

A MOLECULAR INSIGHT INTO HARMFUL
ALGAL BLOOMS OF UTAH LAKE

by

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A dissertation submitted to the faculty of
The University of Utah
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Civil and Environmental Engineering

The University of Utah

December 2021

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The University of Utah Graduate School

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ABSTRACT

Harmful algal blooms (HABs) have become a significant issue in inland lakes across the US and other countries due to the external nutrient input from human societies and global temperature changes. Cyanobacteria (commonly known as green-blue algae) is the dominant phylum of bacteria that causes bloom and produces cyanotoxin, mostly during summer seasons. The reasons that trigger their bloom remain the focus of ecologists and limnologists. Microscopic techniques are traditionally applied to identify and quantify algae, while next-generation sequencing methods and the development of bioinformatics tools (e.g., QIIME) have significantly facilitated the study of HABs, which revealed the holistic identity of cyanobacteria and other flanking microbial community present in-situ.

Utah Lake is the largest freshwater lake located in Utah and experienced severe CyanoHABs in recent summers. Key questions are raised about the taxonomy, the nutrient limitation conditions and utilization strategies of the lake bacterial community. Moreover, bacterial community and metabolisms that could affect algal blooms' tipping point are scarcely studied in the lake. To tackle the existing issues with Utah Lake HABs, molecular methods are used to (1) study the holistic picture of bacteria community in the lake by sequencing the samples using 16S rRNA gene primers, (2) study the cyanobacterial community shift and the trends of N/P associated genes, (3) apply metagenomics or transcriptomics to have a whole community and individual study of

gene or gene expressions at bloom and non-bloom period.

Some key findings suggested that primary cyanobacteria genus shifted from pico-cyanobacteria before the bloom, towards N₂-fixers (*Aphanizomenon* and *Dolichospermum*) during the bloom, then non-fixers (e.g., *Planktotrix*) after the bloom. Microcystin and *Microcystis* were detected as cyanotoxins and toxin-producers. The relative abundance of bacterioplankton was negatively correlated with cyanobacteria. Cyanobacteria has activated several functions to tackle the relative nutrient starvation conditions. The N₂-fixation (encoded by *nif*) and high-affinity inorganic phosphorus assimilation (encoded by *pst*) gene were highly expressed during the bloom. Similarly, genes related to the poly-P storage and stringent response alarmone (p)ppGpp synthesis/hydrolysis were also activated during the bloom. These findings suggest that resolving cyanobacterial blooms probably takes more than just the reduction of external nutrient loadings.

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ACKNOWLEDGEMENTS

First, I would like to thank my advisor, Dr. Ramesh Goel, for providing me the opportunity to work under his guidance, all the options offered, and tremendous support. He is always optimistic and soothes us from headache research problems by suggesting thoughtful pathways for solutions. I am also grateful to EPA STAR program for funding our research.

I am grateful to my other committee members Dr. Michael Barber, Dr. Andy Hong, Dr. Alan Wilson, and Mr. Carl Adams for their time, very nice attitude, and valuable contributions to this dissertation.

I am grateful to all the labmates I met through Ph.D. life for their willingness to share and help. We also shared our feelings and encouraged each other to go through a hard time. I am also grateful to the staff in our department and the entire campus for their sincere help with my academic achievement.

Last but not least, I am grateful to my family back in China for their sincere support and care throughout the Ph.D. and friends I met in Utah for their emotional support.

CHAPTER 1

INTRODUCTION

Eutrophication of inland waters is a global environmental problem and a global cause for water quality impairment due to the input of excess nutrients from various point and nonpoint sources (Keck and Lepori, 2012). Global climate change has further exacerbated eutrophication in surface waters (Deng et al., 2014; Pimentel and Giani, 2014). Harmful algal blooms, abbreviated as HABs, often form a significant component of phytoplankton in eutrophic lakes (Heisler et al., 2008). The HABs mainly consist of cyanobacteria or blue-green algae, a photosynthetic prokaryote that has dominated the Earth since 3 million years ago (Golubic & Seong-Joo, 1999). Cyanobacteria (commonly known as blue-green algae) are gram-negative prokaryotic oxygenic cells that often form an integral part of harmful algal blooms (HABs) known as CyanoHABs (Paerl, 2014; Bertos-Fortis et al., 2016; Feng et al., 2016).

1.1 Project Rationale and Objectives

Cyanobacteria are regarded as complex organisms due to their vast adaptive strategies (Sciuto and Moro, 2016), which is likely a function of their presence in diverse environments (i.e., acidic, marine, freshwater, and terrestrial). How cyanobacteria form blooms and what factors trigger their growth over other phytoplankton has been the focus

of research of ecologists and limnologists for several decades. CyanoHABs have been anecdotally reported in most of the states in the USA (Figure 1.1) (Graham et al., 2017). Very little is known about the genomic content of different bloom-forming and toxin-producing cyanobacteria (Steffen et al., 2012). Although there are several environmental problems associated with CyanoHABs, species capable of producing toxic secondary metabolites, such as the hepatotoxin microcystin, are of particular concern as they have direct negative health implications to humans, pets, livestock, and aquatic food webs (Wiegand and Pflugmacher, 2005; Funari and Testai, 2008). Moreover, CyanoHABs can also cause severe economic losses. In the USA alone, CyanoHABs result in losses of recreational, drinking, and agricultural water resources that are worth ~\$2 billion annually (Dodds et al., 2009).

In light of recent thrusts aimed at identifying novel species of cyanobacteria and new forms of cyanotoxins, the need for new cyanobacterial identification and quantification techniques have been emphasized (Jiang et al., 2017). The current classification of cyanobacteria relies heavily upon morphological observations such as

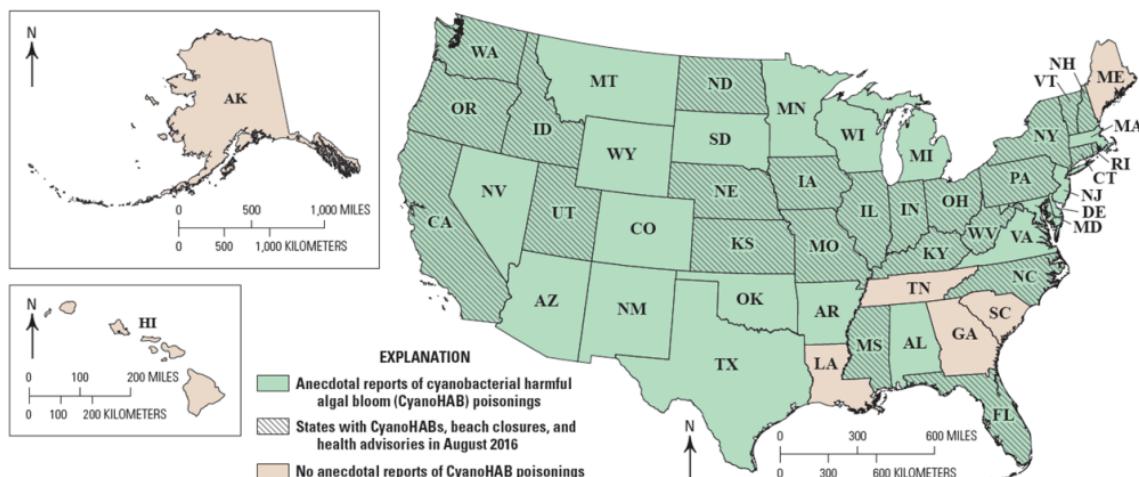


Figure 1.1 Algal blooms occurred at different USA states (USGS, 2016).

cell size, shape and arrangement (filamentous, colonial or single cells), coloration, and the presence of characters such as gas vacuoles and a sheath (Castenholz & Waterbury, 1989). However, typical microscope-based techniques are not adequate to track new species of cyanobacteria, given that many unicellular, non-spore-forming species of cyanobacteria lack distinctive morphological features (Robertson et al. 2001). Additionally, some diagnostic features, such as gas vacuoles, can show variation with different environmental or growth conditions and even be lost during cultivation (Park et al., 2013). For example, picocyanobacteria (<2 µm) are abundant in oceans with the genera *Prochlorococcus* and *Synechococcus*, but escape quantification and identification using conventional techniques because of their size (Diez et al., 2016). For example, HABs were recorded in Utah Lake in 2016 and 2017, and extensive monitoring by the Utah Division of Water Quality (UDWQ) showed the dominance of cyanobacteria belonging to *Aphanizomenon* without showing any evidence of picocyanobacteria. However, high throughput DNA sequencing usually detected the co-occurrence of *Prochlorococcus* or *Synechococcus* with other cyanobacterial communities (Scherer et al., 2017; Hattenrath-Lehmann et al., 2019). Hence, there is a solid need to devise holistic alternative quantification and identification methods.

Despite breakthroughs (Smith and Schindler, 2009; Paerl et al., 2011), there is a great degree of ambiguity about the factors that mediate CyanoHABs in inland waters. A key question facing surface water quality professionals and managers is: which nutrient(s) control CyanoHAB formation in surface waters? Observations for this issue are conflicting, especially when comparing nutrient limitations in freshwaters. Several investigations at lab and field scales have highlighted the importance of these two

nutrients, with differing opinions about which nutrient is generally limiting (Carpenter et al., 2005; Kolzau et al., 2014). The early study recognized P as the primary limiting nutrient in most lakes based on the stoichiometry of N and P in phytoplankton (Lewis and Wurtsbaugh, 2008). However, subsequent studies found that N was often the limiting nutrient in shallow eutrophic lakes, while the oligotrophic deep lake was mostly P limited (Downing and McCauley, 1992; Reynolds, 2006). Recent studies also recognized the dominance of cyanobacteria under low N/P ratios (Søndergaard et al., 2017; Isles et al., 2017). Generally, an N-limitation condition could result from nitrate lost to heterotrophs (e.g., denitrifiers) via denitrification (Chen et al., 2012; Holmroos et al., 2012), while the levels of P were determined by interactions between sediment and water column (Wu et al., 2017; Hogsett et al., 2019). Despite the bioassay that detects the N and P limitation of the lake (Schindler et al., 2008; Kolzau et al., 2014), mechanisms that drive the cyanobacterial activities are usually blurry from most of the study.

Utah Lake is the largest freshwater lake in the state of Utah, which has been impaired by high TP (>0.025 mg/L) and summer CyanoHABs (Psomas & SWCA, 2007). Filamentous cyanobacteria (e.g., *Aphanizomenon* and *Dolichospermum*) are some of the dominant genera identified recently from the Lake (UDWQ, 2016), implying possible N-fixing potentials. Apart from that, MCs and potential toxin-producing strains caused issues in recent years (UDWQ, 2016). Based on the characteristics and current study of the lake, I would like to propose the following three focuses and questions of research: (1) What are the overall ambient water quality, bacteria compositions, and synergy for cyanobacteria with other heterotrophic bacteria in Utah Lake during bloom and non-bloom forming periods? (2) Why would filamentous cyanobacteria dominant the lake? Is

it related to their N-fixation and P utilization pathways and the effects on other non-N fixing populations? And (3) What is the whole community genomic and transcript dynamics based on the metagenomic and metatranscriptomic analysis at the non-bloom and bloom period? In this regard, the following three objectives and tasks are formed.

1.2 Chapters and Objectives

The dissertation is divided into six chapters as follows:

- **Chapter 1: Introduction, Project Rational, Chapter Summary and Objectives.**

It sets out to respond to the following objective.

- 1) To provide general information, introduction, and objectives of the dissertation.

- **Chapter 2: Literature review.** It sets out to respond to the following objectives:

- 1) To provide general knowledge of surfacewater eutrophication, cyanobacteria and toxin production, triggering factors for cyanobacterial bloom, monitoring strategies, metabolic pathways and sites descriptions.

- 2) To place this doctoral study in the wider context of harmful algal bloom study.

- **Chapter 3: Dominance of Pico- and Filamentous Cyanobacteria at Utah**

Lake¹. It sets out to respond to the following objectives:

- 1) To study the holistic picture of the bacteria community in the lake by sequencing the water samples (16S rRNA sequencing) and assigning taxonomy.

¹ This chapter was previously published as Li, H., Alsanea, A., Barber, M., & Goel, R. (2019). *High-throughput DNA sequencing reveals the dominance of pico-and other filamentous cyanobacteria in an urban freshwater Lake*. *Science of the total environment*, 661, 465-480.

- 2) To measure the variants of environmental parameters at different periods and locations.
- 3) To analyze the correlations between surface bacterial community and water quality parameters.

- **Chapter 4: Microbial community successions and their dynamic functions during harmful cyanobacterial blooms in a freshwater lake².** It sets out to respond to the following objectives:

- 1) To study the bacterial community composition at surface water by (1) using amplicon sequencing to study the dominant bacterial communities and (2) applying QIIME 2 to analyze sequencing results.
- 2) To find the correlated gene and gene expression changes that imply the dominant metabolic pathways at various bloom stages by (1) using qPCR to target the functional genes, (2) predicting functions based on the taxonomy analysis using PICRUSt, and (3) quantifying gene expression for specific genes.
- 3) To investigate the correlations among nutrient utilization pathways, toxin-production, and the cyanobacterial community changes.

- **Chapter 5: The nitrogen and phosphate related stringent response of cyanobacterial and bacterioplankton community at different stages of a cyanobacterial bloom.** It sets out to respond to the following objectives:

- 1) To study metagenomics and metatranscriptomics of the lake community, especially the N and P related metabolism at the bloom and non-bloom periods.

² This chapter was previously published as Li, H., Barber, M., Lu, J., & Goel, R. (2020). *Microbial community successions and their dynamic functions during harmful cyanobacterial blooms in a freshwater lake*. *Water Research*, 185, 116292.

- 2) To study the key genes and metabolic pathways of dominant organisms (cyanobacteria and bacterioplankton) by constructing individual bacterial genomes.
- 3) To study the differential expression of samples at varying bloom periods and analyze the primary transcripts and metabolic pathways at different periods.
- **Chaper 6: Conclusions.** This chapter draws together the general conclusions and discussions of this study. It also contains general application and the suggestion for future studies.

Chapters 3 to 5 were written in manuscript format, contain an abstract, an introduction specific to the section, methodology, results, discussion, and references.

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CHAPTER 2

LITERATURE REVIEW

2.1 Surface Water Eutrophication

Excess nutrients, and other environmental conditions such as elevated temperatures support the excessive growth of phytoplankton, leading to eutrophication (Deng et al., 2014; Pimentel and Giani, 2014). Eutrophication is usually described when a body of water becomes overly enriched with minerals and nutrients, together with the excess growth of algae and phytoplankton. An algal bloom is defined as the rapid increase of algae in aquatic systems, which can cause severe outcomes such as oxygen depletion, fish die-offs, smelly odor, or discoloring water. The group of phytoplankton consists of free-floating algae, protists, and cyanobacteria, which are primary producers that use photosynthesis to convert inorganic carbon to organic carbon and generate oxygen (Grafton et al., 2013; Paerl and Otten et al., 2013; Paerl, 2014). Not all of the bloom of phytoplankton is harmful; harmful algal blooms are mainly the result of a type of microorganisms called cyanobacteria or “blue-green algae.” In the past, cyanobacteria were often mistaken for algae as they can both produce dense mats that impede aquatic lives’ activities. However, significant differences exist between cyanobacteria and common algae. In general, cyanobacteria (commonly known as blue-green algae) are gram-negative prokaryotic oxygenic cells that often form an integral part of harmful algal

blooms (HABs) known as CyanoHABs (Grafton et al., 2013; Paerl and Otten et al., 2013; Paerl, 2014; Bertos-Fortis et al., 2016; Feng et al., 2016). Although several environmental problems are associated with CyanoHABs, species capable of producing toxic secondary metabolites (known as cyanotoxins) are of particular concern (Wiegand and Pflugmacher, 2005; Funari and Testai, 2008). Cyanotoxins have been proven to have direct adverse health implications to humans, pets, livestock, and aquatic food webs, causing significant economic losses (Ferrão-Filho and Kozlowsky-Suzuki, 2011; Zanchett and Oliveira-Filho, 2013; Drobac et al., 2013).

2.2 Cyanobacteria and Toxin Production

The vast morphological and genetic diversity among cyanobacteria is one of the main challenges for practitioners and managers to manage CyanoHABs (Kormas et al., 2011; Schirrmeyer et al., 2013). Dated ~3 million years ago, cyanobacteria produced oxygen through photosynthesis to transit the earth's atmosphere from anoxygenic to oxygenic conditions (Schirrmeyer et al., 2011). With time passing by, the cyanobacteria population has evolved into some main groups. Based on morphology, cyanobacteria can mostly be grouped into picocyanobacteria, filamentous, and colonial cyanobacteria. Mainly, picocyanobacteria (e.g., *Synechococcus* and *Cyanobium*) is one of the essential organisms globally, in marine and freshwater systems, due to their crucial roles in aquatic primary production (Śliwińska-Wilczewska et al., 2018). Many filamentous cyanobacteria are typically capable of N fixation by cell differentiation. Under aerobic conditions, the vegetative cells conduct primary production activity, whereas the specialized cells, heterocysts, perform nitrogen fixation by utilizing nitrogenases

(Schindler et al., 2008; Paerl, 2017). Lastly, the colonial cyanobacteria (e.g., *Microcystis* and *Merismopedia*) contain species that commonly produce cyanotoxins. Additionally, the phylogenetic traits again divide cyanobacteria into orders of Chroococcales (*Microcystis*), Nostocales (*Aphanizomenon*), Synechococcales (*Synechococcus*), and Oscillatoriales (*Planktothrix*). Because of the close phylogenetic relationship, some cyanobacteria species (e.g., *Aphanizomenon flos-aquae* and *Dolichospermum flos-aquae*) could only be differentiated by observing under light microscopy (Rajaniemi et al., 2005; Stüken et al., 2005).

