7.5 | 30.4 |
| I | 8.49 | 7.6 | 29.6 |
| J | 8.35 | 7.4 | 31.0 |
| K | 8.27 | 7.7 | 29.0 |
| L | 8.37 | 7.6 | 30.0 |
| M | 8.33 | 7.8 | 28.0 |

### 4.2.2 Physical parameter of Post-treated water

Temperature of the bathroom (Post-treated) water were ranged from 28.0 to 29.2 ˚C, DO were from 7.6 to 7.8 mg/L, pH were from 7.25 to 7.59.

**Table 6** Physical parameters from Equipment Building Complex F2 (F2), Equipment Building Complex F9 (F9), Surasammanakhan Hotel 1 (D1), and Surasammanakhan Hotel 2 (D2).

| Sample name | pH | Dissolved Oxygen (mg/L) | Temperature (˚C) |
|---|---|---|---|
| F2-1 | 7.56 | 7.8 | 28.2 |
| F2-2 | 7.25 | 7.7 | 29.2 |
| F9-1 | 7.51 | 7.8 | 28.3 |
| F9-4 | 7.45 | 7.6 | 28.4 |
| D1 | 7.46 | 7.7 | 29.2 |
| D2 | 7.59 | 7.8 | 28.0 |

## 4.3 Environmental genome extraction

The sample from A and B, C and D, E and F, H and I, J and K, L and M were combined and used for genomic DNA extraction. They were named as sample AB, CD, EF, HI, JK, and LM, respectively. Extraction of genomic DNA from the reservoirs were performed by using FavorPrep$^{TM}$ Soil DNA Isolation Mini Kit (Favorgen, Taiwan). Prior to the DNA extraction from liquid sample, vacuum filtration with cellulose nitrate membrane filter was performed. For all samples, approximately 300 ml of water samples from water reservoirs (Pre-treated) were filtrated. The process of DNA extraction were performed as described in chapter 3 (Material and Method). Five microliter of extracted genome were applied to agarose gel electrophoresis to determine

quantity and quality of the extract. The electrophoresis results showed that all of the samples had genomic DNA were observed above 3 kbp or 10 kbp compared with DNA ladder (Figure 12).

Three-thousand milliliter of water from buildings (Post-treated) were used for genomic DNA extraction. Total of four water samples, two samples were collected from Equipment Building Complex F2 (F2) and Equipment Building Complex F9 (F9), another two were extracted from Surasammanakhan Hotel 1 (D1), and Surasammanakhan Hotel 2 (D2) (Figure 9). Five microliter of extracted DNA were applied to agarose gel electrophoresis to determine the quantity and quality of the samples. According to the results, genomic band was observed above 3 kbp with sample D2 (Figure 13).

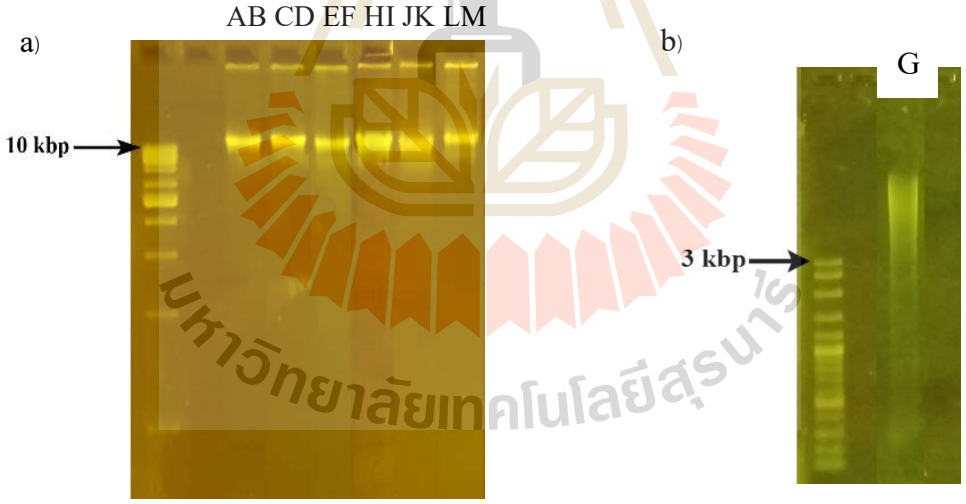



**Figure 12** Electrophoresis results of entire genome from Ang Sura 1 and Ang Sura 2 water reservoirs (Pre-treated). a) The electrophoresis was performed on 1.5 % agarose gel with 1 kb DNA ladder. b) The electrophoresis was performed on 0.7 % agarose gel with 100 bp DNA ladder.

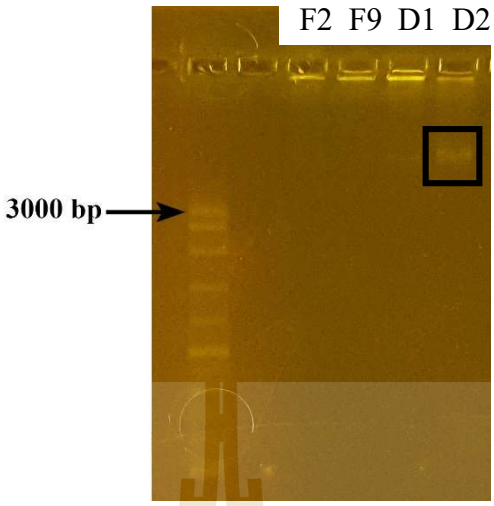



**Figure 13** Electrophoresis results of entire genome from buildings (Post-treated). The electrophoresis was performed on 1.5 % agarose gel with 100 bp ladder.