Among all the cyanobacterial species, the ones that produce cyanotoxins are of particular concern. In general, cyanotoxins can be primarily grouped into neurotoxins (targeting the nervous system), hepatotoxins (targeting the liver), or dermatotoxins (targeting the skin). Due to the toxicities of cyanotoxins, many states have regulated the cyanotoxin standards for drinking and recreational water. In 1998, The World Health Organization (WHO) released a provisional drinking water guideline for microcystin-LR of 1 µg/L, along with the regulations for other cyanotoxins. According to USEPA, cyanobacterial cell numbers and cyanotoxin concentrations higher than 100,000 cells/mL and 20 µg/L in recreational systems are considered of high health risk. The common gene clusters responsible for cyanotoxin production include *mcy* gene clusters for microcystin (Nishizawa et al., 2000), *cyr* gene clusters for cylindrospermopsins (Schembri et al., 2001), *sxt* gene clusters for saxitoxin (Kellmann et al., 2008) and *ana* gene clusters for anatoxin and anatoxin-a (s) (Rantala-Ylinen et al., 2011). The information for each cyanotoxin and correlated gene cluster is detailed in Table 2.1. Because of a detection level of fewer than 10^2 copies per mL, qPCR is more sensitive to detect toxin-producing

Table 2.1 Common Cyanotoxins and Functional Genes

Cyanotoxins	Target organs	Cyanobacteria genera	Functional genes or clusters
Microcystin	Cyclic peptides, liver	<i>Microcystis, Anabaena, Planktothrix, Phormidium</i>	mcy genes (two operons, <i>mcyA-C</i> and <i>mcyD-J</i>)
Cylindrospermopsin	Alkaloids, liver	Filamentous cyanobacteria, <i>Cylindrospermopsis, Aphanizomenon</i>	CYN genes (amidino transferase-CyrA, uracil ring formation-CyrG and CyrH,etc)
Saxitoxin	Alkaloids, Nerve synapse	Many freshwater filamentous, <i>Aphanizomenon</i>	sxt gene (31 ORFs, <i>sxtF</i> and <i>sxtM</i> genes, etc)
Anatoxin-a Anatoxin-a (s)	Alkaloids, Nerve synapse	<i>Anabaena, Oscillatoria, and Aphanizomenon</i>	ana gene clusters, <i>anaA, anaB -G</i>

strains at low concentrations than the microscopic method.

2.3 Factors Triggering CyanoHABs

The formation of algal blooms has been well correlated with anthropic activities and global climate changes as abiotic factors (Paul, 2008; Anderson, 2014; Wells et al., 2015). It is anticipated that increased water temperatures will accompany phytoplankton growth, as it influences mobility, nutrient uptake, photosynthesis, and other physiological processes (Beardall & Raven, 2004; Bissinger et al., 2008). The optimal and inhibition temperature threshold differs among species for biosynthesis (de Boer et al., 2005; Magaña & Villareal, 2006). Hence, the temperature changes favor the dominance of one genus instead of another in the lake. On the other hand, the characterization of aquatic systems' water quality, especially freshwater bodies, has long been described regarding limiting growth nutrients (Dodds et al., 1989; Smith et al., 1999). Nitrogen (N) and

Phosphorus (P) are recognized as the primary limiting nutrients for algal growth (Carpenter et al., 2005). It bears on the work of Redfield, who observed a molar ratio of 106:16:1 on average phytoplankton assimilations (Redfield, 1958). Early study reorganized P as the primary limiting nutrient in most lakes based on the stoichiometry of N and P in phytoplankton (Lewis and Wurtsbaugh, 2008). However, subsequent studies also found that N was often the limiting nutrient in shallow eutrophic lakes, while the oligotrophic deep lake was mostly P limited (Downing and McCauley, 1992; Reynolds, 2006). Nutrients act as a selection pressure because different species may favor various forms of nutrients (Chaffin & Bridgeman, 2014). The formation of blooms could also be affected by light, wind speed, turnovers, or stratifications, as well as other physicochemical parameters (Jeffrey et al., 1999; Peacock & Kudela, 2014). Additionally, many studies have addressed the influence of biotic factors such as grazer effects (Ventelä et al., 2002; Ger et al., 2016), fungal infection (Agha et al., 2018) and cyanophages (Yoshida et al., 2006; Ni and Zeng, 2016) on shaping community structures.

2.4 Monitoring Strategies

The microscopy method has long been applied as the primary way to identify and enumerate species (Utermohl 1958; Alvarez et al., 2013). Although it is specific to some extent, microscopic methods are considered to have problems of time-consuming, neglect of picoplankton, and no differentiation between toxic/nontoxic strains (Otsuka et al., 1999; Callieri, 2008; Whitton and Potts, 2012). Specifically, microscope-based identification of picocyanobacteria (e.g., *Synechococcus*), which are in the range of 0.2 to 2.0 μm and abundant in many cases (Ouellette et al., 2006; Callieri, 2008), is very

demanding. Furthermore, the counting and identification of filamentous cyanobacteria could be compute-intensive and inaccurate because many filamentous cyanobacteria (e.g., *Aphanizomenon*, *Dolichospermum*, *Anabaena*, *Planktothrix*, etc.) tend to present together and can form involute clusters (Tamulonis & Kaandorp, 2014).

All of the above issues make it difficult for environmental scientists to report the appearance of cyanobacteria and prevent early blooms. The next-generation sequencing and bioinformatics methods have been developed in recent years and demonstrated high efficiency and accuracy for environmental sample analysis. Genetic identification of cyanobacteria using DNA-based high-throughput sequencing has been proven to not only reveal the holistic identity of CyanoHABs but also provide information about other flanking microbial community presents in-situ (Woodhouse et al., 2016; Tromas et al., 2017; Parulekar et al., 2017; Scherer et al., 2017). Additionally, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (Langille et al., 2013) has been recently applied for the prediction of metabolisms in aquatic systems with relatively low cost (Aguinaga et al., 2018; Zhang et al., 2018). The N and P-related metabolisms can be accurately identified by KOs and metabolic pathways from KEGG. Metagenomics and transcriptomics are increasingly applied to study N and P-related metabolisms under nutrient stress (Harke et al., 2015; Harke et al., 2017; Affe et al., 2018; Lu et al., 2019). Additionally, it can be further utilized to analyze viruses, eukaryotes, and bacterial signaling transportations, which cannot be studied through 16S rRNA sequencing (Kimura, 2014; Uyaguri-Diaz et al., 2016; Tang et al., 2018).

2.5 Metabolic Pathways

As an ancient organism appeared on earth, cyanobacteria own unique characteristics, as mentioned earlier in the context. Apart from the secondary metabolites that produce cyanotoxins, other metabolic pathways are critical for the formation of blooms and dominance in the lake. One of the unique traits is a vital capability for nutrient utilization. For instance, to overcome summer nutrient-limiting conditions, cyanobacteria developed strategies such as N₂-fixation and high-affinity phosphate scavenging systems (Pho regulon) (Adams et al., 2008; Santos-Beneit, 2015). Except for a few N-fixers, most cyanobacteria utilize nitrate, ammonia, and simple organic compounds like urea, amino acids, and some nitrogen-containing bases (Herrero et al., 2001). Among all the nitrogenases, the best-known one is Mo nitrogenase, which is encoded by the structural genes *nifHDK* and prevalent in N₂-fixing organisms (Bothe et al., 2010). Additionally, N regulatory genes (e.g., *ntrA*, *ntrC*) and PII signal transduction proteins are widely spread in bacteria and regulate the N assimilations under N starvations (Hirschman et al., 1985; Herrero et al., 2004; Huergo et al., 2013). The Pho regulon includes members as the high-affinity inorganic P transport systems (encoded by *pst* genes) (Makino et al., 1988; Pitt et al., 2010), enzymes polyphosphate kinase (PPK) (Brown and Kornberg, 2004), exopolyphosphatase (PPX) (Gomez-Garcia et al., 2003) and others. Many species were detected to have them upregulated under P starvation conditions. For example, in low phosphorus regions of the lake, the genes associated with P scavenging in *Microcystis* were observed to be upregulated (Harke et al., 2015). Moreover, the gene cluster has been identified in *Synechococcus* sp. strain WH8102 (Su et al., 2003) and some *Nostocales*, such as *Anabaena* (Lu et al., 2019). Under high P

conditions, the expression of N fixation activity can also be enhanced (Harke et al., 2015). The up-regulation of these two gene clusters (N-fixation and Pho regulon) has been observed to alter the dominance of cyanobacteria communities and enhance the toxicity of blooms (Berversdorf et al., 2013; Harke et al., 2015; Lu et al., 2019). The P correlated metabolisms are even more complicated to study, as many phosphorus contained gene/gene products in cells are tightly correlated with carbohydrates assimilations (Harke et al., 2012; Harke & Gobler, 2013) or the formation of heterocyst (Summers et al., 1995; Zhang et al., 2013; Hood et al., 2016). However, studies are still scarce for a full picture monitor of CyanoHABs successions at the metabolic level.

In addition to activating the above metabolism, bacteria can also activate stringent response regulatory systems, changing the cell conditions from fast growth to growth arrest (Masuda 2012). The alarmones involved in stringent response are guanosine tetraphosphate and guanosine pentaphosphate (collectively referred to as (p)ppGpp) (Hsieh & Wanner, 2010). The(p)ppGpp has been proved to be sensitive to many types of stress, including but not limited to light-dark (Puszynska & O'Shea, 2017), nutrition deprivation (Jain et al., 2006), heat shock (Schäfer et al., 2002), and oxidative stress (Fritsch et al., 2020). Recently, more works have brought to light links between the production of (p)ppGpp and N/P starvation response. For instance, close relationships were found between the activation of Pho regulon and (p)ppGpp (Lamarche et al., 2008; de Almeida et al., 2015). Additionally, it seems that stringent response is widely distributed in cyanobacteria and responded to N limitation (Atkinson et al., 2011; Jin et al., 2020). For non-N fixers, *Microcystis* upregulated stress-related genes and exerted protective effects by promoting colony formation and reducing oxidative damage (Jin et

al., 2020). The ppGpp commonly accumulated at the initial stage of heterocyst formation for N-fixers (Zhang et al., 2013). Regardless, studies are still lacking to study the larger scale's stringent response during harmful cyanobacterial blooms. Whether the ppGpp production is activated during a harmful cyanobacterial bloom and its associations with the N and Pi starvation metabolisms is still questioned.

2.6 Overview of Sampling Site

Utah Lake is the largest natural freshwater lake in the western United States, with a maximum length of 24 miles and a maximum width of 13 miles (Figure 2.1) (Hogsett et al., 2019). Its surface area is roughly 145 square miles and has nearly a million acre-feet (902,400 ac-ft). It is a shallow lake with an average depth of approximately 9-10 feet during standard reservoir operating conditions and primarily contains calcium-rich sediments. Utah Lake does not undergo seasonal stratification/turnover. Roughly 42% of the water is lost to evaporation before discharging to the Jordan River, which drains to the terminal Great Salt Lake (Psomas & SWCA, 2007). It is considered hypereutrophic in terms of its trophic status, and extreme algal blooms appear in the late summer and fall (Psomas & SWCA, 2007).

With climate change and population increase, the shallow Utah Lake has been experiencing extreme algal blooms in the summer in recent years, with the detected cyanobacterial numbers have gone up to 36 million cells per mL. Moreover, toxin-producing strains were supposed to present in the lake, as cyanotoxins were detectable during blooms. According to the local environmental agency, Utah Lake was detected to have microcystin concentrations above the recreational threshold of 20 µg/L. The lake

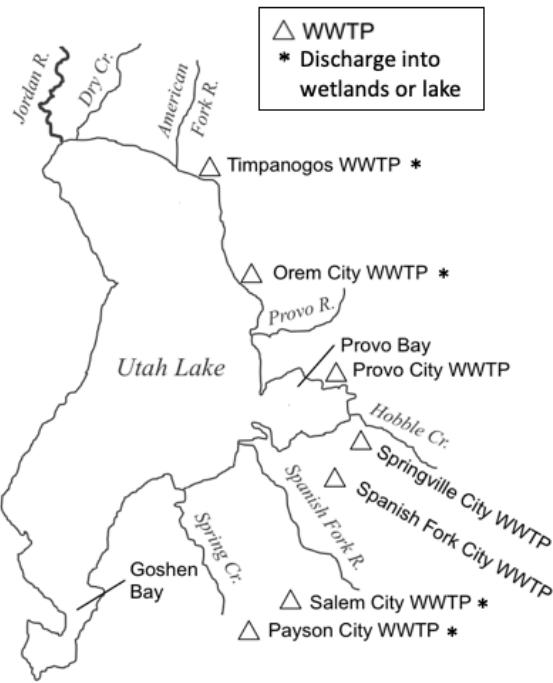


Figure 2.1 Utah Lake schematic map.

was shut down several times during summers for recreational and agricultural usages. It is expected that the population increase over the next 40 years will add to more nutrient loads associated with stormwater, municipal, and industrial wastewater discharges (Utah Foundation, 2014).

The primary concern of Utah Lake's eutrophication is related to overloading phosphorus into the lake. In recent years, over 75% of the 297.6 tons/year of phosphorus loading into the lake was attributed to WWTP discharges (Psomas & SWCA, 2007). Eight wastewater treatment plants near the eastern shore discharge effluent to tributaries (Powell Slough, Mill Race Creek, Spring Creek, Dry Creek, Benjamin Slough, and Minnie creek) (Figure 2.1) and wetlands or directly into the lake. The lake also acts as a sink for over 70% of the phosphorus loading, with only 30% (83.5 tons/year) being

known to exit the lake via the Jordan River. Low phosphorus concentrations in the water column are believed to associate with a high amount of calcium in the sediment (Brimhall & Merritt, 1981). It primarily exists in the forms of phytoplankton and sestonic matter during the summer months (Psomas & SWCA, 2007). Thus, total phosphorus concentrations in the Utah Lake water column are typically found to be higher than 0.025 mg P/L, which is a pollution indicator of excessive phosphorus entering or sediment phosphorous fluxing into the lake (Psomas & SWCA, 2007). It is estimated that the internal P-cycling was supported by P-rich sediment (up to 1767 mg P/kg dry mass) and continuous external loading. This accounted for 1,500 tons P per year, five times higher than the external P loading (Hogsett et al., 2019). Due to the rich sediment P and internal recycling, combating eutrophication in Utah Lake would take time.

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CHAPTER 3

DOMINANCE OF PICO- AND FILAMENTOUS CYANOBACTERIA AT UTAH LAKE

3.1 Abstract

The current study presents findings related to algal blooms in a fresh water lake, which has been experiencing severe cyanobacterial blooms (CyanoHABs). Primarily, picocyanobacteria belonging to the genus *Synechococcus* and filamentous cyanobacterial group belonging to *Aphanizomenon* and *Dolichospermum* dominated top water column during non-bloom and bloom periods respectively. The dominance of *Synechococcus* in early summer informs that blooming in Utah Lake starts in early summer and then later is taken over by other bloom-forming cyanobacteria, such as species belonging to the genus *Aphanizomenon*. A strong negative correlation ($r = -0.9, p < 0.001$) was found between the occurrence of *Aphanizomenon* and *Synechococcus* which correlates very well with the fact that the blooms of these two different cyanobacteria never coexisted. The predominance of cyanobacteria in 2017 was attributed more to temperature ($r = 0.18, p < 0.001$).

The Actinobacteria was negatively correlated with primary production and high chlorophyll *a* concentration. *Flavobacterium* and *Limnohabitans* were the main phytoplankton colonizers and predators detected that could secrete extracellular

enzymes to degrade algal exudates (such as proteins and polysaccharides). Additionally, cyanotoxins producers *Microcystis aeruginosa* and *Planktothrix* accounted for up to 12.43% and 7.04% of total cyanobacteria abundance during blooms. The relative abundance of chloroplast reads was overall lower than the cyanobacteria reads, except for the May 5th sampling in 2017. There was interannual variability in the bloom-associated heterotrophic bacterial populations, but these populations were consistent with bloom-associated bacterial populations found in other lakes. Community diversity analysis for both Shannon and Simpson indices indicated lower community diversity during the bloom period. The beta diversity conducted by PCoA and UPGMA trees suggested the significant temporal rather than spatial impacts on shaping the phytoplankton community structures during the summer season.

3.2 Keywords

Harmful algal blooms, high-throughput sequencing, filamentous cyanobacteria, picocyanobacteria, bacterioplankton, environmental factors.

3.3 Introduction

Excess nutrients, along with other environmental conditions such as elevated temperatures, support the excessive growth of phytoplankton, leading to eutrophication (Deng et al., 2014; Pimentel and Giani, 2014). Phytoplankton—which consists of free-floating algae, protists, and cyanobacteria—are primary producers that use photosynthesis to convert inorganic carbon to organic carbon and generate oxygen (Grafton et al., 2013; Paerl and Otten, 2013; Paerl, 2014). Characterization of aquatic

systems' water quality, especially freshwater bodies, has long been described in terms of limiting growth nutrients (Dodds et al., 1998; Smith et al., 1999). Nitrogen (N) and Phosphorus (P) are two vital nutrients that limit the growth of terrestrial and aquatic plants (Elser et al., 2007; Conley et al., 2009; Paerl, 2014; Dodds and Smith, 2016). Excess growth of algae and micro-algae phytoplankton causes oxygen depletion in the water column following the decay of algal biomass (Anderson et al., 2002).

Cyanobacteria (commonly known as blue-green algae) are gram-negative prokaryotic oxygenic cells that often form an integral part of harmful algal blooms (HABs) known as CyanoHABs (Grafton et al., 2013; Paerl and Otten, 2013; Paerl, 2014; Bertos-Fortis et al., 2016; Feng et al., 2016). Although there are several environmental problems associated with CyanoHABs, species capable of producing toxic secondary metabolites (known as cyanotoxins) are of particular concern (Wiegand and Pflugmacher, 2005; Funari and Testai, 2008). Cyanotoxins have been proven to have direct negative health implications to humans, pets, livestock, and aquatic food webs (Ferrão-Filho and Kozlowsky-Suzuki, 2011; Zanchett and Oliveira-Filho, 2013; Drobac et al., 2013). Moreover, CyanoHABs can also cause serious economic losses (Hoagland et al., 2002; Hoagland and Scatasta, 2006). In the United States alone, CyanoHABs result in losses of recreational, drinking, and agricultural water resources worth ~\$2 billion annually (Dodds et al., 2009). Furthermore, CyanoHABs also disturb food chain dynamics and can cause unwanted changes in ecosystem processes (Havens, 2008; Rigosi et al., 2014).

The vast morphological and genetic diversity among cyanobacteria is one of the main challenges for practitioners and managers to manage CyanoHABs (Kormas et al.,

2011; Schirrmeister et al., 2013). Microscopic techniques are still being used to identify and enumerate many planktonic species (Utermöhl, 1958; Álvarez et al., 2013). Although these methods are specific to some extent, they are time-consuming and require specialized training (Whitton and Potts, 2012). Specifically, Microscope-based identification of picocyanobacteria (e.g., *Synechococcus*), with an average size range of 0.2 to 2.0 µm (Callieri, 2008; Ouellette et al., 2006), is very demanding. Furthermore, the counting and identification of filamentous cyanobacteria could be compute-intensive and inaccurate, because many filamentous cyanobacteria (e.g., *Aphanizomenon*, *Dolichospermum*, *Anabaena*, *Planktothrix*, etc.) tend to present together and can form involute clusters (Tamulonis and Kaandorp, 2014). It is also difficult to differentiate between toxin- and non-toxin-producing strains using microscopy (Otsuka et al., 1999). Additionally, cyanobacteria co-flourish with algae and other heterotrophic bacteria populations in surface waters (Buchan et al., 2014; Louati et al., 2015; Ramanan et al., 2016). The interaction dynamics among them may inform intrinsic reasons for CyanoHABs formation, which is challenging to understand by solely studying cyanobacteria phylum.