## 4.4 PCR amplification of 16S rRNA gene.

The 16S rRNA genes of environmental genome obtained from the water reservoirs (Pre-treated) and buildings (Post-treated) were amplified with Cyanobacteria specific primers, Cya 106F and Cya 781Ra. The amplified bands from reservoirs (Pre-treated) were ranged from 600 bp to 700 bp (Figure 14). On the other hand, samples from buildings (Post-treated) did not show amplified band with Cya 106F and Cya 781Ra primers (Figure not shown). In order to verify whether or not Cyanobacteria could amplify with other set of primer, Bacterial universal primer 27F and 1525R were used to amplify 16S rRNA gene of Bacteria. According to the result, samples from buildings (Post-treated) did not show amplified band with the primers. Since the universal primers also could amplify 16S rRNA of Cyanobacteria, there were no bacterial genome including Cyanobacteria. Thus, the genomic DNA from buildings

(Post-treated) may come from other microorganisms or too low to detect the 16S rRNA gene from PCR.

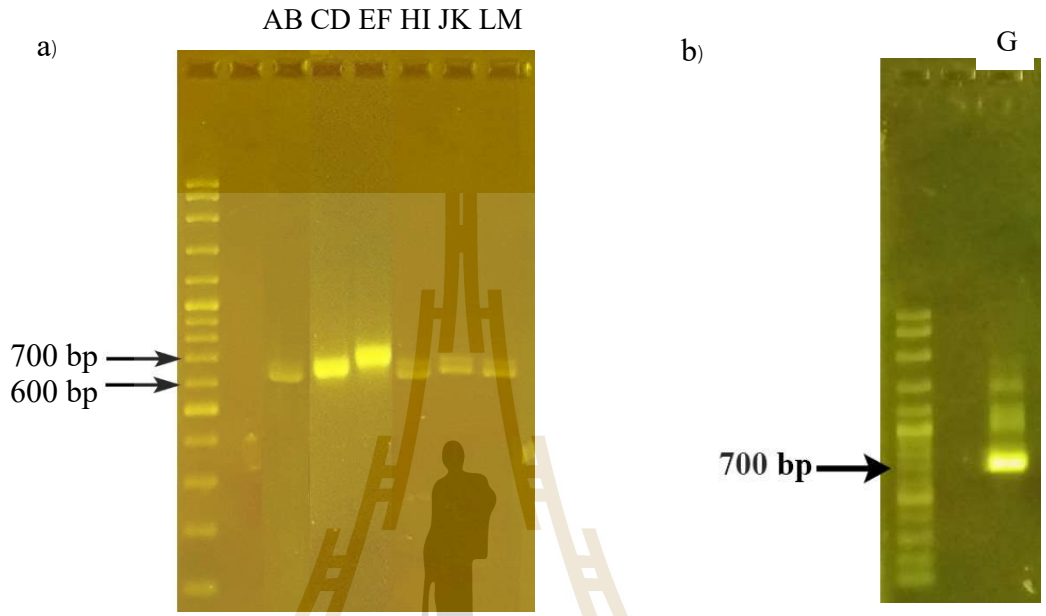



**Figure 14** PCR product of entire genome from reservoirs (Pre-treated) on agarose gel electrophoresis. The electrophoresis was performed on a) 1.5 % and b) 0.7 % agarose gel with 100 bp DNA ladder.

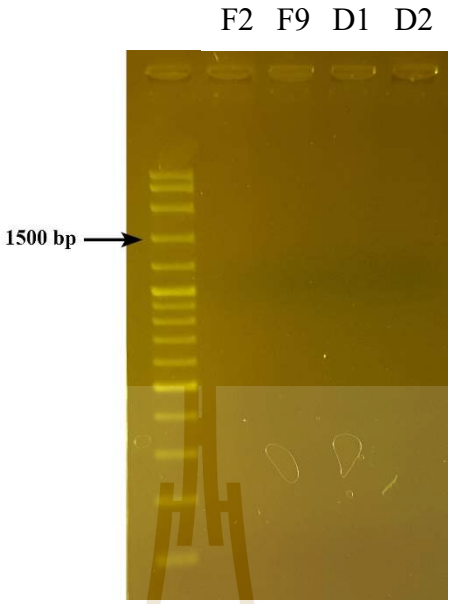



**Figure 15** The PCR product of entire genome from buildings (Post-treated) on agarose gel electrophoresis. The electrophoresis was performed on 1.5 % agarose gel with 100 bp DNA ladder.

## 4.5 Purification of Cyanobacterial 16S rRNA gene

The 16S rRNA gene from the reservoirs (Pre-treated) amplified with Cya106F and Cya 781Ra primers obtained approximately 600 bp to 700 bp fragment size from the result of electrophoresis (Figure 14). In this study amplification of the sample was performed directly from environmental genome and the PCR products were not pure. Since the product was mixed with multiple species of 16S rRNA gene, PCR product was cloned to obtain pure product. Thus, the amplified 16S rRNA gene fragments were cut and purified by using FavoPrep$^{TM}$ GEL/PCR Purification Kit (Favorgen, Taiwan) prior for cloning.

The purified 16S rRNA gene sequence obtained from PCR were ligated into pTG19-T vector and transformed into *E.coli* DH5α. Positive clones which show as

white colony was selected from LA medium with ampicillin and X-gal. The positive clones containing 16S rRNA gene were randomly selected. Total of twenty-two clones were selected and sequenced. They were named as AB3, AB8, AB10, CD1, CD5, CD6, EF1, EF4, EF7, G1, G2, G3, G4, G5, G6, HI1, HI2, HI9, JK25, JK60, LM16, and LM40. These sequences were search for closest relatives from the NCBI database (Table 7).

The clones EF7, HI1, JK25, and LM40 showed 99 % and 100 % identity with *Cylindrospermopsis raciborskii* CHAB3445 strain and JK60 showed 100 % relative to *C. raciborskii* strain LJ. The closest relative for clones AB3 and AB10 was *Prochlorothrix hollandica* strain PCC9006 with 95 % and 96 %, respectively. Clones of G1, G4, and G5 had 98 % identity with *Ancyclothrix terrestris* strain 10PC. The clone CD5 was 99 % relative to *Synechococcus* sp. The closest relatives for LM16 clone was Cyanobacterium clone SGSO631 with similarity percentage of 91 %. Clones AB8, CD6, EF1, EF4, and HI2 were closely related to an uncultured Bacterium clone A-113 with 99 % similarity. Clone CD1 had 94 % relative to *Verrucomicrobia* Bacterium SGCG AAA204-G18 strain and HI9 showed 99 % relative to an uncultured *Verrucomicrobia* Bacterium clone LiUU-9-291. Clones G2, G3, and G6 were closely related to 16s rRNA genes of *Thalassiosira weissflogit* chloroplast with 93 % and 99 % similarity (Table 7).