Genetic identification of cyanobacteria using DNA-based high-throughput sequencing has been proven to not only reveal the holistic identity of CyanoHABs, but also provide information about other flanking microbial community members present in-situ (Woodhouse et al., 2016; Tromas et al., 2017; Parulekar et al., 2017; Scherer et al., 2017). So far, quite a few studies have applied high-throughput sequencing for studying cyanobacteria along with other bacterioplankton (Woodhouse et al., 2016; Tromas et al., 2017; Berry et al., 2017; Parulekar et al., 2017; Scherer et al., 2017). However, the

interactions among harmful CyanoHABs, heterotrophic bacterioplankton, and environmental parameters in freshwater systems still required further interpretation (Beversdorff et al., 2013; Berry et al., 2017; Scherer et al., 2017).

The primary objective of this study was to decipher the cyanobacterial composition with the aim of “nothing left behind of the planktonic microbial community” and to correlate this composition with water quality parameters. This overarching objective was accomplished by sampling both nearshore and deepwater locations around Utah Lake during the summers of 2016 and 2017. Utah Lake is the largest freshwater lake in the state of Utah, which has been listed as impaired due to the excess total phosphorus ($TP > 0.025 \text{ mg/L}$). Utah Lake, with a long history of eutrophic status, has been experiencing frequent CyanoHABs in recent years (Strong, 1974). Utah Lake received various discharges of nutrients from point and nonpoint sources with tremendous population growth occurring in its watershed. Hence, Utah Lake is a good model system to study and is representative of many other shallow lakes around the globe. This manuscript reports results from this study, which employed Illumina sequencing (16S rRNA V4 region) to decipher the linkages among bacterial groups and with water quality parameters. This study presents significant findings in the context of freshwater algal blooms in high altitude lakes in arid climates.

3.4 Materials and Methods

3.4.1 Details of the Surface Water Body and Study Sites

Utah Lake was the model water body that was sampled during the summers of 2016 and 2017. Located near the city of Provo, Utah Lake is the largest freshwater lake in

the state of Utah. It is located in Utah Valley and has a surface area of 376 km² and an average depth of 3 m (PSOMAS and SWCA, 2007). Due to its shallow depth, the lake undergoes quick turnovers, and the water column is generally well-mixed. Due to the large surface area of the lake, generalizing the water quality to a specific attribute is a challenge. According to the Utah Division of Water Quality (UDWQ), Utah Lake experienced a heavy algal bloom on July 14th, 2016, where cyanobacterial cell counts were the highest ever measured. Utah Lake was closed to the public on July 15th, 2016 to avoid human health risk as cyanobacterial concentrations reached 36 million cells per mL.

Surface water sampling was conducted at four sampling sites during the summer of 2016 (Saratoga Spring, Lindon Marina, Mouth of Provo Bay, and Mouth of Goshen Bay). In the summer of 2017, three additional sampling sites (with buoys installed by the UDWQ) were included (Bird Island Buoy, Provo Buoy, and Vineyard Buoy) (Figure 3.1). Site locations were chosen based on UDWQ's regular sampling sites. The Lindon Marina, Vineyard Buoy, Provo Buoy, and Mouth of Provo Bay sites are located near major inlet tributaries. Sites located further offshore include Saratoga Springs, Mouth of Goshen Bay, and Bird Island Buoy. Provo Bay is a shallow eutrophic bay located on the east side of the lake. Its shallow and relatively stagnant nature makes it a concern for HAB origins. Several replicates of water samples were collected from the top 10-cm water column and mixed together to get approximately 1-L surface water sample for each site on a monthly basis from May to August in HDPE sampling bottles following the Standard Operating Procedure for collection of lake water samples by Utah's Division of Water Quality (UDWQ, 2014). Samples for Chlorophyll a (Chl a) were collected in opaque plastic bottles (wrapped with aluminum foil) to prevent Chl a degradation by



Figure 3.1 Geographic locations of sampling sites in 2016 (4 sites) and 2017 (7 sites). The sampling sites are Lindon Marina (LM), Saratoga Spring (SS), Mouth of Goshen Bay (MGB), and Mouth of Provo Bay (MPB) for 2016. Three additional sites with buoys sampled in 2017 include Vineyard Buoy (VB), Provo Buoy (PB), and Bird Island Buoy (BIB).

light. In 2016, Utah Lake experienced a HAB event that started around the middle of July. Hence, water samples were collected twice during July bloom.

3.4.2 Water Quality Monitoring

Temperature, pH, and Total Dissolved Solids (TDS) were measured using an on-field environmental monitoring system (YSI SONDE 600 XL). The collected water samples were immediately transferred on ice to the Environmental Engineering and Microbiology Lab at the University of Utah for further analysis. The sampling of seven

sites across the Utah Lake typically took nearly 4-h. For Chl a, 150 mL sampled water was filtered through 0.45 µm filter papers (HPLV 4700) and was kept at –20 °C for no longer than 2 weeks before analysis. Chl a was measured spectrophotometrically and corrected for pheophytin after extraction with acetone solution, following the standard methods of water and wastewater (Chlorophyll) by the American Public Health Association (Apha, 1999). Nutrients (nitrate-N, nitrite-N, and orthophosphate-P) were analyzed by Ion Chromatography (IC) (Metrohm 883 Basic IC plus) for anions detection (EPA method 300) (Pfaff, 1993). The ion sample preparation and preservation were according to EPA method 300 (Pfaff, 1993). The dissolved organic carbon was measured by the standard carbonaceous biochemical oxygen demand (cBOD₅) bottle test (Method EPA 450.1).

3.4.3 Genomic DNA Extraction, Quality Control, and Sequencing

DNA was extracted from surface water samples using the PowerWater DNA isolation kit (Qiagen Inc., Valencia, California) according to manufacturer's instructions. Genomic DNA concentrations were measured using a Thermo NanoDrop 2000c at 260/280. The samples with 260/280 ratios higher than 1.80 were used for further analysis. Each DNA sample was normalized to have a final concentration of 20 ng/µL for a total volume of 20 µL before sending the DNA sample to the core facility for sequencing. The V4 region of the 16S rRNA gene was amplified using universal bacterial/archaea region-specific primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTCTAAT-3') (Caporaso et al., 2011). A unique eight-base barcode was prepared for each sample. To perform the PCR amplification, each reaction

contained 14 µL Qiagen HotStar Taq master mix (Qiagen Inc., Valencia, California), 9 µL nuclease free water, 1 µL of each 5 µM primer, and 1 µL of the template. Reactions were performed on an ABI Veriti Thermos Cycler (Applied Biosystems, Carlsbad, California) under the following thermal profile: 95 °C for 5 min, then 25 cycles of 94 °C for 30 s, 54 °C for 40 s, 72 °C for 1 min, followed by a final extension at 72 °C for 10 min.

Amplification products were visualized with eGels (Life Technologies, Grand Island, New York). They were then pooled in an equimolar fashion. Each pool size was selected in two rounds using SPRIselect (Beckman Coulter, Indianapolis, Indiana) at a ratio of 0.7. Size-selected pools were then run on a Fragment Analyzer (Advanced Analytical) to assess the size distribution, quantified using a Qubit 2.0 fluorometer (Life Technologies), and loaded on an Illumina MiSeq (Illumina, Inc. San Diego, California) 2 × 300 flow cell at 10 pM.

3.4.4 Sequencing Data Analysis

For sequence and statistical analysis, two different approaches were used, which required two separate processing of trimmed and quality filtered sequences. After the quality filtering of raw sequences, operational taxonomic unit (OTUs) formation was done using the UPARSE algorithm (Edgar, 2013), and taxonomic classification was performed using the USEARCH global alignment (Edgar, 2010) against the NCBI 16S rRNA gene database. Poor sequences such as those with short reads, ambiguous bases, and mismatches with primers were removed from the analysis using the UCHIME chimera detection software executed in de novo mode (Edgar et al., 2011; Kuczynski et

al., 2012). Raw reads were trimmed, and paired-end joined using the forward and reverse reads in a FASTQ format merger (Zhang et al., 2013). For denoising, reads were trimmed to have a Phred quality score higher than 25. Chimera checking was conducted using UCHIME chimera detection software executed in de novo mode (Edgar et al., 2011). The denoised and chimera-checked reads were grouped into OTUs using the UPARSE algorithm (Edgar, 2013). Taxonomic identification was performed by running through the USEARCH global alignment (Edgar, 2010) against the NCBI 16S rRNA gene database. Files generated contained taxonomic levels of kingdom, phylum, class, order, family, genus, and species.

Different diversity indices were calculated using the QIIME pipeline version 1.9.1 with the reference of a mapping file that contained unique barcodes for each sample (Caporaso et al., 2010). The quality-filtered fastq file containing good sequences was used as an input file in QIIME. Basically, QIIME calculates OTUs and performs taxonomic classification first before calculating different diversity indices. After quality control, the quality-filtered sequences were clustered into Operational Taxonomic Units (OTUs) at a 97% similarity by applying UCLUST (Edgar, 2010). After picking the most representative sequence from each OTU, QIIME used a default UCLUST classifier to assign the taxonomy against the Greengenes database (GG V13.8) (DeSantis et al., 2006). The alignment of representative sequences was performed using PyNAST (Caporaso et al., 2009) for the purpose of phylogenetic and diversity analysis. In previous studies, chloroplast 16S rRNA genes allowed a preliminary classification of phototrophic eukaryote (Kong et al., 2017; Scherer et al., 2017). The inferred taxonomy for the eukaryotic group only was classified to order level due to the resolution of the reference

database, which is a default database applied in QIIME.

Both the alpha and beta diversities were calculated using QIIME version 1.9.1. In alpha diversity, the rarefaction curves for observed OTUs and indices (Chao1, Shannon, Simpson, and Good's Coverage) were generated to evaluate the sequencing depth and community diversities. It is noted that QIIME calculates Shannon Index using log base 2 rather than natural log, which can be considered as Shannon entropy with units of "bits". In beta diversity, QIIME performs a jackknifing analysis by choosing and comparing a random number of sequences from each sample. Differences among samples were analyzed using a hierarchical cluster tree created by the Unweighted Pair Group Method with Arithmetic mean (UPGMA). Three-dimensional principal coordinates analysis (PCoA) plots were obtained based on the weighted and unweighted Unifrac using the Emperor tool. Both the alpha and beta diversities were calculated based on an OTU table at a 97% similarity level.

3.4.5 Data and Statistical Analysis

The relative abundance of bacterial sequences at the phylum and genus levels (derived from NCBI) was plotted as bar graphs. NCBI was used as a reference database because it provided a better taxonomic resolution than Greengenes for the current study. Venn diagrams were plotted to compare the unique and similar OTUs at the phylum and genus levels using R package "eulerr" (R Development Core Team, 2008).

Principal Component Analysis (PCA) was performed to observe the directions of maximum variations through the dominant bacterial communities and water quality parameters using the R package "FactoMineR" and "Factoextra." Five principal

components accounting for the highest proportion of variances were calculated. Biplots were plotted for the visualization of the two most significant principal components Dimension 1 (Dim 1) and Dimension 2 (Dim 2). Spearman correlation analysis was further applied to find the correlations between dominant bacteria phyla/cyanobacteria groups (e.g., picocyanobacteria, filamentous, colonial, and toxin-production cyanobacteria groups) and environmental factors. Corrplots were made through R package “Corrplot” to show the correlation coefficients and *p*-values. All statistical analyses were conducted in QIIME 1.9.1 VirtualBox and R studio 1.1.419.

3.5 Results

3.5.1 The Dynamics of Environmental Factors

During the Summer Seasons

Water temperatures were highest during the months of July and August and were below 20 °C in early May. The temperature increased to 23–25 °C in 2016 and 26–29 °C in 2017 during late summer. The pH was measured to be highest in July during both years, which is an important indicator of primary production. Provo Bay had higher primary production rates with pH values of 9 or above and measured Chl *a* values ten times higher in July (20–230 µg/L) than the Chl *a* values measured in early May and August. Three DWQ buoy sites also reported Chl *a* up to 50–60 µg/L in July and usually up to 10–30 µg/L in the other three months (iUTAH GAMUT data, 2017). According to the trophic state classification of lakes (Nürnberg, 1996), Utah Lake could be classified as a mesotrophic to eutrophic lake in May, June, and August and a hypereutrophic lake during July or blooms, based on the sampling and buoy data.

Different patterns of dissolved nitrate-N ($p < 0.05$, ANOVA) were observed in 2016 and 2017. In 2016, the dissolved nitrate-N and orthophosphate-P concentrations were significantly higher during the bloom than during other months. Higher concentrations of nitrite-N were also discovered during the bloom. Comparatively, nitrate-N decreased from 0.30 mg/L to almost below detection limits from June to July in 2017. In 2016, nitrate-N concentrations did follow a specific trend and fluctuated across sampled sites across sampling periods. Nitrite-N concentrations were the highest during the bloom in July of 2016. Orthophosphate-P concentrations were the highest during the bloom; however, Orthophosphate-P concentration still remained relatively elevated in August in comparison to orthophosphate-P concentrations in May and June of 2016. The decreased nitrate-N: orthophosphate-P ratios revealed that nitrate was highly consumed during the 2017 bloom. The highest carbonaceous biochemical oxygen demand (cBOD₅) values were measured in July 2017 when HAB and dissolutions of massive phytoplankton debris occurred.

3.5.2 Sequencing Quality Control and Overall

Community Diversity Indices

Microbial community diversities and phylogenetic structures of the different water column lake samples were analyzed by Illumina high-throughput sequencing. A total of 16 and 33 samples were sequenced in 2016 and 2017, respectively. High-throughput sequencing yielded 674,477 and 859,990 raw sequences for 2016 and 2017, respectively. Finally, only 7 sequences in 2016 and 66 sequences in 2017 did not pass quality filtering after the initial removal of questionable reads. More than 99% sequences

had an average read length of 250 bp. The total number of assigned reads ranged between 6681 and 54,553, with a mean of 32,575 for all samples sequenced (Table 3.1). The sequence number of each sample was normalized, and after taxonomic classification, 2630 and 1434 OTUs were identified in 2016 and 2017, respectively (Table 3.1) with a threshold of 0.95. All rarefaction curves tended to approach the saturation plateau (Figure 3.2), signifying that a reasonable number of individual samples had been taken. As for the richness and diversity indices, the mean values were 6962 and 3221 for Chao1, 7.00 and 6.77 for Shannon, and 0.961 and 0.961 for Simpson in 2016 and 2017, respectively (Table 3.1). Specifically, no significant change in the Chao1 Index was observed during the bloom period. The Shannon Index ranged from 6 to 9 during both years. The exceptions were two sites in 2016 (July 26th) and three sites in 2017 (July 11th) that had a Shannon Index below 6. Similarly, the Simpson diversity (1-D) ranged from 0.9 to 1, except for two sites in 2017 (July 11th) that had indices below 0.9. The above results generally indicate a trend of lower community diversity but greater richness during the bloom period. Good's Coverage (above 0.93 for all of the samples) suggested sufficient sequencing depth and coverage of OTUs (Table 3.1). Finally, the Venn diagrams showed a shared 17 phyla and 13 cyanobacteria species between two years (excluding any chloroplast 16S rRNA sequences) (Figure 3.2).

3.5.3 Overall Bacterial Taxonomy at the Phylum Level

Taxonomic classifications of the bacterial communities present in the lake water column at the phylum level are shown in the top panel in Figure 3.3 for 2016 and Figure 3.4 for 2017, respectively. At the phylum level, the overall microbial community was

Table 3.1 Alpha Diversity Indices – 2016 and 2017

2016 Parameters							
Month	Site	Reads	OTUs	Chao1	Shannon	Simpson	Coverage
June 30 th	MPB	36699	1336	4889.55	0.97	6.39	0.97
	MGB	39715	2739	5965.78	0.95	6.72	0.96
	LM	39676	2671	6206.09	0.97	7.02	0.96
	SS	33535	1144	4324.21	0.98	6.32	0.98
July 20 th	MPB	42919	1887	7042.35	0.96	7.06	0.97
	MGB	44451	3762	10117.82	0.97	7.53	0.95
	LM	54553	3031	7299.11	0.96	7.05	0.97
	SS	30015	3407	7839.60	0.99	8.73	0.93
July 26 th	MPB	52827	2542	7367.07	0.93	5.53	0.97
	MGB	52926	2900	7715.14	0.97	6.56	0.96
	LM	37637	2178	5912.22	0.93	5.67	0.96
	SS	39367	2304	6065.03	0.95	6.22	0.96
Aug 4 th	MPB	43097	3345	7355.07	0.98	7.65	0.95
	MGB	50862	3354	9421.23	0.95	6.51	0.96
	LM	33928	2642	6634.67	0.95	6.57	0.95
	SS	42263	2830	7240.06	0.96	6.84	0.96
2017 Parameters							
Month	Site	Reads	OTUs	Chao1	Shannon	Simpson	Coverage
May 5 th	LM	30259	1546	3180.76	0.98	6.70	0.97
	SS	29984	856	2041.15	0.97	6.17	0.98
	VB	36359	2530	6107.89	0.98	7.15	0.96
	PB	47681	2241	4798.56	0.97	6.58	0.97
	MPB	25176	1222	3226.26	0.96	6.45	0.97
	BIB	21808	1321	2812.39	0.96	6.38	0.96
	MGB	36790	1679	3428.63	0.97	6.63	0.97
June 1 st	LM	2551	6	16.00	0.00	0.03	1.00
	VB	810	5	5.00	0.02	0.10	1.00
	SS	24543	1289	2870.84	0.98	6.82	0.97
	MGB	25239	1572	3376.06	0.97	6.63	0.96
	BIB	24377	1433	2806.64	0.97	6.67	0.97
	MPB	25039	1803	3527.69	0.98	7.44	0.96
	PB	6681	804	1740.01	0.98	7.37	0.94
June 15 th	LM	41487	2030	3980.72	0.97	6.77	0.97
	VB	20317	822	1588.51	0.97	6.14	0.98
	BIB	16251	1640	3321.57	0.97	7.25	0.94
	MPB	22620	1709	3476.39	0.97	7.15	0.96
	PB	24755	1212	2398.01	0.98	6.86	0.97
July 11 th	LM	23511	2014	3969.04	0.98	7.49	0.95
	VB	35981	1366	2753.49	0.87	5.61	0.98
	SS	15977	1604	3826.11	0.96	6.90	0.94
	MGB	41387	1504	3269.75	0.91	6.06	0.98
	BIB	23595	1214	2643.70	0.88	5.69	0.97

Table 3.1 Continued

Month	Site	Reads	OTUs	Chao1	Shannon	Simpson	Coverage
July 11 th	MPB	29688	1834	3792.22	0.94	6.73	0.97
	PB	27307	896	2115.68	0.92	5.59	0.98
Aug 3 rd	LM	26998	1578	3066.37	0.97	6.78	0.97
	VB	25692	1651	3565.01	0.98	7.14	0.96
	SS	20797	820	1732.20	0.94	6.18	0.98
	MGB	37616	1744	3329.23	0.98	6.85	0.98
	BIB	24758	1444	2792.17	0.97	6.69	0.97
	MPB	37377	2667	5971.19	0.98	7.70	0.96
	PB	26513	1257	2337.63	0.98	6.89	0.98