**Table 7** Table of 16S rRNA gene fragments from water reservoirs determined with NCBI database.

| Sample name | 16S rRNA gene fragment length (bp) | Identity (%) | Closest match in NCBI database (accession no.) |
|---|---|---|---|
| AB3 | 661 | 95 | *Prochlorothrix hollandica* strain PCC 9006(NR_126312.1) |
| AB8 | 709 | 99 | Uncultured bacterium clone A-113(HQ860471.1) |
| AB10 | 662 | 96 | *Prochlorothrix hollandica* strain PCC 9006(NR_126312.1) |
| CD1 | 716 | 94 | *Verrucomicrobia* bacterium SCGC AAA204-G18(JF488114.1) |
| CD5 | 662 | 99 | *Synechococcus* sp. BS2(HM346183.1) |
| CD6 | 709 | 99 | Uncultured bacterium clone A-113(HQ860471.1) |
| EF1 | 709 | 99 | Uncultured bacterium clone A-113(HQ860471.1) |
| EF4 | 709 | 99 | Uncultured bacterium clone A-113(HQ860471.1) |
| EF7 | 663 | 100 | *Cylindrospermopsis raciborskii* CHAB3445 (KU360032.1) |
| G1 | 664 | 98 | *Ancylothrix terrestris* strain 10PC (NR149292.1) |
| G2 | 667 | 99 | *Thalassiosira weissflogii* chloroplast (KJ958485.1) |
| G3 | 663 | 93 | *Thalassiosira weissflogii* chloroplast (KJ958485.1) |

**Table 7 (**Continued**).**

| | | | |
|---|---|---|---|
| G4 | 663 | 98 | *Ancylothrix terrestris* strain 10PC (NR149292.1) |
| G5 | 663 | 98 | *Ancylothrix terrestris* strain 10PC (NR149292.1) |
| G6 | 666 | 99 | *Thalassiosira weissflogii* chloroplast (KJ958485.1) |
| HI1 | 663 | 99 | *Cylindrospermopsis raciborskii* CHAB3445 (KU360032.1) |
| HI2 | 709 | 99 | Uncultured bacterium clone A-113(HQ860471.1) |
| HI9 | 751 | 99 | Uncultured *Verrucomicrobia* bacterium clone LiUU-9-291(AY509507.1) |
| JK25 | 663 | 99 | *Cylindrospermopsis raciborskii* CHAB3445 (KU360032.1) |
| JK60 | 663 | 100 | *Cylindrospermopsis raciborskii* strain LJ (AF516725.1) |
| LM16 | 670 | 91 | Uncultured Cyanobacterium clone SGSO631(GQ347873.1) |
| LM40 | 663 | 100 | *Cylindrospermopsis raciborskii* CHAB3445 (KU360032.1) |

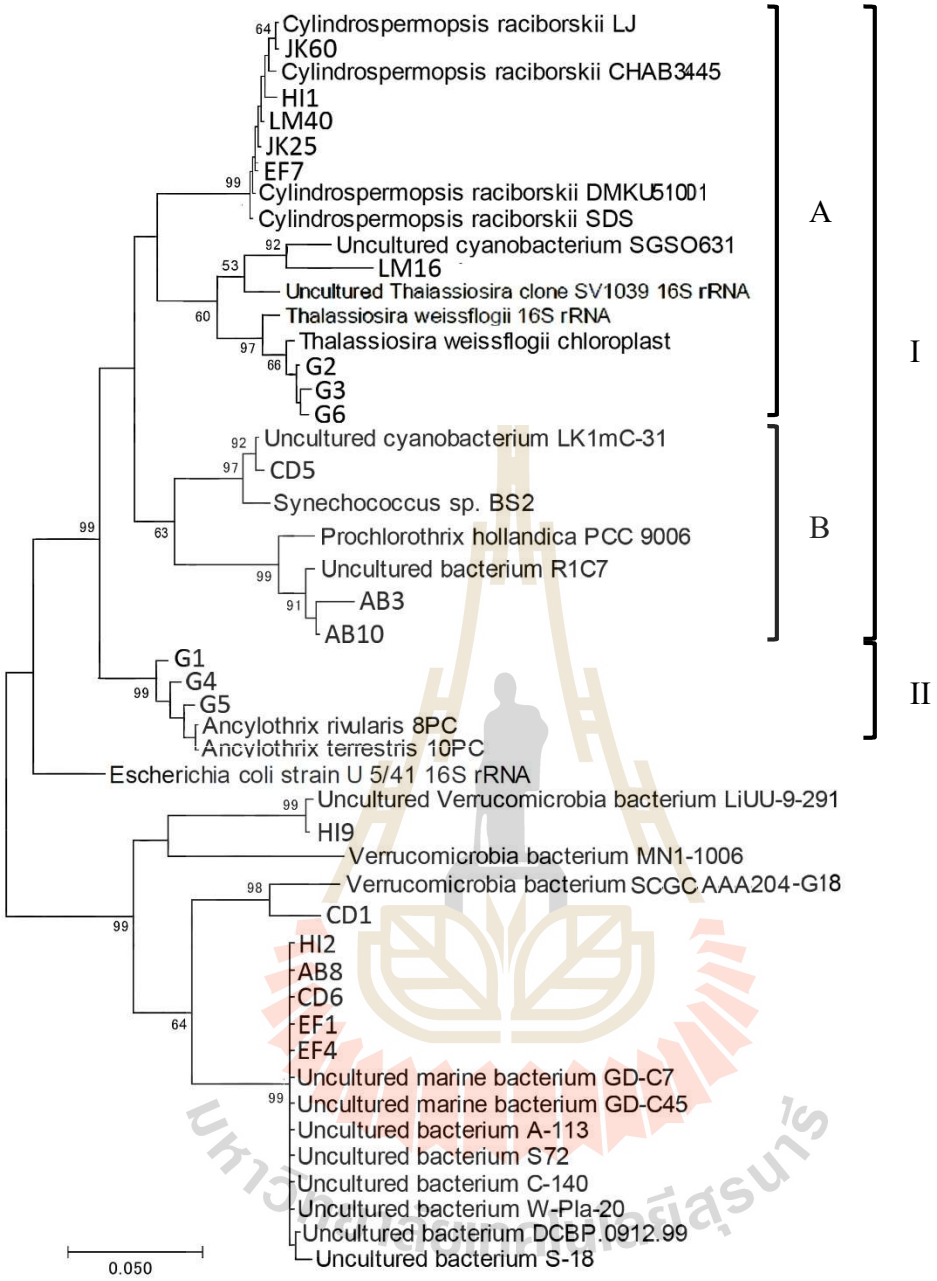



**Figure 16** Phylogenetic tree constructed from 16S rRNA gene sequences using neighbor joining method. The 16S rRNA gene region was aligned by ClustalW and the tree was generated by using MEGA 7.0. Bootstrap percentages calculated from 500 resamplings are indicated at nodes.

## 4.6 Phylogenetic Tree

Phylogenetic relationship of the twenty two 16S rRNA gene sequences were determined with their closely related type strains from the NCBI database. The tree was constructed by using neighbor-joining method (Figure 16).

The phylogenetic tree was constructed from an alignment of 48 sequences, including four *Cylindrospermopsis* sp., two *Ancylothrix* sp., one *Synechococcus* sp., one *Prochlorothrix* sp., two *Verrucomicrobia* sp., and two *Thalassiosira* sp. with *Escherichia coli* as the outgroup using neighbor-joining method (Figure 16). Cyanobacterial genus in the phylogenetic tree was separated into two major clusters. The cluster I was further separated into two subclusters. The subcluster A included *Cylindrospermopsis* sp. at high bootstrap value of 99 %. The subcluster B included *Synechococcus* sp. and *Prochlorothrix* sp. at bootstrap value of 63 %. The cluster II included only Ancylothrix sp. Other prokaryote formed a cluster with either *Verrucomicrobia* bacterium or uncultured bacterium at high bootstrap value of 98 and 99 %. *Thalassiosira* sp. 16S rRNA from chloroplast of algae was included into subcluster A.

# CHAPTER V

# DISCUSSION AND CONCLUSION

The average pH, dissolved oxygen (DO), and temperature of Ang Sura 1 water reservoir were 8.65±0.15, 7.67±0.05, and 29.16±0.60 ˚C, respectively. The average physical parameters of Ang Sura 2 water reservoir were pH 8.40±0.09, DO 7.59±0.14, and temperature 29.71±0.98 ˚C (Table 8). For water from buildings (Post-treated), average pH 7.45±0.12, DO 7.72±0.08, and temperature 28.66±0.50 ˚C. According to World Health Organization (WHO), water quality for aquatic species should range from pH 6.5 to 9.0, DO is 5 to 9.5 mg, and water temperature is 0 to 30 ˚C (Helmer and Hespanhol, 1997). Therefore, the water quality of the SUT reservoirs were in normal range according to the results.

**Table 8** Average value of pH, DO, and temperature from Ang Sura 1 and Ang Sura 2 water reservoirs (Pre-treated) and bathroom (Post-treated).

| | **Ang Sura 1** | **Ang Sura 2** | **Buildings** |
|---|---|---|---|
| Average pH | 8.65±0.15 | 8.40±0.09 | 7.45±0.12 |
| Average DO (mg/L) | 7.67±0.05 | 7.59±0.14 | 7.72±0.08 |
| Average temperature (˚C) | 29.16±0.60 | 29.71±0.98 | 28.66±0.50 |

Since the cyanobacterial bloom decreases concentration of DO, there were no indication of bloom from SUT reservoirs. Thus, the quality of water in SUT reservoirs were in normal range for aquatic organisms to survive. Although the chemical parameters of the reservoirs were not determined, amount of essential nutrients such as nitrogen and phosphorous might be low since no bloom was observed (Prasertsin and Peerapornpisal, 2015).

Total of twenty-two clones, twelve clones were highly relative to Cyanobacterial genus. The relative genus were *Cylindrospermopsis*, *Anclylothrix*, *Prochlorothrix*, and *Synechococcus*. Other clones were related to *Thalassiosira*, *Verrucomicrobia*, and uncultured Bacterium. It has been reported that Cya106F and Cya781Ra primers could amplify product from 600 bp to 700 bp in length (Garcia-Pichel, F., López-Cortés, A., and Nübel, U. 2001; Nübel et al., 1997). However, this study showed that most of 16S rRNA gene with fragment length between 663 bp to 670 bp were identified as Cyanobacteria and all samples with fragment length around 700 bp were identified as other bacteria.

**Table 9** Relationship between genus and 16S rRNA gene fragment length size.

| Microbes | Genus | 16S rRNA gene fragment length (bp) |
|---|---|---|
| Cyanobacteria | *Cylindrospermopsis* | 663 |
| | *Ancylothrix* | 663, 664 |
| | *Prochlorothrix* | 661, 662 |
| | *Synechococcus* | 662 |
| | Uncultured Cyanobacteria | 670 |
| Other Bacteria | *Verrucomicrobia* | 716, 751 |
| | Uncultured bacterium | 709 |
| Algae | *Thalassiosira* | 663, 666, 667 |

The phylogenetic tree results from figure 15 showed that EF7, HI1, JK25, JK60, and LM40 shared same ancestor with *Cylindrospermopsis raciborskii* species with high bootstrap value of 99 %. *C. raciborskii* is a second prevalent species in tropical Asia and belong to *Nostocale* Cyanobacteria. The species was first observed in island of Java, Indonesia in 1899 and currently observed all around the world (Antunes, Leão, and Vasconcelos, 2015). Growth temperature of *C. raciborskii* is between 11 to 35 ˚C and preference pH for growth is between 8.1 to 9.4 with median value 8.2. Since optimal bloom temperature for *C. raciborskii* is between 25 to 32 ˚C, it is often observed in tropical, subtropical, and temperate climate zones. (Antunes, Leão, and Vasconcelos, 2015).