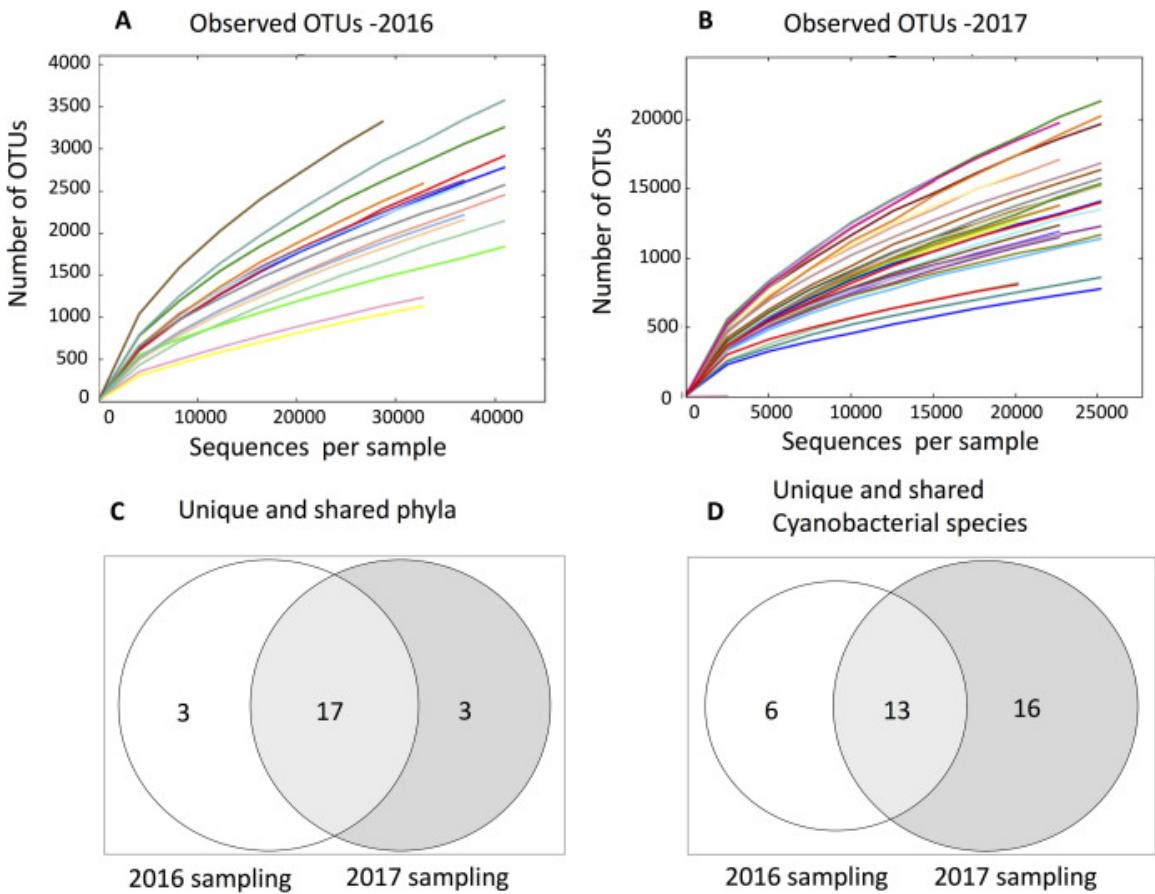


Figure 3.2 Observed OTUs clustered at a 97% sequence similarity. (A, B) Refraction analysis of observed OTUs from Illumina V4 MiSeq sequence reads in 2016 and 2017, respectively. (C) Unique and shared phyla between 2016 and 2017. (D) Number of unique and shared cyanobacteria genera or species between 2016 and 2017.

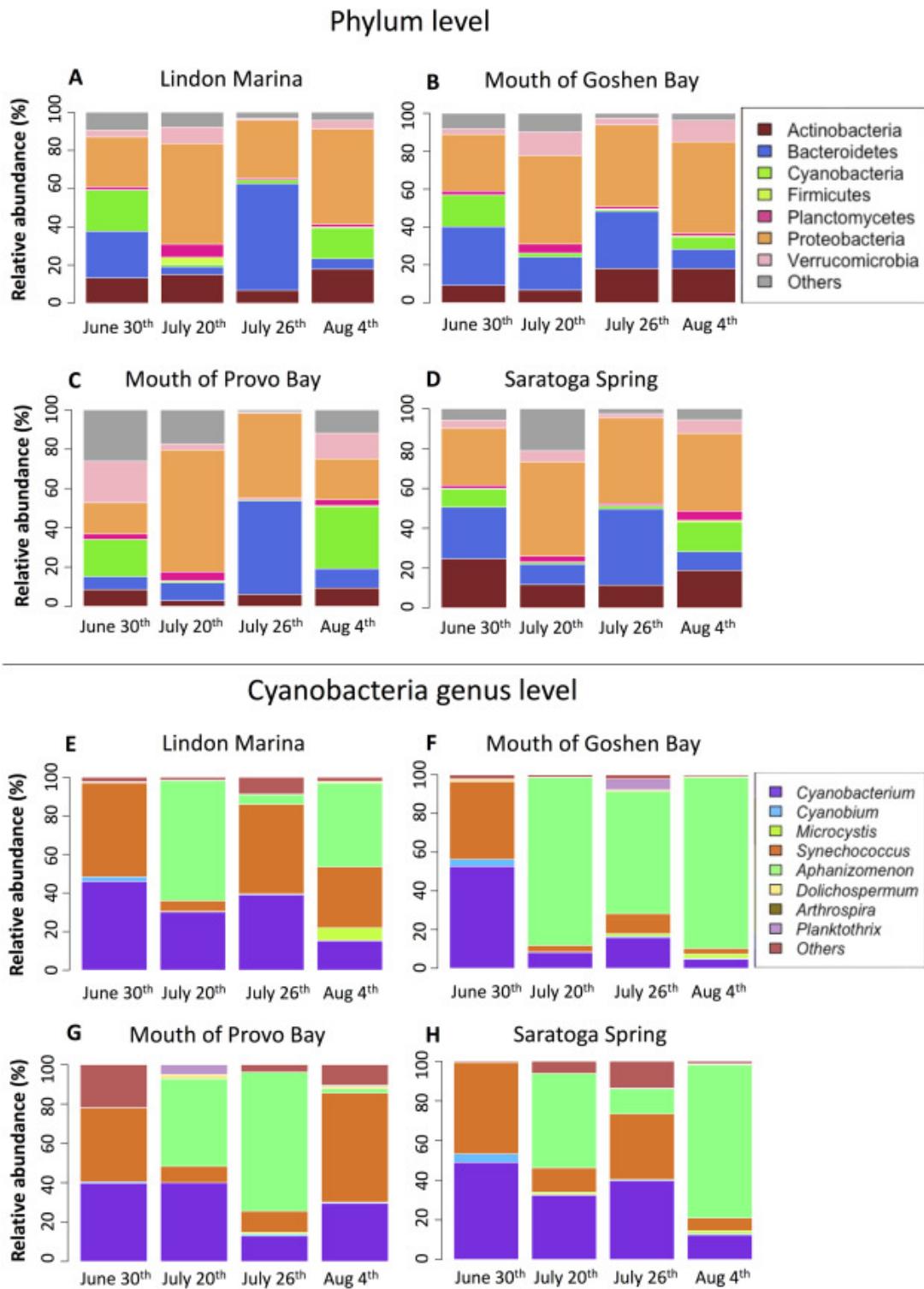


Figure 3.3 Relative abundance of 16S rRNA gene sequences at the phylum and cyanobacteria genus level in 2016. (A–D) Bacteria taxa composition at the phylum level. (E–H) Cyanobacteria taxa composition at the genus and species level. “Others” contains “unknown,” “unclassified,” and taxa with small relative abundance. “Cyanobacterium” contains strains that are unnamed.

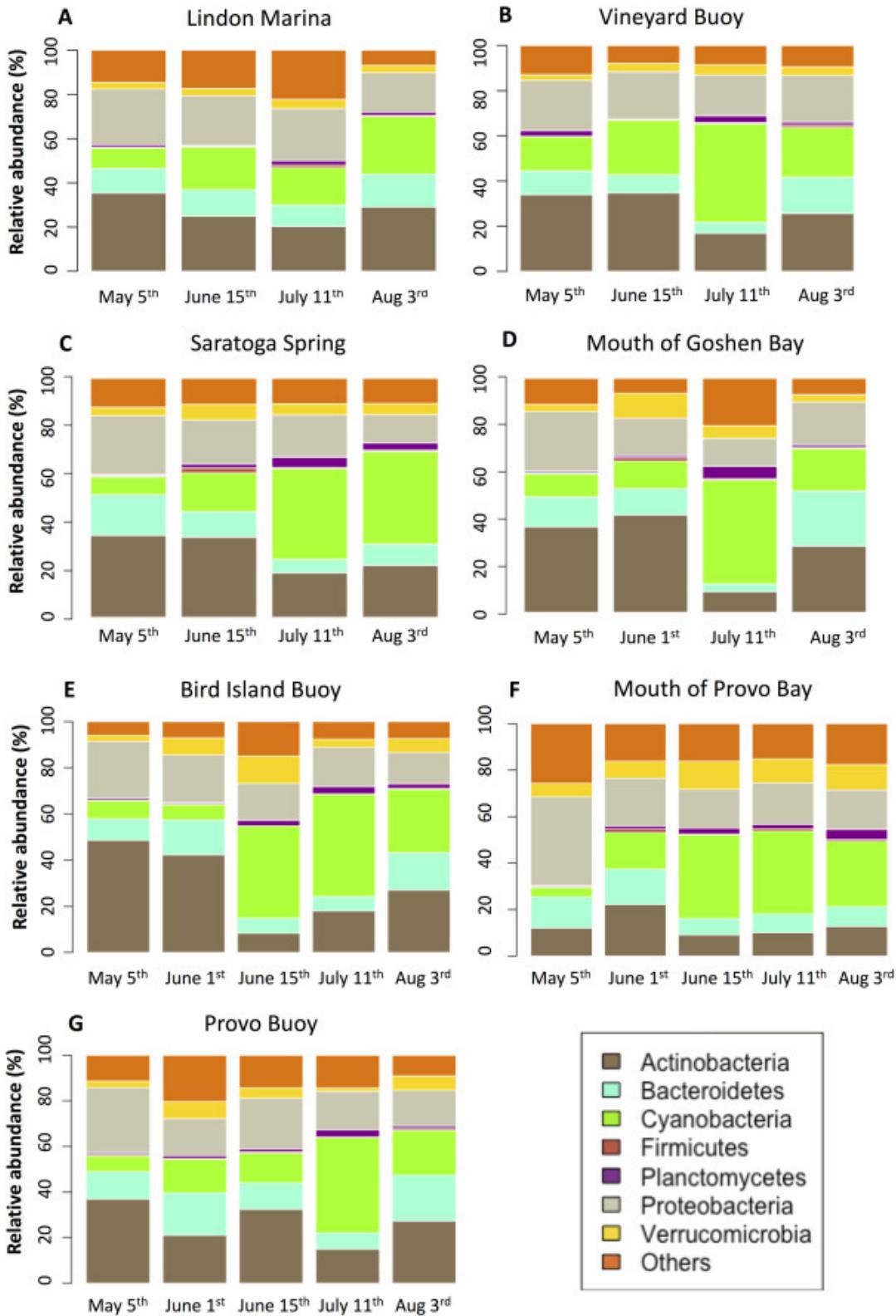


Figure 3.4 Microbial community composition at the phylum level in 2017 from different sampling sites.

primarily represented by the phyla Actinobacteria, Proteobacteria, Bacteroidetes, Firmicutes, Planctomycetes, and Verrucomicrobia in both years. Proteobacteria accounted for almost 50% of the total bacterial abundance in 2016 (Figure 3.3 A–D). Bacteroidetes was the second-largest phylum during the bloom in 2016. Surprisingly, Cyanobacteria was not the dominant phylum during the bloom. In fact, the relative abundance of cyanobacteria decreased from 10 to 30% (depending upon the site) before bloom to <1% during the bloom in 2016. The relative abundance of cyanobacteria increased to 7–33%, depending upon the sampled site, after the bloom in 2016. On the contrary, Cyanobacteria was the dominant phylum in 2017, especially during the bloom period. Almost 45% of the total number of sequence reads were classified as Cyanobacteria phylum (Figure 3.4). On the other hand, Actinobacteria and Proteobacteria were the dominant phyla before the occurrence of the bloom. It is also interesting to note that Actinobacteria replaced Proteobacteria as the most dominant bacterial phylum at many of the sampled sites in 2017.

3.5.4 Overall Cyanobacterial Taxonomy at the Genus Level

Synechococcus (up to 77% of total cyanobacteria) and *Aphanizomenon* (up to 88% of total cyanobacteria) were the most dominant genera during non-bloom and bloom periods respectively (Figure 3.3 E–H for 2016, and Figure 3.5 for 2017). *Cyanobium* and *Prochlorococcus* were also detected at all sites, albeit in relatively small abundances. The bloom of *Aphanizomenon* (especially *Aphanizomenon flos-aquae*) was often associated with the presence of other filamentous cyanobacteria, such as *Dolichospermum* (e.g., *Dolichospermum flos-aquae*), *Planktothrix* (e.g., *Planktothrix pseudagardhii*), and

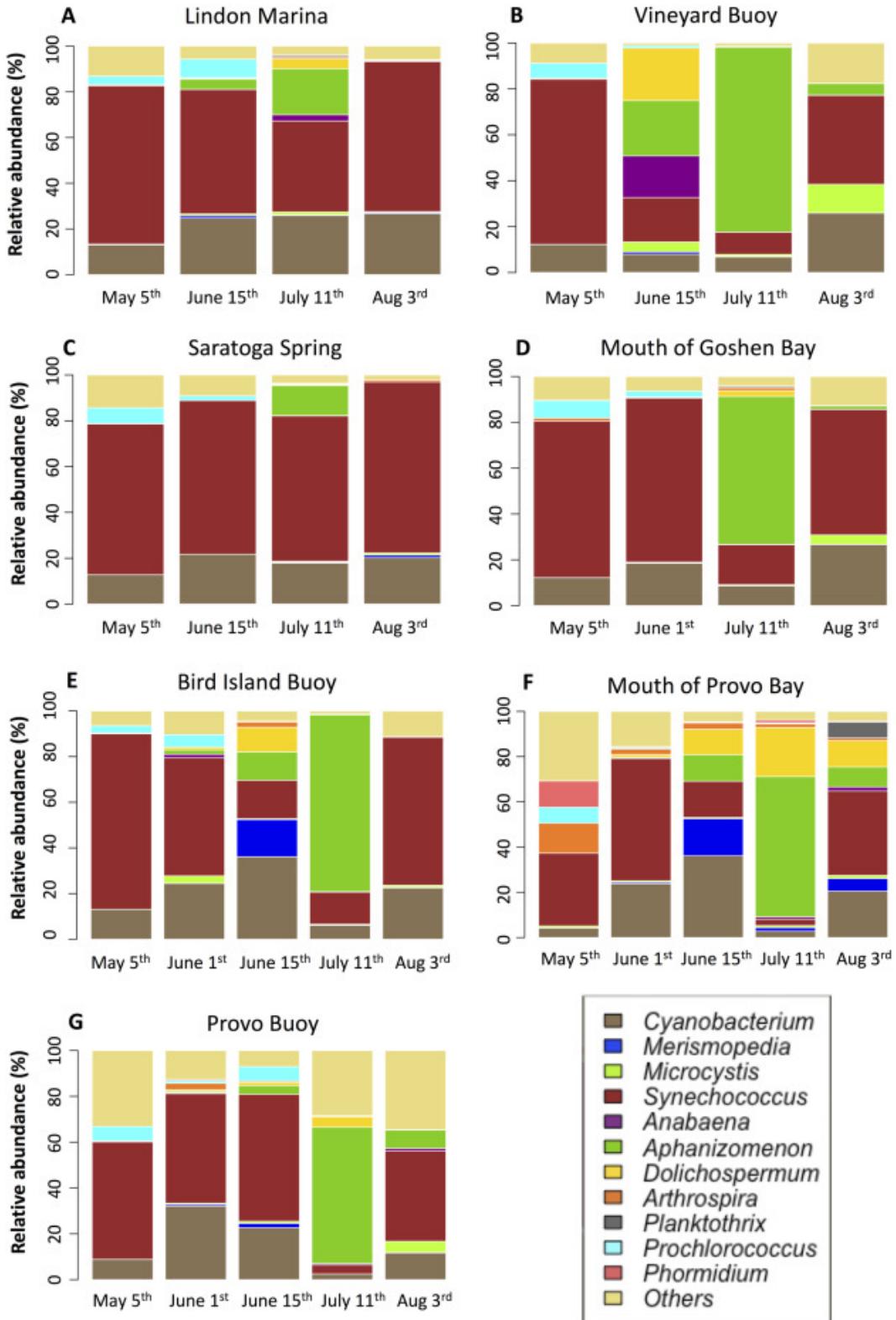


Figure 3.5 Microbial community composition at the genus level in 2017 from different sampling sites.

Arthospira. *Arthospira platensis*, a nontoxic floating filamentous species, was the most abundant one under the genus *Arthospira* among other filamentous cyanobacteria. Two more new genera detected in 2017 with relatively higher abundance were *Anabaena* (Vineyard Buoy site) and *Phormidium* (Mouth of Provo Bay site). The dominant colonial cyanobacteria detected were *Microcystis* and *Merismopedia*. Microcystin-producing *Microcystis aeruginosa* was detected in both years. Unlike *Microcystis*, which can travel all over the lake, *Merismopedia* was mostly found at the Bird Island Buoy and Provo Buoy sites. Heavy *Aphanizomenon* blooms generally began from the end of June and had vanished by early August. The presence of *Synechococcus* was negatively correlated with most filamentous cyanobacteria, including *Leptolyngbya*, *Dolichospermum*, *Anabaena*, *Phormidium*, and *Aphanizomenon*.