AB3 and AB10 shared same ancestor with *Prochlorothrix hollandica* species with high bootstrap value of 99 %. *Prochlorothrix hollandica* is belong to Nostocale Cyanobacteria and usually observed from eutrophic freshwater or brackish habitats (Pinevich, Velichko, and Ivanikova, 2012). This genus is not a common toxin producer of Cyanobacteria and growth temperature is between 20 to 30 ˚C and pH is 8 to 9 (Burger-Wiersma, Stal , and Mur , 1989; World Health Organization, 2003)

CD5 shared a same ancestor with *Synechococcus* genus with high bootstrap value of 97 %. *Synechococcus* belongs to Synechococcales Cyanobacteria and it has been observed from oceanic surface and freshwater environment (Kim et al., 2018). This genus can be either toxin or non-toxin producer but also one of the important contributors of global primary production since they found globally and capable to perform $CO_2$ fixation (Coutinho, Tschoeke, Thompson, and Thompson, 2016; Kim et al., 2018; World Health Organization, 2003). The optimum temperature range for *Synechococcus* to grow is from 15 to 28 ˚C and they are often observed in mesotrophic and eutrophic habitats (Moore, Goericke, and Chisholm, 1995; Varkey, 2015).

G1, G4, and G5 shared a same ancestor with *Ancylothrix* genus with high bootstrap value of 99 %. *Anclylothrix* is a newly found genus of filamentous Cyanobacteria isolated from wet soils and tree trunks in Brazil (Martins, Rigonato, Taboga, and Branco, 2016). The species was found in recent years and detail have not been studied.

LM16 formed a clade with uncultured Cyanobacterium SGSO631 strain and shared a same ancestor with bootstrap value of 60 %. The information of the strain was limited yet the uncultured Cyanobacterium clone was found from oxygen minimum zones in ocean where nutrition is low (Walsh et al., 2009).

CD1 and HI9 shared a same ancestor with *Verrucomicrobia* Bacterium with high bootstrap value of 98 % and 99 %. *Verrucomicrobia* does not belong to Cyanobacteria and first observed in aquatic habitat (Dworkin, Falkow, Rosenberg, Schleifer, and Stackebrandt, 2006). This bacterium was found from wide range of temperature such as cold temperature in deep sea and hot spring (75 to 95 ˚C) (Dworkin et al., 2006).

AB8, CD6, EF1, EF4, and HI2 did not form a cluster with Cyanobacteria and shared a same ancestor with uncultured bacterium and uncultured marine bacterium with high bootstrap value of 99 %. The detail of uncultured bacterium was not published and the detail was not informed in online database.

G2, G3, and G6 shared a same ancestor with 16s rRNA gene of *Thalassiosira* chloroplast which is belong to algae. Evolutionally, chloroplast of algae is derived from Cyanobacteria by endosymbiosis and chloroplast carry their own DNA (OpenStax College, 2012). Therefore, since 16S rRNA gene of chloroplast from Algae was derived from Cyanobacteria, Cyanobacterial specific primer used in this study maybe amplified the 16S rRNA gene from Algae. Also this maybe reason that Thalassiosira 16S rRNA was formed a same cluster with Cyanobacteria.

From twelve clones relative to Cyanobacteria genus, seven belonged to order *Nostocales*, three were order *Oscillatoriales*, and one was *Synechococcales*, and one was uncultured Cyanobacteria (Table 10). Although number of the samples sequenced

as Cyanobacteria in this study was small, seven clones belonged to order *Nostocales* from reservoirs. One report indicated that water movement significantly affect survival of Cyanobacteria. Cyanobacteria which do not perform nitrogen fixing such as *Microcystis* are likely to observe in lakes. On the other hand, nitrogen fixing Cyanobacteria such as *Cylindrospermopsis* are likely to observe in reservoirs (Dokulil and Teubner, 2000). The difference of dominant Cyanobacterial genus in lake and reservoir is affected by type of water movement and nutrients in water (Figure 17). In lakes, usually there is no extreme fluctuation of water and discharge water occur only from surface. Since the water releases from surface, accumulated nutrients remains in lake water body and Cyanobacteria that do not perform nitrogen fixation is able to acquire nutrients from the water. On the other hand, reservoirs usually discharge water from different depth, either from side or from bottom. Accumulated nutrients from water body of reservoir also release when water discharge which decrease the nutrients in reservoir (Mowe et al., 2015). Nitrogen fixing genera such as *Cylindrospermopsis* and *Anabaena* are capable to fix atmospheric nitrogen and store in akinetes for their growth. The nitrogen fixing and storing functions of these Cyanobacteria help them to grow with low nutrition. (Figueredo and Giani, 2009). Although number of clones sequenced in this study was small, sequenced Cyanobacterial genus from SUT reservoirs were maybe affected from water movement and amounts of nutrients in water. Decrease of nutrients in the reservoirs reduce survival of Cyanobacteria which do not have nitrogen fixing functions. Thus reservoirs increase in chance to amplify 16S rRNA gene of nitrogen fixing cyanobacteria such as *Cylindrospermopsis*.