3.5.5 Presence of Potentially Toxin-Producing Cyanobacteria

It is a well-established fact that some genera of Cyanobacteria, such as *Cylindrospermopsis*, *Microcystis*, and *Planktothrix* are capable of producing secondary metabolites in the form of toxins known as cyanotoxins (Pearson et al., 2010). Hence, the sequence reads were analyzed for the presence of toxin-producing cyanobacteria under the phylum Cyanobacteria. Compared to the nontoxic cyanobacterial population, most potentially toxin-producing cyanobacteria (not including the *Aphanizomenon flos-aquae*) were in relatively small abundance and were sparsely distributed. Additionally, the dominance of potentially toxin-producing species was observed during times of heavy algal blooms, especially in 2017 sampling. *Microcystis aeruginosa* (0–6.51% of all cyanobacteria) was the potentially microcystin-producing species detected in 2016's HAB

(Figure 3.4 E-H) when relatively elevated concentrations of hepatotoxins were measured by local regulatory agencies (data not shown). Apart from *Microcystis*, a small relative abundance of *Dolichospermum* (0–2.22%) and *Planktothrix* (0–5.60%) were also detected.

A more complicated, potentially toxin-producing cyanobacteria community composition was detected in 2017 (Figure 3.5). Microcystin, anatoxin-a, and cylindrospermopsin were the cyanotoxins that were measured in water samples by the local regulatory agency. Specifically, *Microcystis aeruginosa* and *Planktothrix* accounted for up to 12.43% and 7.04% of total cyanobacteria abundance in the August 3rd sampling. A higher percentage of *Anabaena flos-aquae* (18.17%) (a potentially anatoxin-a producer) was found in the June 15th sampling at Vineyard Buoy. *Dolichospermum* (0–22.91%), the potential microcystin and anatoxin producers, presented at all of the sites except for Saratoga Spring. A bloom of *Dolichospermum flos-aquae*, together with *Aphanizomenon flos-aquae*, was detected at the Mouth of Provo Bay from mid-June to early August. Additionally, *Merismopedia* (0.10–16.37%) and *Phormidium* (0.38–11.62%) were cyanobacteria genera discovered near the Provo Bay area in 2017. *Phormidium* is mostly benthic in nature (McAllister et al., 2016). However, water column agitation due to the shallow nature of Utah Lake may have caused some sloughing of the benthic community members.

3.5.6 Dominant Heterotrophic Bacterioplankton

The three main heterotrophic phyla detected in both years were Actinobacteria, Bacteroidetes, and Proteobacteria. For both years, a similar decreasing trend throughout

the sampling period was observed for Actinobacteria, while increasing trends were detected for Bacteroidetes and Proteobacteria. The percentage of reads at the level of order was plotted for each phylum (Figure 3.6). Corynebacteriales and Micrococcales were two orders that dominated the Actinobacteria community in both years (Figure 3.6 A, B). *Mycobacterium* (order Corynebacteriales) was the dominant genus that had a lower relative abundance during the bloom. It is noted that the unclassified reads for Actinobacteria in 2017 accounted for 5.50% to 37% of the total reads. Bacteroidetes were

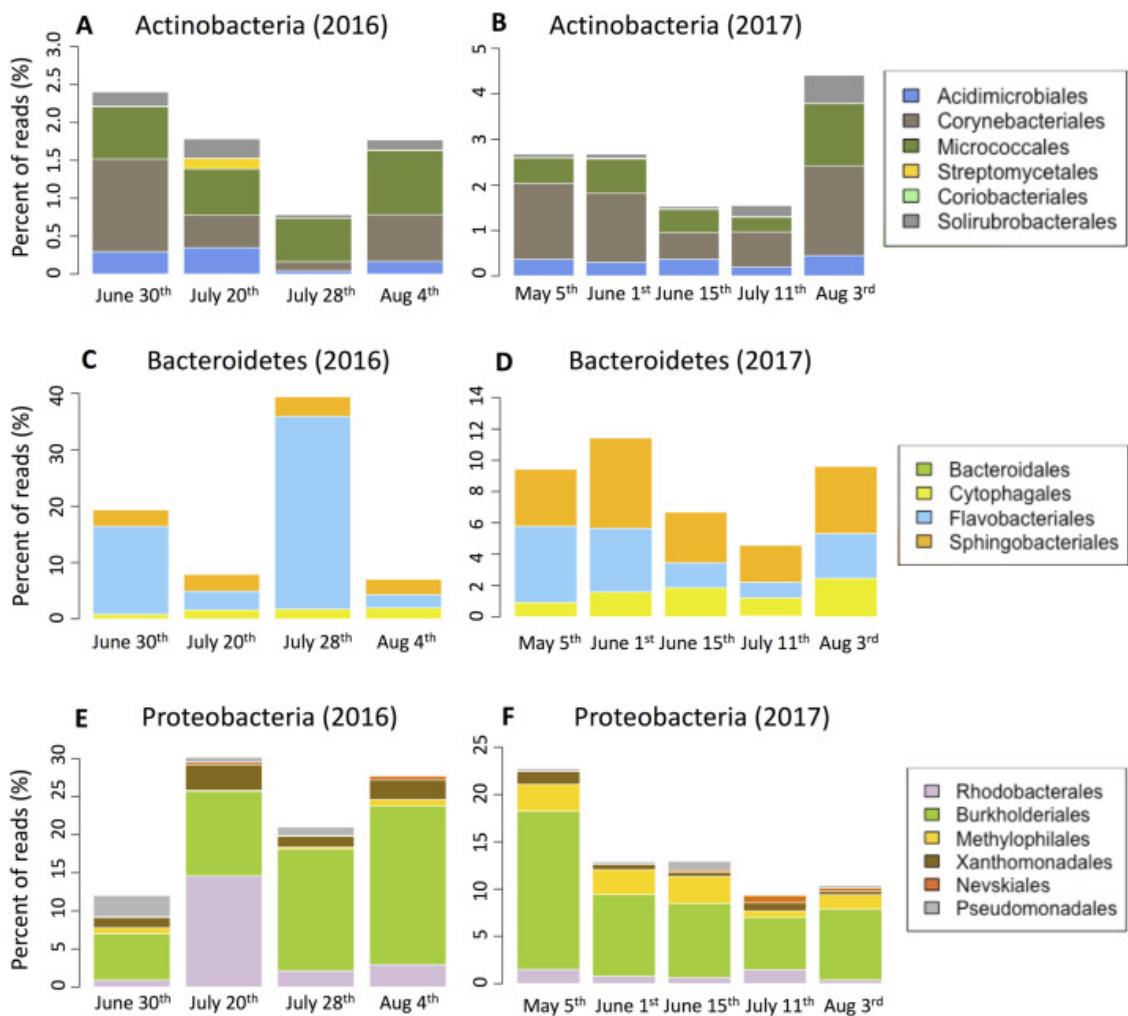


Figure 3.6 Dominant heterotrophic bacterioplankton community.

mainly dominated by *Flavobacterium* (order Flavobacterales), *Haliscomenobacter* (order Sphingobacterales), and Cytophagaceae (order Cytophagales) (Figure 3.6 C, D). The relative abundance of Flavobacterales was elevated during the 2016 HAB as compared Flavobacterales abundance in 2017. It accounted for 35% of the total reads, which was seven times its maximum percentage (5.80%) in 2017. Moreover, the decrease of Flavobacterales made Sphingobacterales the largest order in 2017. Proteobacteria also showed an opposite trend in 2016 and 2017 (Figure 3.6 E, F). The class of Alpha- and Betaproteobacteria represented most of the Proteobacteria. *Limnohabitans* (order Burkholderiales) was the most dominant genus observed in Betaproteobacteria. The order of Rhodobacterales (Alphaproteobacteria) occurred at a high relative abundance (15%) on July 20th, 2016. Besides, Methylophilales (Betaproteobacteria) showed a lower relative abundance during the bloom.

3.5.7 Inferred taxa of chloroplast community

In both years, Chlorophyta and Stramenopiles were the dominant eukaryotic phytoplankton orders detected in the lake. In general, the relative abundance of Chlorophyta decreased on days of heavy algal blooms (Figure 3.7 A, B). Instead, an increased relative abundance of Stramenopiles was observed in 2016's bloom period. A more notable abundance of other communities was detected in 2017. Streptophyta (20.56%) suddenly appeared in the sampling of July 11th. It is also noted that the enhanced abundance of Cryptophyta occurred after the peak of the bloom (July 11th), when the relative abundance of cyanobacteria was the highest at most of the sites. The dominant families detected for Chlorophyta were Chlamydomonadaceae and

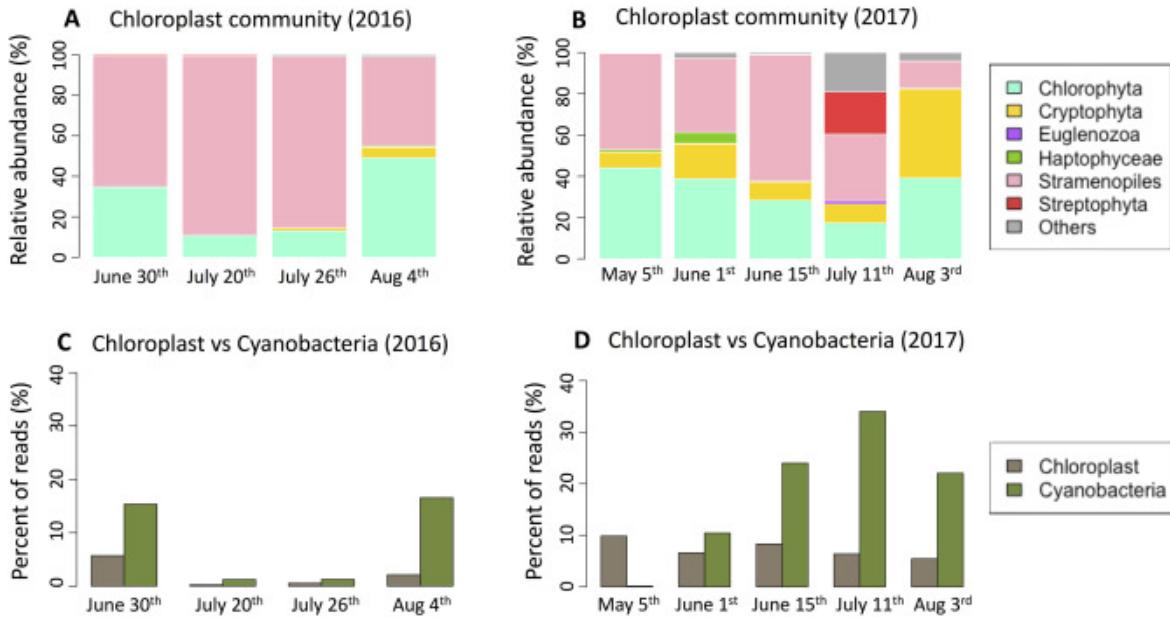


Figure 3.7 Eukaryotic phytoplankton communities analyzed by chloroplast 16S rRNA gene. (A, B) The relative abundance of chloroplast community at the order level. (C, D) The comparison of sequence reads percent between chloroplast and cyanobacteria communities. Note the differences in y-axis scales.

Trebouxiophyceae. In 2017, the relative abundance of the chloroplast community reads was overall lower than the cyanobacteria reads (Figure 3.7 C, D), except for early summer (May 5th). The lowered chloroplast and cyanobacteria communities suggested a bloom of heterotrophic bacterioplankton during the 2016 bloom.

3.5.8 Statistical Analysis of Environmental Effects on Bacterial Community Compositions

The two methods used for comparisons of beta diversity among samples were the UPGMA phylogenetic method and 3D PCoA. The UPMGA phylogenetic tree showed that samples were primarily clustered by time series in both years. Particularly, samples from 2016 were grouped by dates except for the Mouth of Provo Bay, where samples

tended to group more by location. In 2017, most samples from May or early June were located at the farthest part of the tree, which implies distinct patterns of microbial community compared with the bloom period. By contrast, samples collected during the bloom season (June 15th, July 11th, and August 3rd) were closely related. Samples from the Mouth of Provo Bay tend to be clustered in 2017. The 3D PCoA plots for unweighted and weighted Frac further confirmed the relative distance among samples. Except for a few outliers, most samples were significantly assembled based on sampling periods. The first three eigenvalues explained 36.38% and 72.85% of the observed variations in 2016, as well as 28.88% and 63.16% of the variations in 2017. The results of those two methods demonstrated the significance of time series rather than geographical locations in the phytoplankton community change.

3.6 Discussion

3.6.1 Principal Component Analysis Using Sequencing and Water Quality Data

The principal component analysis (PCA) was conducted with environmental parameters (e.g., temperature, pH, TDS, and Chl *a*) and the relative abundance of bacterial groups (cyanobacteria, heterotroph bacteria, and chloroplast community) (Figure 3.8). Two principal components (Dim 1 and Dim 2) with the highest percentages were shown on the plots, which explained 58.0% and 58.4% of the variations for 2016 and 2017. For variance analysis, the appearance of cyanobacteria was positively correlated with temperature ($r = 0.18, p < 0.001$, Spearman) and negatively related to nitrate-N ($r = -0.54, p < 0.01$, Spearman) (Figure 3.9-top panel). In contrast, dominant

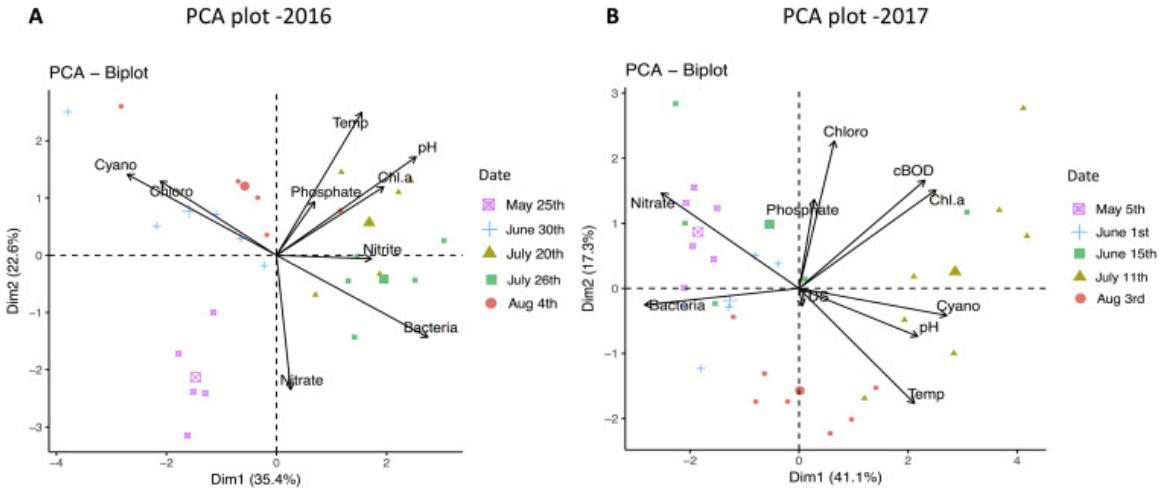


Figure 3.8 Two-dimensional principal components analysis (PCA) biplots linking dominant bacterial communities with environmental factors. (A) Biplot for 2016. (B) Biplot for 2017. Temperature (Temp), Chlorophyll a (Chl.a), total dissolved solids (TDS), carbonaceous biochemical oxygen demand (cBOD), Cyanobacteria (Cyano), Chloroplast (Chloro) and Heterotrophs (Bacteria).

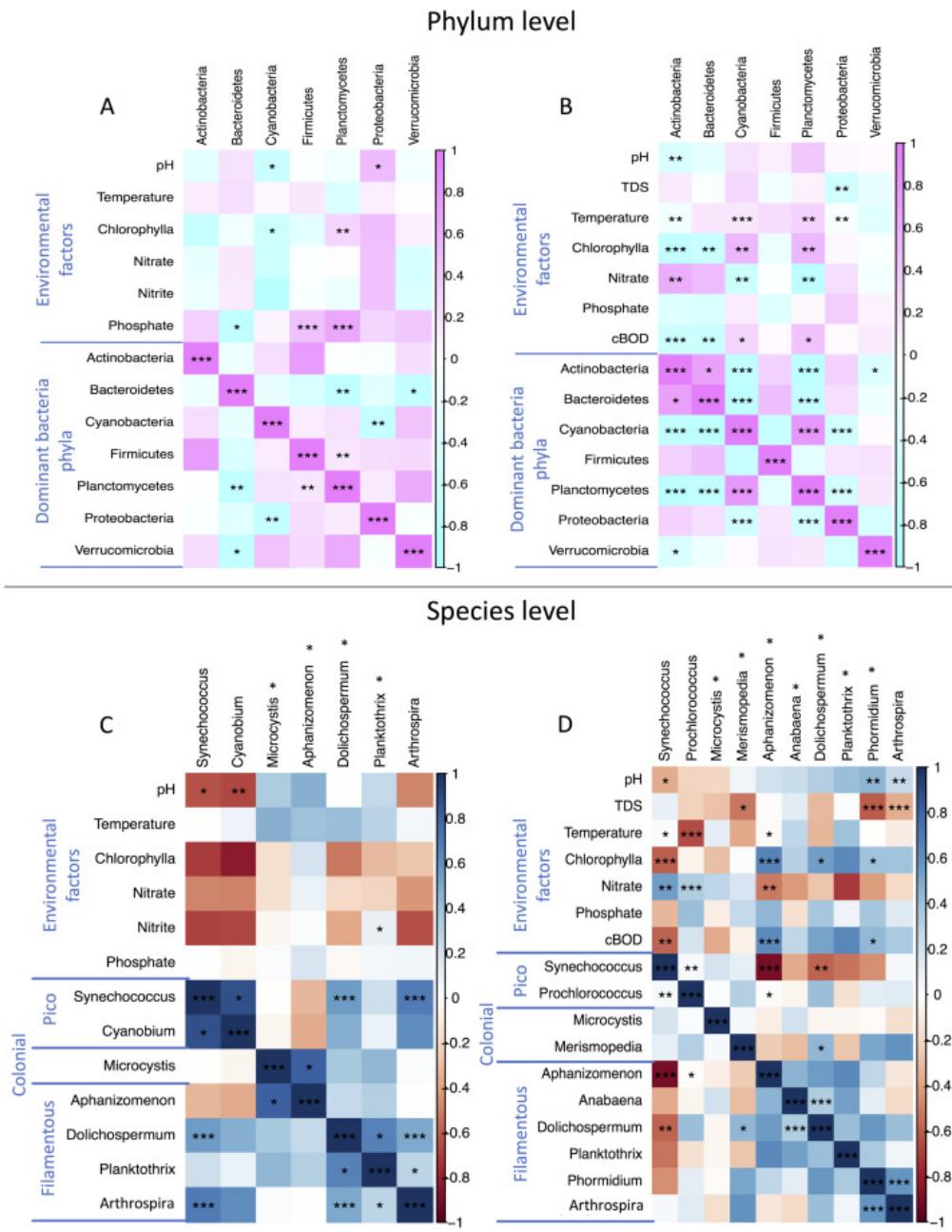


Figure 3.9 Spearman coefficients between the most dominant bacterial phyla and environmental parameters. Top panel: Spearman coefficients between the most dominant bacterial phyla and environmental parameters. (A) Corrplot in 2016. (B) Corrplot in 2017. The significance levels were symbolled as: *: $p \leq 0.05$, **: $p \leq 0.01$ and ***: $p \leq 0.001$. The confidence interval is 95%, Bottom panel. Spearman coefficients between the most dominant cyanobacterial taxa and environmental parameters. (C) Corrplot in 2016. (D) Corrplot in 2017. The significance levels were symbolled as: *: $p \leq 0.05$, **: $p \leq 0.01$ and ***: $p \leq 0.001$. The confidence interval is 95%.

Cyanobacterial taxa were grouped based on both morphological and phylogenetic differences. Potentially Toxin-producing species are indicated by asterisks.

heterotrophic phytoplankton (Actinobacteria, Bacteroidetes, and Proteobacteria) were positively linked to nitrate-N and negatively correlated with cyanobacteria.

Environmental factors such as Chl *a*, pH, temperature, and cBOD were positively correlated with each other. The occurrence of chloroplast communities was observed to have the same trend as cyanobacteria.

3.6.2 Overall Microbial Community

Interactions among different bacterial community members, phytoplankton, and other organisms support aquatic food webs and control nutrient dynamics in aquatic environments. An important step towards identifying these interactions is to understand the relative abundance of different bacterial community members to the precision.

Traditional microscope-based techniques often underestimate bacterioplankton and cyanobacterial diversity (Xiao et al., 2014) and routine molecular techniques, such as restricted fragment length polymorphism and denature gradient gel electrophoresis, do not provide much-needed resolution (Samarajeewa et al., 2015). On the contrary, the use of 16S rDNA based high throughput sequencing allowed us to study different cyanobacterial species as a greater resolution as a function of different water quality parameters. For example, *Synechococcus* (up to 77% of total cyanobacteria) and *Aphanizomenon* (up to 88% of total cyanobacteria) were the most dominant genera during the non-bloom and bloom periods respectively (Figure 3.3 E-H for 2016, and Figure 3.5 for 2017) based on high throughput sequencing. Cased on microscopic identification and quantification by UDWQ, *Aphanizomenon* was also the dominant genus identified during bloom periods, which is consistent with what we found.

However, *Synechococcus* was neglected from their study due to its relatively small sizes but was shown to be the dominant cyanobacterial species during non-bloom period in our 16S rDNA-based analysis. The significance of this work lies in the fact that all components of the overall bacterial community were analyzed thoroughly and a comprehensive statistical analysis linked community diversity with water quality parameters using ecological theory.

At the genus level, a similar trend was observed for the same cyanobacterial phenotype (Figure 3.9-bottom panel). In general, picocyanobacteria showed a negative correlation with pH and Chl *a*, while filamentous bacteria had a positive correlation with pH, Chl *a*, and cBOD. Additionally, the presence of filamentous bacteria was negatively associated with nitrate-N ($r = -0.2$ to -0.71 , Spearman) in 2017 (Figure 3.9-bottom panel). By contrast, picocyanobacteria and colonial cyanobacteria were positively correlated with nitrogen. A clear pattern was observed when individuals are grouped based on months rather than geographical locations. In 2016, heterotrophs dominated the algal bloom in July and were linked to nitrogen sources (Figure 3.8 A). Cyanobacteria and the chloroplast community accounted for higher relative abundance before and after the bloom, while the dominance of cyanobacteria in July 2017 was mostly related to temperature (Figure 3.8 B).

Strong negative correlations between cyanobacteria and proteobacteria ($r > -0.52$, $p < 0.01$, Spearman) were observed in both years. (Figure 3.9-top panel). Besides, the occurrence of Planctomycetes tended to be positively linked to cyanobacteria, while negatively correlated with dominant heterotrophs. The most significant negative correlation ($r = -0.9$, $p < 0.001$) was observed between *Synechococcus* and

Aphanizomenon in 2017 (Figure 3.9-bottom panel), which further emphasized the seasonal community succession from picocyanobacteria to filamentous cyanobacteria. Colonial bacteria (*Microcystis* and *Merismopedia*) generally appeared before or after the bloom of filamentous bacteria.

Our results showed that the bacterial community varied highly at the phylum and genus levels in both years at multiple sites (Figure 3.3, Figure 3.4, and Figure 3.5). These results were consistent with previous studies, which suggested a temporary composition change reacting to a bloom (Michaloudi et al., 2008; Parulekar et al., 2017; Scherer et al., 2017). Although many of the bacterial phyla were shared between both years, the community distribution changes were evident. The most significant change at the phylum level was the relative abundance between the main heterotrophic bacterioplankton (Actinobacteria, Bacteroidetes, and Proteobacteria) and cyanobacteria communities. This indicates possible correlations and network connections among communities.

All three dominant heterotrophic phyla and cyanobacteria showed temporal variations during the bloom, and also between the two sampling years. Lower alpha diversity during blooms was suggested by the Shannon and Simpson index (Table 3.1). The decreased evenness and richness suggested by the two indexes demonstrated that the bacterial communities were affected by the bloom. Nevertheless, the Chao1 Index reflected no obvious change in bacterial richness due to a bloom. This result was consistent with the study that found no significant Chao1 difference in lakes with and without cyanobacteria blooms (Eiler and Bertilsson, 2004). In general, our result is consistent with the study that found greater effects on community evenness rather than richness during a bloom (Berry et al., 2017). The decreased evenness could result from

the dominance of bloom specialists (e.g., *Aphanizomenon flos-aquae*) due to increased temperatures and scarce nutrient conditions in 2017. With the relatively abundant nutrients in 2016, heterotrophic bacteria were the dominant groups affecting community evenness.

Distinct correlations and relative abundance were found between cyanobacteria and each heterotrophic phylum. Specifically, a slightly decreasing trend for Actinobacteria abundance was linked to a bloom (Figure 3.6 A, B). *Mycobacterium* from order Corynebacteriales was the most representative genus identified that could be a potential N-fixer (Gtari et al., 2012; Sellstedt and Richau, 2013). Interestingly, a large percentage of unknown orders of Actinobacteria were detected in 2017. This phylum's flourishing could be caused by their abilities to fix nitrogen and to live in low-nutrient conditions (Newton et al., 2011).

The Bacteroidetes sequences showed an increased relative abundance in 2016 and a decreased abundance in 2017 (Figure 3.6 C, D), which could be most affected by available nitrogen and carbon sources in the lake. Similar to many previous studies, Flavobacteriales and Sphingobacteriales were the orders that dominated Bacteroidetes in a cyanobacterial bloom (Parulekar et al., 2017; Scherer et al., 2017). *Flavobacterium* was the most abundant genus detected from the order Flavobacteriales. The sudden rise of *Flavobacterium* on July 20th in 2016 could be more driven by its capacity of degrading complex organic matter (Eiler and Bertilsson, 2004) and less by chemically stable hepatotoxins (Berg et al., 2009). Certain species of *Flavobacterium* was also enhanced in nutrient-rich conditions (Madetoja et al., 2003). As for Sphingobacteriales, *Haliscomenobacter* (family Saprospiraceae) was the most abundant genus found that

commonly caused bulking in activated sludge (Eikelboom, 1975). Members of Saprospiraceae were also known for scavenging cyanobacteria (Ashton and Robarts, 1987; Lewin, 1997) and cyanobacterial secondary metabolites (Mcilroy and Nielsen, 2014). As a result, Bacteroidetes were negatively correlated with cyanobacteria due to the tentative predator-prey relationships between the two phyla, as described above (Figure 3.9-top panel).

Similar to Bacteroidetes, an inverse trend was observed for the temporal variations of Proteobacteria in 2016 and 2017 (Figure 3.6 E, F). In 2016, Proteobacteria accounted for a significant portion of the total bacterial community, which was consistent with previous studies on eutrophic lakes (Shi et al., 2012; Cai et al., 2013; Parulekar et al., 2017). *Limnohabitans* (Betaproteobacteria) was the predominant genus detected (Hahn et al., 2010). Its proliferation in Utah Lake could attribute to the alkaline environment (Hahn et al., 2010), and the efficient utilization of *Dolichospermum* exudates as organic carbon sources (Šimek et al., 2011). The typical pH in Utah Lake is above 8.0 and can be enhanced to around 9.0 during a heavy bloom. Additionally, the high representative of Rhodobacterales (Alphaproteobacteria) on July 20th also emphasized the presence of organic carbon, which could greatly promote photoheterotrophic growth (Androga et al., 2012). Some members in the Methylophilales (Betaproteobacteria) could also suggest better microcystin degradation ability than Sphingomonadales of Alphaproteobacteria (Mou et al., 2013). Differing from cyanobacteria, community structures of Proteobacteria were greatly affected by the presence of dissolved organic matter (Cottrell and Kirchman, 2000; Alonso-Sáez et al., 2009). Although the cBOD was measured high during the peak bloom in 2017, there is a

hypothesis that the proliferation of proteobacteria was limited by nutrients (Peura et al., 2012). Significant negative correlations between Proteobacteria and Cyanobacteria were probably a result of their distinct metabolic pathways (Figure 3.9-top panel).

Furthermore, Verrucomicrobia and Planctomycetes were common freshwater phyla that represented relatively larger percentages in Utah Lake. Specifically, Verrucomicrobia was expected to be present because they are good degraders of algal polysaccharides (Martinez-Garcia et al., 2012; Cardman et al., 2014). In addition to organic matter, their community dynamics were greatly affected by inorganic nutrients (Parveen et al., 2013). Planctomycetes contain ANAMMOX bacteria that oxidize ammonium to dinitrogen in the absence of oxygen (anaerobic conditions) (Fuerst and Sagulenko, 2011). The newly-discovered nitrogen-fixation community also revealed its significant participation in the nitrogen cycle (Delmont et al., 2017). Besides, Planctomycetes were reported to be important members of cyanobacterial assemblages (Cai et al., 2013). These facts helped explain their significant negative correlations with nitrate in 2017 and positive correlations with cyanobacteria (Figure 3.9). Because heterotrophic bacterioplankton is closely associated with either cyanobacterial assemblages or environmental factors, it is important for us to trace them for an understanding of bloom dynamics.

3.6.3 The Presence of Dominant and Potentially Toxin-Producing Cyanobacteria in Cyanobacterial Assemblages

At the cyanobacteria genus level, *Synechococcus* and *Aphanizomenon* were the dominant genera that experienced community shifts (Figure 3.4, Figure 3.5). The

increased cyanobacteria genera in 2017 mostly belonged to potentially cyanotoxin-producers. In contrast with lakes that had one dominant toxin-producer, most potentially toxic cyanobacteria in Utah Lake were either low or high in relative diversities (Dadheeck et al., 2014; Scherer et al., 2017; Malazarte et al., 2017). Our study also suggested that cyanobacteria grouped by phenotype (filamentous, colonial, and pico-cyanobacteria) shared common seasonal successions. These results were consistent with previous studies on the Baltic Sea, which mainly focused on predominant phenotypes/genotypes (Mazur-Marzec et al., 2013; Bertos-Fortis et al., 2016), as well as toxic seasonal cyanobacteria OTUs (Bertos-Fortis et al., 2016).

Picocyanobacteria is regarded as one of the most important organisms globally, in both marine and freshwater systems, due to their essential roles in aquatic primary production (Śliwińska-Wilczewska et al., 2018). The study of picocyanobacteria was conventionally limited by microscopy due to their relatively small sizes (Waterbury et al., 1986). Until now, freshwater picocyanobacteria, including *Synechococcus* have been studied less compared to their marine members (Jakubowska and Szeląg-Wasielewska, 2015; Cabello-Yeves et al., 2017). *Synechococcus* is adapted to warm water and is capable of quick nutrient uptake due to a larger surface-to-volume ratio (Iturriaga and Mitchell, 1986), making it easier for them to adapt to summer's nutrient limitation conditions (Agawin et al., 2000; Moisan et al., 2010). The dominance of *Synechococcus* was also reported in other freshwater eutrophic lakes in China (Ji et al., 2018). Compared with picocyanobacteria, wide varieties of filamentous cyanobacteria genera were detected during the bloom seasons (Figure 3.5). The low genetic resolution between some genera (e.g., *Aphanizomenon* and *Dolichospermum*) also emphasized the importance of study

according to their phenotypes/genotypes (Lyra et al., 2001; Gugger et al., 2002). Both *Aphanizomenon* and *Dolichospermum* belong to planktonic Nostocales (Driscoll et al., 2018). Conventionally, the decline of filamentous cyanobacteria was thought to be due to P limitations (Walve and Larsson, 2007). With the P-rich sediment and potential for internal P-loadings in Utah Lake (Abu-Hmeidan et al., 2018), the bloom of filamentous cyanobacteria could be attributed more to their diazotrophic nature (Vahtera et al., 2007; Beversdorf et al., 2013). The positive correlations between filamentous and colonial groups argued that their synthesized N source supported the bloom of toxic *Microcystis* (Beversdorf et al., 2013) (Figure 3.9-bottom panel). By contrast, *Synechococcus* had a significant negative correlation with *Aphanizomenon*, which demonstrated weak or no metabolic correlations between them (Mazur-Marzec et al., 2013).

In addition to dominant picocyanobacteria and filamentous cyanobacteria, most of the potential toxin producers were considered to be opportunists (highly temporal or spatial-specified OTUs) (Bertos-Fortis et al., 2016). New toxic colonial and filamentous genera were detected in 2017 (Figure 3.5). To specify, *Merismopedia* is known to produce lipopolysaccharides that caused severe skin irritation (Jakubowska and Szeląg-Wasielewska, 2015). *Phormidium* was proved to be a potential anatoxin-producer (Heath et al., 2016). Finding *Phormidium* in the water column was unexpected, as this species is commonly known as benthic alga (McAllister et al., 2016). Its presence in the water column could be a result of floating debris decay and mixing. Conventionally, the distribution patterns of opportunists are mostly explained by the variations of environmental factors. However, the correlation with biotic factors is still vague,

especially when it comes to species present in relatively small abundances and with sparse distributions. In some cases, their presence and disappearance may be top-down controlled more by surrounding organisms. Currently, more studies have addressed the influence of biotic factors such as grazer effects (Ventelä et al., 2002; Ger et al., 2016), fungal infection (Agha et al., 2018) and cyanophages (Yoshida et al., 2006; Ni and Zeng, 2016) on shaping community structures. Due to varying selective feeding (Zwirglmaier et al., 2009) and infection pressures (Ni and Zeng, 2016), the diversity of cyanobacteria can be controlled top-down by their predators and infected viruses. Additionally, the algicidal activities owned by some heterotrophic bacterioplankton (mainly Bacteroidetes and Proteobacteria) also played potential roles for cyanobacterial community regulations, as discussed previously.

3.6.4 The Inferred Chloroplast Community During Blooms

Interestingly, chloroplast ribosomal RNA gene sequences suggested the presence of algae in the lake, mostly Chlorophyta and Stramenopiles groups (Figure 3.7). Previously, sequences from chloroplast have been successfully used for the identification of eukaryotic phytoplankton (Burja et al., 2001; Kong et al., 2017; Scherer et al., 2017). More reads were found for cyanobacteria compared with chloroplasts, which agreed with studies of other eutrophic lakes (Scherer et al., 2017). Besides, the chloroplast community showed close correlations with cyanobacteria, especially in 2016 (Figure 3.8). This trend could be a result of the same evolutionary origins (Okamoto and Inouye, 2005) and similar ecological niches (Scherer et al., 2017). Among all groups, the abundant chloroplast sequences suggested that Chlamydomonadaceae could be the predominant

family, while the interpretation of relative cell abundance was still hindered by the viable numbers of chloroplasts within and among species. Their quick growth under nutrient-rich conditions typically helps them to dominate a spring bloom (Similä, 1988).

3.6.5 Bacterial Community Composition in the Context of Environmental Parameters

The beta diversity analysis demonstrated a significant effect of time series rather than spatial variance on community structures. Specifically, our results suggested minor effects of geographical locations on dominant bacteria structures near tributaries to the deep water, except for the Provo Bay area. Conventionally, Provo Bay is listed as the most eutrophic area in the lake. The UPGMA tree also demonstrated a relative cluster of samples from the Mouth of Provo Bay. In contrast, toxic-producing opportunists were more temporal or location-specified. For example, *Anabaena* mainly occurred at the North Lake (site Vineyard Buoy and Lindon Marina), while *Merismopedia* and *Phormidium* were more abundant near the Provo Bay area (Bird Island Buoy, Mouth of Provo Bay, and Provo Buoy sites) in 2017 (Figure 3.5). Since the blooms were spreading to the entire lake in both years, it was consistent with studies that observed larger effects of time/environmental factors than locations on shaping community structures (Bertos-Fortis et al., 2016; Berry et al., 2017).

The PCA plots and Spearman correlations also suggested a cluster of environmental factors linked to monthly bacterial community changes and strong correlations among some of them (Figure 3.8, Figure 3.9). Heterotrophic bacteria communities were close correlated with nitrate, emphasizing their high requirements for

nutrients (Vrede, 2005; Bernhard et al., 2005) and possible opposite nutrient utilization strategies from phytoplankton (Xu et al., 2014). Particularly, Actinobacteria was more affected by primary productions (Claire Horner-Devine et al., 2003; Berry et al., 2017), while Proteobacteria and Bacteroidetes were highly sensitive to nutrients (Bernhard et al., 2005) and cyanobacteria communities (Shi et al., 2011; Scherer et al., 2017). Since many proteobacteria and Bacteroidetes are phytoplankton colonizers and predators, they can form tight associations with phytoplankton and/or their aggregates (Dang and Lovell, 2016). Besides, Bacteroidetes were found to secrete enzymes that could degrade and utilize particulate organic matters (POMs), such as proteins and polysaccharides (Buchan et al., 2014; Dang and Lovell, 2016). Additionally, pH and temperature could be strong factors shaping a bacterioplankton community (Lindström et al., 2005). Previously, the temperature has been found to be the single largest factor affecting bacterial communities in many freshwater systems (Crump and Hobbie, 2005; Shade et al., 2007). Our data also showed that temperature was crucial for cyanobacteria to dominate the bacterioplankton community in 2017, especially with the depletion of nutrients. In addition to temperature, the depletion of nitrate-N was probably the most important reason for the dominance of filamentous cyanobacteria community (Ploug et al., 2011; Bertos-Fortis et al., 2016). The correlations between cBOD and Chl *a* during the bloom season suggested strong effects of primary production on dissolved carbon concentrations (Stets and Cotner, 2008).

Above all, the variations of temperature and nutrient conditions were possible reasons causing the temporal community change in Utah Lake. Instead of only studying cyanobacteria, our results added to studies that investigated environmental effects on the whole-community scale (Bertos-Fortis et al., 2016; Parulekar et al., 2017; Berry et al.,

2017). The dominance of heterotrophs during a 2016 bloom season is probably triggered by nutrients (Prieto et al., 2015; Soares et al., 2017). In contrast, the temperature may be the drivers promoting the dominance of cyanobacteria in 2017 (Paerl and Huisman, 2008; Paul, 2008). At the genus level, filamentous bacteria may take advantage of the nitrogen-limiting conditions and dominate the cyanobacteria group (Niemi, 1979; Kangro et al., 2007; Walve and Larsson, 2010). All of the above results emphasized the importance of studying the interactions of multiple factors on cyanobacterial temporal changes.