**Table 10** Number of Cyanobacterial genus sequenced from reservoirs.

| Genus | Ang Sura 1 | Ang Sura 2 | Total |
|---|---|---|---|
| *Cylindrospermopsis* | 1 | 4 | 5 |
| *Prochlorothrix* | 2 | - | 2 |
| *Anclylothrix* | - | 3 | 3 |
| *Synechococcus* | 1 | - | 1 |
| Uncultured Cyanobacteria | - | 1 | 1 |

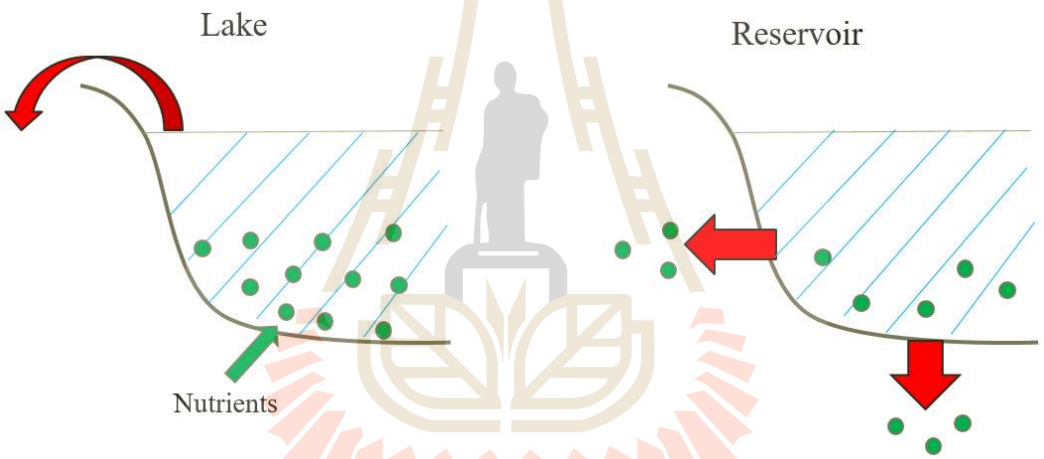



**Figure 17** Illustration of moving water between lake and reservoir. Lake water is discharge from surface (red arrow) and reservoir water discharge from side or bottom (red arrow). Nutrients are easier to release from the reservoir during discharge of water (Dokulil and Teubner, 2000).

For the post-treated water from SUT campus, ten times amount of water sample compared with pre-treated water was filtrated for genomic extraction. Total of four sampling sites, entire genome was obtained only from Surasammanakhan Hotel 2 (D2) (Figure 13). Although whole genome was observed from electrophoresis, 16s rRNA gene of Cyanobacteria was not amplified from the primer used in this study. Since the soil extraction kit used in this study is capable to extract total DNA which include Bacteria, Archaea, Fungi and Algae. This could be suggested that the genomic band obtained from the kit did not contain any Cyanobacterial genome.

Globally, number of microorganism contamination from tap water was reported and those microorganisms were such as *Escherichia coli*, *Legionella pneumophila*, *Acanthamoeba keratitis*, and parasites (Kilvington et al., 2004; Uga et al., 2003). The major reason of detecting microorganisms in tap water is from contamination of faucet (Kilvington et al., 2004; Liu et al., 2012). Only few cases that cyanobacterial contamination were reported from inadequate treatment of water from industry (Zhang et al., 2010). In this study genomic DNA of Cyanobacteria was not observed from all buildings (Post-treated). Thus genomic DNA of Cyanobacteria from post-treated water in SUT was too low to detect with the method at the moment.

In this research, culture-independent molecular surveys using 16S rRNA gene of Cyanobacteria was studied from reservoirs in Suranaree University of Technology, Thailand. Based on 16S rRNA gene analysis, twelve clones were highly related to four Cyanobacterial genus which were *Cylindrospermopsis*, *Anclylothrix*, *Prochlorothrix*, and *Synechococcus.* Among four genus the most abundant Cyanobacterial population found in this study was belong to the species *Cylindrospermopsis raciborskii*. This species can be either toxin or non-toxin producing Cyanobacteria which could grow

well in reservoirs since it has ability to fix nitrogen. Beside *C. raciborskii*, one clone of *Synechococcus* was observed from the SUT reservoirs which is another possible toxin producer. However, nitrogen fixing Cyanobacteria, *C. raciborskii*, may have more chance to increase in number than *Synechococcus*. Discharge of nutrients from water reservoirs reduce number of *Synechococcus* since it does not produce nitrogen.

In this study, sequenced *C. raciborskii* from the reservoir was highly related to LJ strain which is non-toxin producer (Neilan et al., 2003). In order to approve toxin production, amplification of toxin gene with toxin specific primers such as CYLAT-F and CYLAT-R are required which target the gene involves in the biosynthesis of cylindrospermopsin (Hoff-Risseti et al., 2013). From post-treated water, samples from buildings (Post-treated) could not detect any 16S rRNA gene of Bacteria include toxin producing Cyanobacteria. Thus, in Cyanobacterial viewpoint, the water seems safe to use for daily uses even though the post-treated water is not recommended for drinking. However, chemical contents and other microorganisms including Archaea, Fungi, Algae, Protozoa, and other common water contaminating Bacteria (*Escherichia coli*, *Legionella pneumophila*, and *Acanthamoeba keratitis*) were not known since they were not studied in this research. In conclusion, biodiversity of Cyanobacteria from pre-treated and post-treated water from SUT were successfully studied by culture-independent molecular surveys using 16S rRNA gene sequencing.

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# APPENDIX

# APPENDIX

# SEQUENCES OF ENVIRONMENTAL SAMPLES FROM SUT WATER RESERVOIR

## 1. Sequences from reservoirs in SUT

**AB3**

CGGACGGGTGAGTAACGCGTGCGGACCTGCCCTTAGGTCGGGGACAACAG
TTGGAAACGACTGCTAATACCGGATGAGCCGAGAGGTAAAAGATTTATCG
CCTAAGGATGGACTCGCGTCAGATTAGCTAGTTGGTGTGGTAACGGCATA
CCAAGGCGACGATCTGTAGCTGTTCTGAGAGGATGATCAGCCACACTGGG
ATGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCG
CAATGGGCGACAGCCTGACGCAGCAACGCCGCATGCGGGAGGACGGTTTT
AGGACTGTACACCGCTTTTCTCAGGGAAGAAGATCTGACGGTACCTGAGG
AATCAGCATCGGCTAATTCCGTGCCAGCAGCCGCGGTAATACGGGAGATG
CAAGCGTTATCCGGAATTATTGGGCGTAAAGCGTCCGCAGGCGGGCTATC
AAGTCTGCTGTCAAATCACACAGCTTAACTGTGGGGCGGCAGTGGAAACT
GAAAGCCTAGAGTGCGGTAGGGGTAGGGGGAATTCCCGGTGTAGCGGTG
AAATGCGTAGATATCGGGAAGAACACCAGCGGCGAAAGCGCCCTACTGG
GCCGCAACTGACGCTCATGGACGAAAGCTAGGGGAGCGAATGGGATTAG
ATACCCCAGTAGTC