3.7 Conclusions

For the first time, high-throughput sequencing was applied by us for the study of bacterial communities at Utah Lake. The results of two years' sampling suggested significant abiotic/biotic effects on cyanobacteria community structures. It also overcame the resolution problem associated with microscopic-based methods for the characterization of pico- and potentially toxin-producing cyanobacteria during CyanoHABs. We successfully demonstrated the presence (and, in some cases, the dominance) of filamentous cyanobacteria and *Synechococcus* in the lake. Moreover, genetic identification of cyanobacteria using DNA-based high-throughput sequencing has not only revealed the holistic identity of CyanoHABs but also demonstrated the potential correlations with heterotrophic bacterioplankton and to some extent, the inferred phototrophic eukaryotic community. Results obtained from this study can be further utilized by local agencies for policy-making and research on an impaired ecosystem. Particularly, the bacterioplankton community and environmental factors detected can be

applied for the prediction of potential CyanoHABs blooms and the assessment of ecosystem risks.

3.8 References

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CHAPTER 4

MICROBIAL COMMUNITY SUCCESSIONS AND THEIR DYNAMIC FUNCTIONS DURING HARMFUL CYANOBACTERIAL BLOOMS IN A FRESHWATER LAKE

4.1 Abstract

The current study reports the community succession of different toxin and non-toxin producing cyanobacteria at different stages of cyanobacterial harmful algal blooms (CyanoHABs) and their connectivity with nitrogen and phosphorus cycles in a freshwater lake using an ecogenomics framework. Comprehensive high throughput DNA sequencing, water quality parameter measurements, and functional gene expressions over temporal and spatial scales were employed. Among the cyanobacterial community, the lake was initially dominated by *Cyanobium* during the months of May, June, and early July, and later primarily by *Aphanizomenon* and *Dolichospermum* depicting functional redundancy. Finally, *Planktothrix* appeared in late August and then the dominance switched to *Planktothrix* in September. *Microcystis aeruginosa* and *Microcystis panniformis*; two species responsible for cyanotoxin production, were also present in August and September, but in significantly smaller relative abundance. MC-LR (0.06–1.32 µg/L) and MC-RR (0.01–0.26 µg/L) were two major types of cyanotoxins detected. The presence of MC-LR and MC-RR were significantly correlated with the *Microcystis*-related genes (*16SMic/mcyA/mcyG*) and their expressions ($r = 0.33$ to 0.8 , $p < 0.05$). The

metabolic analyses further linked the presence of different cyanobacterial groups with distinct functions. The nitrogen metabolisms detected a relatively higher abundance of nitrite/nitrate reductase in early summer, indicating significant denitrification activity and the activation of N-fixation in the blooms dominated by *Aphanizomenon*/ *Dolichospermum* (community richness) during nutrient-limited conditions. The phosphorus and carbohydrate metabolisms detected a trend to initiate a nutrient starvation alert and store nutrients from early summer, while utilizing the stored polyphosphate and carbohydrate (PPX and F6PPK) during the extreme ortho-P scarcity period, mostly in August or September. Specifically, the abundance of *Aphanizomenon* and *Dolichospermum* was positively correlated with the nitrogen-fixing *nif* gene and ($p < 0.001$) and the PPX enzyme for the stored polyphosphate utilization ($r = 0.77, p < 0.001$). Interestingly, the lake experienced a longer N-fixing period (2–3 months) before non-fixing cyanobacteria (*Planktothrix*) dominated the entire lake in late summer. The Provo Bay site, which is known to be nutrient-rich historically, had early episodes of filamentous cyanobacteria blooms compared to the rest of the lake.

4.2 Keywords

Harmful algal blooms, cyanobium, *Aphanizomenon*, *Dolichospermum*, nitrogen fixation, P Scavenging genes, cyanotoxins.

4.3 Introduction

The input of excess nutrients, primarily nitrogen and phosphorus, causes eutrophication in surface water bodies, leading to harmful algal blooms (HABs) in many

freshwater lakes (Heisler et al., 2008; Dodds et al., 2009; Keck and Lepori, 2012; Drobac et al., 2013). Nitrogen (N) and phosphorus (P) are two of the most important nutrients of concern, although their relative contribution to eutrophication is always debatable (Carpenter, 2005; Conley et al., 2009; Kolzau et al., 2014; Paerl et al., 2017). Early studies recognized P as the primary limiting nutrient in most lakes based on the stoichiometry of N and P in phytoplankton (Schindler, 1977; Hecky and Kilham, 1988; Lewis and Wurtsbaugh, 2008). P addition-based bioassays have shown that P addition enhanced the growth of toxin-producing *Microcystis* (Davis et al., 2009). However, subsequent studies also found that N was often the limiting nutrient in shallow eutrophic lakes, while the oligotrophic deep lake was mostly P limited (Downing and McCauley, 1992; Reynolds, 2006). A switch from spring P to summer N limitation has also been demonstrated in some locations (Conley, 1999). Recent studies also recognized the dominance of cyanobacteria under low N/P ratios (Søndergaard et al., 2017; Isles et al., 2017). Generally, an N-limitation condition could result from nitrate lost to heterotrophs (e.g., denitrifiers) via assimilation, denitrification and other biochemical processes (Allen et al., 2005; Chen et al., 2012; Holmroos et al., 2012), while the levels of P were determined by interactions between sediment and water column of seasonal hydrological processes (Armon and Starovetsky, 2015; Hogsett et al., 2019; Ma et al., 2019). Nevertheless, none of the past efforts or recent literature have denied the importance of nitrogen and phosphorus in supporting surface water eutrophication (Downing et al., 2001; Håkanson et al., 2007).

With new species of cyanobacteria being identified, the paradigm that surface water is either N limited or P limited is fast changing because nutrient limitations also

depend on which cyanobacterial species dominate the bloom (Cottingham et al., 2015). Under N stress conditions, many filamentous cyanobacteria (e.g., *Aphanizomenon*, *Dolichospermum*) can conduct both nitrogen fixation and photosynthesis by cell differentiation. It is well-known that vegetative cells conduct primary productivity, whereas the specialized cells, heterocysts, perform nitrogen fixation by utilizing nitrogenases (encoded by *nif* genes; Schindler et al., 2008; Paerl, 2017). Additionally, N regulatory genes (e.g., *ntrA*, *ntrC*) and PII signal transduction proteins are widely spread in bacteria that regulate the N assimilations under N starvations (Hirschman et al., 1985; Herrero et al., 2004; Huergo et al., 2013).

Similar to N systems, one of the commonly recognized strategies for bacteria to enhance phosphate assimilation is inducing the high-affinity inorganic phosphate (Pi) scavenging system- Pho regulon (Adams et al., 2008; Santos-Beneit, 2015), which includes members having the high-affinity Pi transport systems (encoded by *pst* genes; Makino et al., 1988; Pitt et al., 2010), enzymes polyphosphate kinase (PPK; Brown and Kornberg, 2004), exopolyphosphatase (PPX; Gomez-Garcia et al., 2003), and others. The P correlated metabolisms are even more complex to study, as many P-containing compounds in cells are tightly linked with carbohydrates assimilations (Harke et al., 2012; Harke and Gobler, 2013) or the stringent conditions alert (Abranches et al., 2009; Santos-Beneit, 2015). It is reported that phosphate bioavailability for diazotrophs was one of the constraint factors for nitrogen fixation rates as an interaction between N and P (Ward et al., 2013; Wu et al., 2018).

Recent studies have suggested that cyanobacterial N₂-fixation and Pi-scavenging also play important roles in promoting and sustaining cyanobacterial harmful algal

blooms (CyanoHABs) (Beversdorf et al., 2013; Harke et al., 2015). A very recent meta-transcriptomic based study by Lu et al. (2019) revealed that expressions of genes involved in N₂-fixation (*nifDKH*) and high-affinity Pi transporter (*pstSABC*) were significantly upregulated during the bloom compared to pre-bloom in Harsha Lake. In this study, these researchers found that the temporal action of N₂-fixation (*nifDKH*) and high-affinity Pi transporter genes (*pstSABC*) controlled the ecology of cyanobacterial populations in Harsha Lake. Interestingly, many studies have observed the co-presence or succession of N-fixers (or *nif* genes) and toxin-producing strains at different stages of blooms (Elser et al., 2000; Beversdorf et al., 2013; Chia et al., 2018; Lu et al., 2019).

Eutrophication is a dynamic process where harmful toxin-producing and nontoxic blooms coexist, although their relative abundance may vary. Additionally, the transition of a lake ecosystem from being N limited to P limited or vice versa would not only depend on the exogenous input of nutrients but the relative expressions of N₂-fixing and Pi-affinity genes. Lastly, the presence of toxic cyanobacteria identified taxonomically does not necessarily mean that they are expressing their toxin-producing functional genes. Recent studies successfully linked the dynamics of certain cyanobacterial species with their metabolic activities (Beversdorf et al., 2013; Harke et al., 2015; Lu et al., 2019). However, studies are still scarce for a whole-picture investigation into nutrient utilization pathways and toxin-producing functional genes at the entire bacterial community level during HABs.

The overall objective of this research was to take a holistic approach to illustrate the interdependency of CyanoHABs with several factors, including water quality parameters and genomic contents in a freshwater peri-urban lake. This study fills an

important gap elated to the dynamics of P and N cycles during CyanoHABs. Unlike a previous publication from this group on the ecology of cyanobacteria in Utah Lake (Li et al., 2019), the objective also included studying N-fixing, P-regulating, and toxin-producing functional genes before the onset, during and after CyanoHABs in addition to spatial and temporal variations in the abundances of different cyanobacteria. The general N and P metabolic pathways and functions were predicted by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (Langille et al., 2013); the functional gene/gene expressions for nutrient scavenging (*nif/pst*) and microcystin-producing (*mcy*) were precisely targeted by qPCR and Reverse-transcript qPCR (Freeman et al., 1999). The main objectives of the study were to: (1) detect the microbial community shift and predict key functional dynamics related to N and P; (2) study the dynamic behavior of *nif* genes, *pst* genes, and *mcy* genes during the CyanoHABs; (3) investigate the correlations among bacterial community presence, functions, and environmental factors.

4.4 Materials and Methods

4.4.1 Sampling Sites

Freshwater Utah Lake, which is located nearly 50 miles south of Salt Lake City, was considered as the model freshwater lake. Utah Lake is the largest natural freshwater lake in the western United States, with a maximum length of 38.6 km and a maximum width of 20.9 km. As a shallow alkaline lake, it has an average depth of 3.0–3.4 m in open water during standard reservoir operating conditions and has calcium-rich sediments. Utah Lake has experienced frequent CyanoHABs in recent years, with the

cyanobacteria cell numbers up to 36 million cells per mL in 2016 and *Aphanizomenon flos-aquae* as the primary species (UDWQ, 2016a). Utah Lake was also shut down for recreational and irrigation purposes as MCs concentrations were detected to be much higher at some locations than the recreational exposure guideline of 10 µg/L by the World Health Organization (Bartram and Chorus, 1999). Site selections were based on the Utah Division of Water Quality's (UDWQ's) regular sampling. All of the sites are in open water and are located near major tributaries with varying depths (Figure 4.1). Deepwater sites (depth > 1 m) included Saratoga Springs, Geneva Discharge, Vineyard Buoy, Provo Buoy, and Bird Island Buoy, while Entrance to Provo Bay and Goshen Bay were the shallow sites, with water levels less than 0.5 m during the regular summertime (e.g., early May to September). Sampling was conducted on a monthly basis from early May to late September in 2018, except for sampling twice in June when CyanoHABs typically occurred, based on historical observation updated warnings issued by UDWQ. During sampling, water samples were collected at three depths at each site and combined onsite into one composite sample. For most in-lab analysis, samples were collected in HDPE sampling bottles following the Standard Operating Procedure for the collection of phytoplankton to detect harmful algal blooms (UDWQ, 2016b). Two exceptions were cyanotoxin and chlorophyll a, measurements of which required the use of glass amber bottles to prevent sunlight exposure. The containers were immediately stored in coolers and transferred to the Environmental Engineering and Microbiology Lab at the University of Utah for further physicochemical and biological analysis.

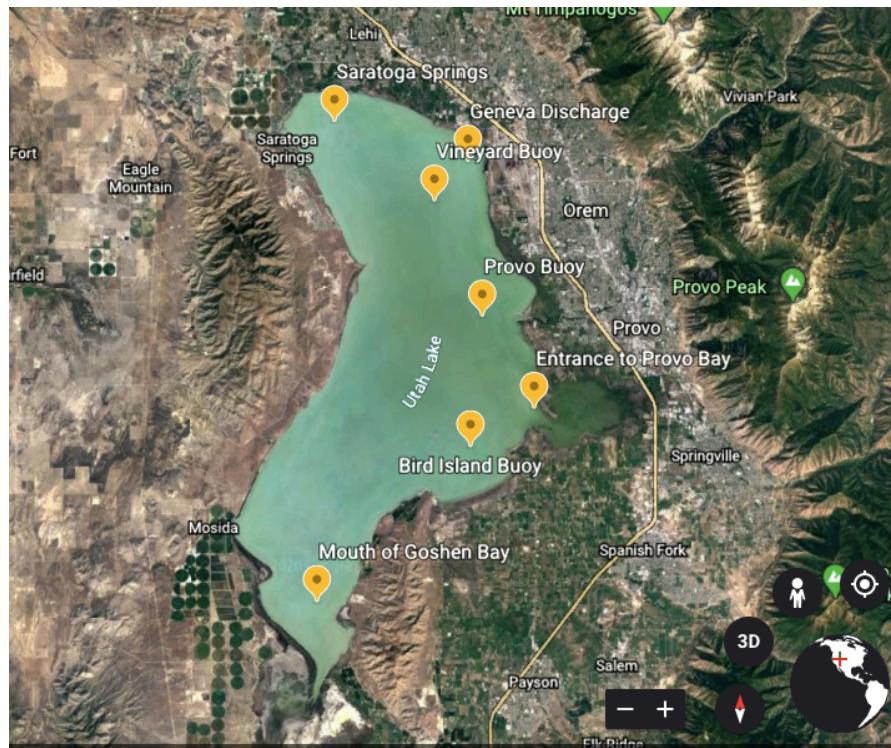


Figure 4.1 Sampling locations.

4.4.2 Measurement of Physicochemical Parameters

Temperature, pH, dissolved oxygen, conductivity, and depth were measured in situ. Samples were directly filtered through 0.22 µm filters (HPLV 4700, Fisher Scientific) before the measurement of soluble nutrients. The filters with planktonic biomass were kept at -20 °C before genomic (Sections 4.2.3 and 4.2.4) analysis and in Invitrogen® RNALater stabilization solution before gene expression (Section 4.2.4) analysis. Dissolved nutrient anions (nitrate-N, nitrite- N, and orthophosphate-P) in the filtrate were analyzed using Ion Chromatography (IC) (Metrohm 883 Basic IC plus) following EPA method 300 (Pfaff, 1993). The ammonia-N, total dissolved nitrogen (TDN), and total dissolved phosphorus (TDP) were measured using Low Range Ammonia TNTplus Vial Test (TNT830, Hach, USA), Total Nitrogen TNT Reagent Set

(LR, Hach), and Total Phosphorus TNT Regent Set (LR, Hach), respectively. Chl a was measured spectrophotometrically and corrected for pheophytin following the standard methods of water and wastewater (APHA, 1999). The dissolved organic carbon was measured by the standard carbonaceous biochemical oxygen demand (cBOD₅) bottle test following EPA 450.1. The microcystin concentrations (MCs) were measured starting in June according to EPA method 544. More precisely, microcystins were extracted from both filtrate (lake water) and filter (algal biomass), concentrated by solid-phase extraction, and detected on a Waters® ACQUITY UPLC with TQD mass spectrometry.

4.4.3 Genomic Analysis of Blooms

4.4.3.1 Genomic DNA Extraction and High-Throughput Sequencing

Genomic DNA was extracted from the filtered biomass using a PowerWater® DNA isolation kit (Qiagen Inc, Valencia, California) according to the manufacturer's instructions. Concentrations were determined on a Thermo® NanoDrop 2000c, and samples with 260/280 ratios higher than 1.80 were selected for further analysis. Before being sent for Illumina® MiSeq sequencing, samples were diluted to 10 ng/µL for a total volume of 10 µL. The amplicon library preparation of the bacterial 16S rRNA gene V4 gene region was conducted in the RTSF Genomics Core at Michigan State University using primer set 515F/806R, following the protocol described by Kozich et al., 2013. The final PCR products obtained from the protocol were batch normalized using Invitrogen® SequalPrep DNA Normalization plates and pooled into each well. The pool was cleaned up using Ampure XP beads, quantified using a combination of Qubit® dsDNA HS, Agilent® Bioanalyzer DNA 1000, and Illumina® Kapa Library Quantification qPCR

assays. It was then loaded onto an Illumina® MiSeq Standard v2 flow cell and sequenced in a 2×250 bp paired-end format using a v2 Standard 500 cycle MiSeq reagent cartridge. Custom sequencing and index primers were added to appropriate wells of the reagent cartridge as described. The Base calling was done by Illumina® RealTime Analysis (RTA) v1.18.54, and the output of RTA was demultiplexed and converted to Fastq format with Illumina® Bcl2fastq v2.19.1.

4.4.3.2 The Analysis of Microbial Community and the Prediction of Metabolic Pathways and Functional Groups

The amplicon sequencing results were analyzed according to QIIME 2 “moving picture” tutorials (<https://docs.qiime2.org/2018.11/tutorials/moving-pictures/>) (Vázquez-Baeza et al., 2013; Bolyen et al., 2018). The overall analysis with read quality filtering, demultiplexing, truncating of paired-end reads, operational taxonomic units (OTUs) formation, alpha diversity analysis, and other analyses were conducted similar to our earlier protocol (Li et al., 2019). The predicted metabolic functions of the microbial community were determined by PICRUSt v1.1.3 (Langille et al., 2013). To do so, features (OTUs or sequence variants) were closed-reference picked using “qiime vsearch cluster-features-closed-reference” against the database that was trained on the Greengenes version 13_5 with 99% identity cluster OTUs from the 515F/806R region of sequences (McDonald et al., 2012). The percent identity at which clustering should be performed (–p-perc-identity) was set at 1 to prevent any mismatches. The generated feature table was exported as a biom format file, which was then used as the input for PICRUSt to predict metabolic function counts by referencing the Kyoto Encyclopedia of

Genes and Genome (KEGG) Orthology (KO) Database (Kanehisa and Goto, 2000; Kanehisa et al., 2014). The function prediction was achieved by processing through scripts of “normalize_by_copy_number.py” and “predict_metagenomes.py.” The accuracy of metagenomic prediction was estimated by the Nearest Sequenced Taxon Index (NSTI) value, which is considered the standard method for validation of KEGG functional groups (Langille et al., 2013; Koo et al., 2017). A lower NSTI value (< 0.15) implies that samples are phylogenetically close in relationship and suitable for PICRUSt analysis. In our analysis, the weighted NSTI values ranged from 0.105 to 0.169 with a mean value of 0.127 ± 0.014 . Thousands of predicted functions were further collapsed into KEGG pathways by “categorize_by_function.py.” The KEGG Orthology (KO) counts of N and P/carbohydrate metabolisms related pathways were plotted using the heatmap option at the log scale in STAMP (Parks et al., 2014).