**AB8**

CGGACGGGTGAGTAACGCGTGAGCAACCTGCCGTGAAGTGGGGGATAGC

TCGCCGAAAGGCGAATTAATACCGCGTGTGGCCAAGGAAGACATCTTCCT

GACGCCAAAGCCGGGGCAACCTGGCGCTTCTCGAGGGGCTCGCGGCCTAT

CAGCTTGTTGGTGAGGTAACGGCTCACCAAGGCTATGACGGGTAGCTGGT

CTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGTCCAGACACCT

ACGGGTGGCAGCAGTCGAGAATTTTTCACAATGGGGGAAACCCTGATGGA

GCGACGCCGCGTGGAGGATGAAGGTTTTCGGATTGTAAACTCCTGTCATT

AGAGAACAAGGCACACGGTTTAACTGGCCGTGTGTTGATAGTATCTGAAG

AGGAAGAGACGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGTCT

CAAGCGTTGTTCGGATTCATTGGGCGTAAAGGGTGCGTAGGTGGCGATGT

AAGTCGGGTGTGAAATCTCCAAGCTTAACTTGGAAACTGCACTCGATACT

GCGTCGCTCGAGGACTGTAGAGGGCATTGGAATTCACGGTGTAGCAGTGA

AATGCGTAGATATCGTGAGGAAGACCAGTGGCGAAGGCGAATGCCTGGG

CAGTTCCTGACACTGAGGCACGAAGGCCAGGGGAGCAAATGGGATTAGA

TACCCCAGTAGTC

**AB10**

CGGACGGGTGAGTAACGCGTGAGGACCTGCCCTTAGGTCGGGGACAACA

GTTGGAAACGACTGCTAATACCGGATGAGCCGAGAGGTAAAAGATTTATC

GCCTAAGGATGGACTCGCGTCAGATTAGCTAGTTGGTGTGGTAACGGCAT

ACCAAGGCGACGATCTGTAGCTGTTCTGAGAGGATGATCAGCCACACTGG

GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTC

CGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGCGGGAGGAAGGT

TTTAGGACTGTAAACCGCTTTTCTCAGGGAAGAAGATCTGACGGTACCTG
AGGAATCAGCATCGGCTAATTCCGTGCCAGCAGCCGCGGTAATACGGGAG
ATGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGTCCGCAGGCGGGCT
ATCAAGTCTGCTGTCAAAGCACACAGCTTAACTGTGGGGAGGCAGTGGAA
ACTGAAAGCCTAGAGTGCGGTAGGGGTAGGGGGAATTCCCGGTGTAGCG
GTGAAATGCGTAGATATCGGGAAGAACACCAGCGGCGAAAGCGCCCTAC
TGGGCCGCAACTGACGCTCATGGACGAAAGCTAGGGGAGCGAATGGGAT
TAGATACCCCAGTAGTC

**CD1**

CGGACGGGTGAGTAACGCGTGAGCAATTTGCCTGAAAGCCCGGGATAACT
CCGAGAAATCGGAGCTAATACCGGATGTGATCCGGGGGTGGCATCACCTC
CAGCTCAAAGTCGGGGACCGCAAGGCCTGACACTTTCAGATAAGCCCGCG
GCCTATCAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCTAAGACGGGTA
GCTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGTCCAG
ACACCTACGGGTGGCAGCAGTCGAGAATTTTTCACAATGGGGGAAACCCT
GATGGAGCGACGCCGCGTGGGGGATGAATGGCTTCGGCCCGTAAACCCCT
GTCATTTGCGAACAAACCTCGGTCTTGAATAGGGATCGAGCTGATAGTAG
CAGAAGAGGAAGGGACGGCTAACTCTGTGCCAGCAGCCGCGGTAAGACA
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GCAAGGTAAGTTCATGGTGAAATCTCCGGGCTCAACCCGGAAAATGCCCC
GAATACTATCTTGCTAGAGGACCGGAGGGGAGACTGGAATTCTCGGTGTA
GCAGTGAAATGCGTAGATATCGAGAGGAACACCAGTGGCGAAGGCGAGT

CTCTGGACGGTACCTGACGCTGAGGCACGAAAGCCAGGGGAGCAAATGG
GATTAGATACCCCAGTAGTC

**CD5**

CGGACGGGTGAGTAACGCGTGAGAATCTGCCCTCAGGAGGGGGATAACG
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GCCTGAGGATGAGCTCGCGTCTGATTAGCTAGTTGGTGGGGTAAGGGCCT
ACCAAGGCTTCGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTC
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GGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGTCCGCAGGCGGCCTC
GTAAGTCTGTCGTTAGAGCGTGGAGCTTAACTCCATTTCAGCGATGGAAA
CTGTGAGGCTTGAGTGTGGTAGGGGCAGAGGGAATTCCCGGTGTAGCGGT
GAAATGCGTAGATATCGGGAAGAACACCAGTGGCGAAGGCGCTCTGCTG
GGCCATAACTGACGCTCATGGACGAAAGCCAGGGGAGCGAATGGGATTA
GATACCCCAGTAGTC

**CD6**

CGGACGGGTGAGTAACGCGTGAGCAACCTGCCGTGAAGTGGGGGATAGC
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GACGCCAAAGCCGGGGCAACCTGGCGCTTCTCGAGGGGCTCGCGGCCTAT
CAGCTTGTTGGTGAGGTAACGGCTCACCAAGGCTATGACGGGTAGCTGGT

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GCGTCGCTCGAGGACTGTAGAGGGCATTGGAATTCACGGTGTAGCAGTGA
AATGCGTAGATATCGTGAGGAAGACCAGTGGCGAAGGCGAATGCCTGGG
CAGTTCCTGACACTGAGGCACGAAGGCCAGGGGAGCAAATGGGATTAGA
TACCCCAGTAGTC

**EF1**

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GACGCCAAAGCCGGGGCAACCTGGCGCTTCTCGAGGGGCTCGCGGCCTAT
CAGCTTGTTGGTGAGGTAACGGCTCACCAAGGCTATGACGGGTAGCTGGT
CTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGTCCAGACACCT
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GCGTCGCTCGAGGACTGTAGAGGGCATTGGAATTCACGGTGTAGCAGTGA
AATGCGTAGATATCGTGAGGAAGACCAGTGGCGAAGGCGAATGCCTGGG
CAGTTCCTGACACTGAGGCACGAAGGCCAGGGGAGCAAATGGGATTAGA
TACCCCAGTAGTC

**EF4**

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CAGCTTGTTGGTGAGGTAACGGCTCACCAAGGCTATGACGGGTAGCTGGT
CTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGTCCAGACACCT
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AATGCGTAGATATCGTGAGGAAGACCAGTGGCGAAGGCGAATGCCTGGG
CAGTTCCTGACACTGAGGCACGAAGGCCAGGGGAGCAAATGGGATTAGA
TACCCCAGTAGTC

**EF7**

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GCCTGGAGATGAGCTCGCGTCTGATTAGCTAGTTGGTGGTGTAAGGGACC
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GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTC
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GATGCAAGCGTTATCCGGAATGATTGGGCGTAAAGGGTCTGCAGGTGGAA
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AACTACAGAACTAGAGTGCGGTAGGGGCAAAAGGAATTCCTGGTGTAGC
GGTGAAATGCGTAGATATCAGGAAGAACACCGGTGGCGAAAGCGTTTTGC
TAGACCGTAACTGACACTGAGGGACGAAAGCTAGGGGAGCGAATGGGAT
TAGATACCCCAGTAGTC

**G1**

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GCGGTGAAATGCGTAGAGATCAGGAAGAACACCGGTGGCGAAAGCGCTT
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**G2**

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**G3**

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**G4**

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AAACTGAAGGTCTAGAGAGGGGCAGGGGCAAAGGGAATTCCTGGTGTAG

CGGTGAAATGCGTAGAGATCAGGAAGAACACCGGTGGCGAAAGCGCTTT

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ATTAGATACCCCAGTAGTC

**G5**

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CGGTGAAATGCGTAGAGATCAGGAAGAACACCGGTGGCGAAAGCGCTTT

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ATTAGATACCCCAGTAGTC

**G6**

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CGCCTAAAGAGAAGCTCGCGGCTGATTAGCTTGTTGGTAAGGTAAGAGCT
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GGGCTATTACTAACACTCAGAGACGAAAGCTAGGGTAGCAAATGGGATTA
GATACCCCAGTAGTC

**HI1**

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GCCTGGAGATGAGCTCGCGTCTGATTAGCTAGTTGGTGGTGTAAGGGACC
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GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTC
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GGTGAAATGCGTAGATATCAGGAAGAACACCGGTGGCGAAAGCGTTTTGC
TAGACCGTAACTGACACTGAGGGACGAAAGCTAGGGGAGCGAATGGGAT
TAGATACCCCAGTAGTC

**HI2**

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GACGCCAAAGCCGGGGCAACCTGGCGCTTCTCGAGGGGCTCGCGGCCTAT
CAGCTTGTTGGTGAGGTAACGGCTCACCAAGGCTATGACGGGTAGCTGGT
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ACGGGTGGCAGCAGTCGAGAATTTTTCACAATGGGGGAAACCCTGATGGA
GCGACGCCGCGTGGAGGATGAAGGTTTTCGGATTGTAAACTCCTGTCATT
AGAGAACAAGGCACACGGTTTAACTGGCCGTGTGTTGATAGTATCTGAAG
AGGAAGAGACGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGTCT
CAAGCGTTGTTCGGATTCATTGGGCGTAAAGGGTGCGTAGATGGCGATGT
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AATGCGTAGATATCGTGAGGAAGACCAGTGGCGAAGGCGAATGCCTGGG
CAGTTCCTGACACTGAGGCACGAAGGCCAGGGGAGCAAATGGGATTAGA
TACCCCAGTAGTC

**HI9**

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TC

**LM16**

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GATTAGATACCCCAGTAGTC

**LM40**

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ACCAAGGCGTCGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTC
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TAGACCGTAACTGACACTGAGGGACGAAAGCTAGGGGAGCGAATGGGAT

TAGATACCCCAGTAGTC

**JK25**

CGGACGGGTGAGTAACGCGTGAGAATCTGGCTCCAGGTCGGGGATAACA

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ACCAAGGCGTCGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACACTGG

GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTC

CGCAATGGGCGAAAGCCTGACGGAGCAATACCGCGTGAGGGAGGAAGGC

TCTTGGGTCGTAAACCTCTTTTCTCAAGGAAGAAGAAAGTGACGGTACTT

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CTGAAAGTCTGCTGTTAAAGAGTTTGGCTTAACCAAATAAAAGCGGTGGA

AACTACAGAACTAGAGTGCGGTAGGGGCAAAAGGAATTCCTGGTGTAGC

GGTGAAATGCGTAGATATCAGGAAGAACACCGGTGGCGAAAGCGTTTTGC

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TAGATACCCCAGTAGTC

**JK60**

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AACTACAGAACTAGAGTGCGGTAGGGGCAAAAGGAATTCCTGGTGTAGC

GGTGAAATGCGTAGATATCAGGAAGAACACCGGTGGCGAAAGCGTTTTGC

TAGACCGTAACTGACACTGAGGGACGAAAGCTAGGGGAGCGAATGGGAT

TAGATACCCCAGTAGTC

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