4.4.4 The Quantitative PCR (qPCR) and Functional Gene Expressions

Using Reverse Transcript (RT)-qPCR

Cloning and sequencing was conducted using mcyAcy/mcyEcya primers (Table 4.1) to identify possible *mcy* gene clusters within the entire bacterial community. Cloning was conducted from the lake biomass using TOPO® TA cloning kit (Invitrogen, USA), and plasmids were extracted by Zippy® Plasmid Miniprep Kit (Zymo Research, USA). The cloned plasmids were sent for Sanger Sequencing at the Health Science Center Cores, University of Utah. The species or target genes were identified by blasting against the National Center for Biotechnology Information (NCBI) database. Based on the cloning sequencing results, real-time quantitative polymerase chain reaction (qPCR) was

Table 4.1 List of Primers Applied

Oligo name	Sequences (5'-3')	Tm (°C)	Target	Length (bp)	Reference
MICf	GCCGC RAGGTGAAA MCT	60	16S rRNA in <i>Microcystis</i>	248	Neilan et al., 1997
MICr	AATCCAAARACCTTC CTCCC				
mcyEcyaf	TTTGGGGTTAAC TTTT TTGGGCATAGTC	56	<i>mcyE</i> or <i>ndaF</i> in	470	Jungblut and
mcyEcyar	AATTCTTGAGGCTGT AAATCGGGTTT		Cyanobacteria		Neilan, 2006
mcyAcyaf	AAAAGTGT TTTATT A GCGGCTCAT	56	<i>mcyA</i> in Cyanobacteria	302	Hisberg ues et al., 2003
mcyAcyaf	ATCCAGCAGTTGAGC AAGC				
mcyAmsf	ATCCAGCAGTTGAGC AA	60	<i>mcyA</i> in <i>Microcystis</i>	171	Furukaw a et al., 2006
mcyAmsr	GCCGATGTTGGCTG TAAAT				
mcyGmicf	CAACCCAACAGGTT C TTAAAGC	60	<i>mcyG</i> in <i>Microcystis</i>	244	Ngwa, 2012
mcyGmicr	TGAGGCAAGGTTCC TCTTG				
nif_nostF3	ATCGTTCAACACGCA GAATTG	60	Ana, Nos, Cyl	90	Lu et al., 2019
nif_nostR3	TCATCCATT CGATA GGTGTGG				
pstSf3	TGGAATGTTACCAGC AGGAATAA	60	A Flos Aq, AFA	110	Lu et al., 2019
pstSr3	AGTGCTGCTTGACGT AAACT				

further performed to quantify total gene copy numbers and reverse transcript qPCR (RT-qPCR) was conducted to estimate expressions of key functional genes responsible for nitrogen fixation, high-affinity Pi-transporter for *Nostocales*, and MCs related genes for *Microcystis*. Primers used to quantify absolute gene copy numbers and genes targeted for expression are listed in Table 4.1. The genomic DNA templates were diluted to 20 ng/ μ L to prevent inhibition at high concentrations. Total RNA was extracted using PureLink[®] RNA Mini Kit (Thermal Fisher, USA) and immediately stored at –80 °C until used. Following RNA extraction, residual genomic DNA was removed from total RNA using an on-column PureLink[®] DNase set (Life Technologies, NY, USA). It was then converted to cDNA by SuperScript[®] VILO mastermix (Thermal Fisher, USA). The qPCR and RT-qPCR reaction mixture are 20 μ L in total, containing 10 μ L 2 \times Power SYBR[®] Green Master Mix (Applied Biosystems, Foster City, CA), 0.5 μ M of each primer, 2 μ L of templates (blank), and 6 μ L nuclease-free water. The quantification cycling was conducted with a QuantStudio[®] 3 Real-Time PCR System (Applied Biosystems) following the process of an initial 2 min at 50 °C and 10 min at 95°C, followed by 40 cycles of 15 s at 95 °C, 30 s at respective annealing temperature, and 30 s at 72 °C. Before running samples, cloned standards for each gene were serially diluted to a range of 10¹ to 10⁶ copies per 20 μ L reaction using 10% purified plasmid DNA and 90% nuclease-free water. After the qPCR process, the template gene copies were directly calculated based on the linear regressions of standards versus the cycle threshold. The copy numbers were multiplied by the diluting factors to gain results of copies/mL of lake water.

4.4.5 Statistical Analysis

Except for the default graph tools in bioinformatics software, all of the figures were created using R studio 1.1.419 (R Development Core Team, 2013). Specifically, the boxplots for predicted metabolic pathways were generated using the R package “ggpubr.” The associations of ambient water parameters, bacterial communities, metabolism prediction results and real-time gene/gene expression quantifications were estimated by Spearman correlation analysis using the R package “corrplot” at a 95% confidence interval.

4.5 Results

4.5.1 Water Quality Parameters

Water parameters were sampled from May to September. Briefly, water temperatures increased from May (16 - 20 °C) to July (25 - 28 °C) and decreased to around 20 °C in September’s. The overall pH increased from May (8.23 – 8.38) to August (8.64 – 8.98) and leveled off in September. The only exception was the Entrance to Provo Bay site when the bloom and pH peaked on June 27th. Chl a and DO results were consistent with pH results. The Entrance to Provo Bay had earlier indicating of bloom in June (Chl a 100 µg/L and DO 10.6 –14.6 mg/L) than the entire lake. The sudden rise in cBOD in the lake was detected in August (5.2 –22.4 mg/L) and September (6.6 –14.3 mg/L). As for cyanotoxins, MC-LR and MC-RR were two main variants of microcystins detected in the lake water, which peaked on June 12th at the sites Mouth of Goshen Bay and Entrance to Provo Bay and another peak on Sep 19th at all sites. The highest concentrations were 0.69 - 1.16 µg/L for MC-LR and 0.15 - 0.26 µg/L for MC-

RR in September.

The nutrient concentrations varied at locations and sampling dates. Generally, ammonium-N, nitrate-N, and ortho-P were measured in the range of 0-0.97 mg N/L, 0-0.09 mg N/L, 0-0.07 mg P/L respectively. Nitrite-N was mostly non-detectable. The south part of the lake showed high concentrations of ammonium-N, as high ammonium concentrations were measured in the Mouth of Goshen Bay (0.97 mg N/L) and Bird Island Buoy (0.63 mg N/L). Nutrients observed a decreasing trend overall except for some sudden increases in ammonium-nitrogen concentrations in July and August. Nitrate-N and ortho-P were non-detectable in August and September's sampling. Because of higher TDN concentrations (0.17 – 7.07 mg N/L) compared with TDP (0.23 - 1.04 mg PO₄³⁻/L), the atomic N:P ratios were generally higher than 16:1, except for some sites in September. Overall, Provo Bay experienced early blooming period in June followed by blooms in other parts of the lake in July, August, and September.

4.5.2 Sequencing Depth and Diversity Analysis

To identify the microbial community composition, a total of 54 samples were collected during different months, from different locations, and were then sequenced. High-throughput sequencing yielded a total of 8,099,216 sequences. A total 6,482,848 sequences remained after quality control and were clustered into 10,163 features. The refraction curves tended to approach the saturation plateau, implying adequate sampling depth and coverages. Shannon's diversity index indicated a relatively lower abundance or evenness of species present in August's sampling. The observed OTU counts were detected moderately lower on May 16th than the other months.

4.5.3 Microbial Community Classification at Phylum and Genus Levels

The taxonomy classification and calculations of relative abundance were based on bacterial 16S sequencing. The microbial community at the phylum level is shown in panel A of Figure 4.2. Actinobacteria, Bacteroidetes, Proteobacteria, and Cyanobacteria were the most observed phyla (Figure 4.2). The relative abundance of cyanobacteria generally increased starting May 16th (7.88–22.7%) onward for subsequent months to 28.2–38.1% in September (depending on the site). However, different sites exhibited varying trends in the relative abundances of Cyanobacteria. For example, the Mouth of Goshen Bay, Bird Island Buoy, and Entrance to Provo Bay sites, which are mostly located toward the south part of the lake, reached the highest relative abundance, varying from 28.6 to 48.3% for cyanobacteria on June 27th when the lake was declared bloom dominated by the UDWQ based on cell counts. On the other hand, sites inside the Provo Buoy, Geneva Discharge, Vineyard Buoy, and Saratoga Springs, which are located toward the north part of the lake, had the highest relative abundance of cyanobacteria varying from 42.6 to 46.3% as observed in the August 9th sampling. Overall, among all sites and sampling occasions, the highest relative abundance for cyanobacteria (48.3%) was detected on June 27th at the Entrance to Provo Bay. Cyanobacteria was the highest phylum in terms of relative abundance during the occurrence of CyanoHABs, while Proteobacteria, Bacteroidetes, and Actinobacteria generally occupied higher relative abundance before or after the blooms (Figure 4.2). A trend of decrease in the relative abundance of heterotrophic bacterioplankton was also observed with increases of cyanobacterial relative abundance.

Due to the focus of this work on lake eutrophication and to further classify the

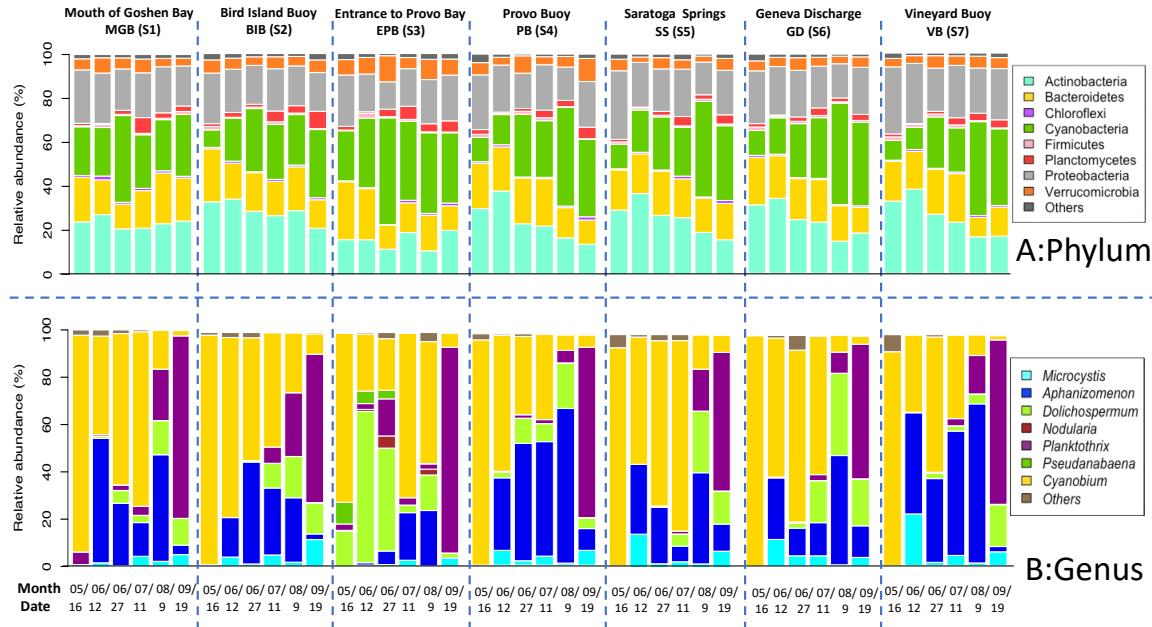


Figure 4.2 Microbial community composition at the phylum (top panel) and cyanobacterial genus level (bottom panel) from different sampling sites. “Others” contains “unknown,” “unclassified,” and taxa with small relative abundance.

taxonomic composition of cyanobacteria, we considered the bacterial assignments at the genus level and compared them within and among sites (Panel B in Figure 4.2).

Cyanobium, *Aphanizomenon*, *Dolichospermum*, *Microcystis*, and *Planktothrix* were the dominant genera present at all sites (Figure 4.2). Initially, picocyanobacteria *Cyanobium* (72.2–99.8%) dominated the lake from May until July. The relative abundances of this genera varied from 91.7% at the Mouth of Goshen Bay to a high percentage of 98.2% Bird Island Buoy and Provo Buoy in May, thus representing itself the dominant cyanobacterial genera. In June and July sampling events, the relative abundances of *Cyanobium* varied between 22.1% and 81.8%, depending upon the site. There is not much information available about the genus *Cyanobium*. One paper by Komárek et al. (1999) suggests a closer relationship between the picocyanobacteria *Synechococcus* and

Cyanobium with the identical thylakoid arrangement.

As for the bloom-forming genera, *Aphanizomenon* and *Dolichospermum* belonging to Nostocales appeared and gradually occupied higher percentages from May end until mid-August. In fact, *Aphanizomenon* was the dominant genera or equally abundant in late June, July, and August samplings at Bird Island Buoy, Provo Buoy, and Vineyard Buoy sites. *Aphanizomenon* and *Dolichospermum* appeared and gradually occupied higher percentages (a maximum of 68.9% for *Aphanizomenon* and 64.6% for *Dolichospermum*) until August's sampling. *Microcystis* was detected at all sites from June, although its relative abundance varied from 0.27–22.3% depending upon the sampling month (e.g., 22.3% at Vineyard Buoy on June 12th). Except for the first peak detected on June 12th, the relative abundance of this genus was generally high in September, varying from 3.66 to 11.4%. *Planktothrix*, mostly a non-diazotroph and common toxin producer in temperate lakes, started to appear in August and dominated in September with relative abundances as high as 58.3–87.9% at all sites. The Entrance to Provo Bay was unique as it formed early filamentous cyanobacteria blooms (mostly *Dolichospermum*) in June, while other sites had a peaked bloom mostly in August.

4.5.4 Function Predictions by PICRUSt

PICRUSt matched 2,961,431 sequences to the Greengenes 13_5 and finally generated 655 features. The weighted NSTI values ranged from 0.105 to 0.169 with a mean value of 0.127 ± 0.014 , suggesting the high reliability of predictions (Koo et al., 2017). Zhu et al., 2018 mentioned the NSTI scores of each sample to be varied from 0.11 to 0.17 (mean=0.14), which were mostly lower than the mean NSTI score (0.17) in soil

samples reported by Langille et al., 2013, suggesting that the predictions were accurate and reliable. Hence, an average NSTI score of 0.127 ± 0.014 seems reasonably reliable. KEGG pathways of cellular process, environmental information processing, genetic information processing, microbial metabolism, and organismal systems at the highest hierarchy were predicted from the sequencing results. The main metabolisms predicted were amino acid (10.3% - 11.7%), carbo- hydrate (9.6% - 11.0%), energy (5.9% - 7.5%), membrane transport (10.3% - 11.4%), and replication and repair (7.1% - 7.6%). However, we primarily focused on nutrient-related pathways as these directly reflect the bacterioplankton assimilations and metabolic dynamics. Figure 4.3 shows these predictions for nitrogen, phosphatase, phosphotransferase, and photosynthesis-related pathways in different panels. Generally, the “nitrogen metabolism” contains genes that regulate the N-fixation (*nif*) and inorganic N reductions (Figure 4.3A). All of them, as well as some N transporters and regulation genes, regulated N cycles in the lake. As for phosphate and carbohydrate assimilations, two of the important pathways for bacterioplankton are “Phosphonate (*phn*) and phosphinate metabolism” and “Phosphotransferase system (PTS)” (Figure 4.3B, C) (Kotrba et al., 2001; Gomez-Garcia et al., 2011). Both pathways are highly correlated with the transferase and utilization of P and carbohydrate in phytoplankton cells. The PTS system enables bacteria to import sugars by forming P derivatives. However, no pathways were reported for the “Inorganic phosphorus scavenging system (*pst*)” due to the limitations of the database whereas our functional gene expressions show high abundances of *pst* between samples (see results later). Photosynthesis was counted relatively higher in August (Figure 4.3D). PICRUSt can only predict gene families that are already known and included in the orthology

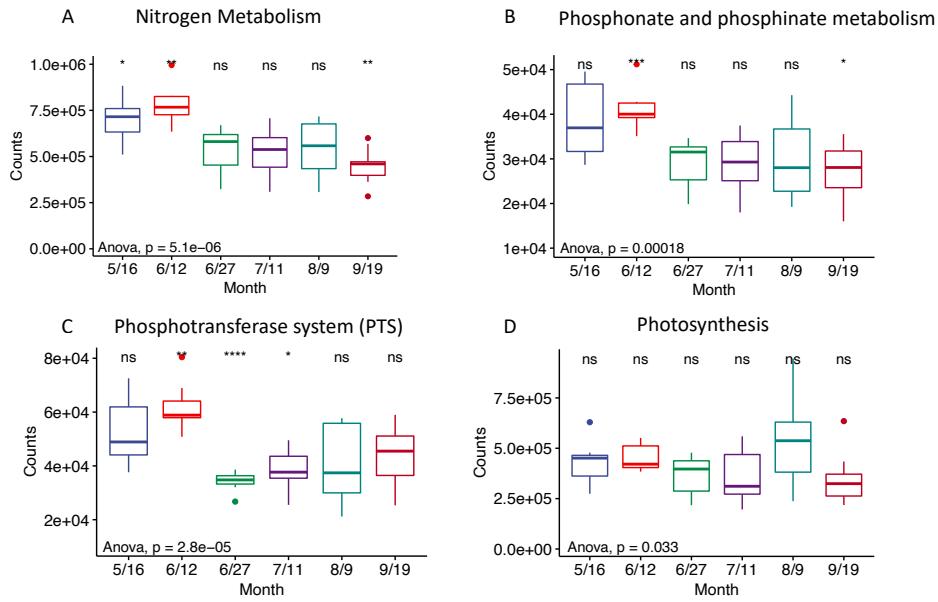


Figure 4.3 Predicted metabolic pathways at a 99% sequence similarity. (A) Nitrogen Metabolism. (B, C) Carbohydrate/Phosphorus related metabolism. (D) Photosynthesis. Note the differences of y-axis scales. Reference group is “all”. The significance levels were symbolled as: ns: $p > 0.05$, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$ ****: $p \leq 0.0001$.

reference used (KEGG KOs) by default. This could be considered as one of the limitations of PICRUSt. Another limitation of PICRUSt includes any gaps or inaccuracies in pathway annotation or assignments of gene function. For example, many KEGG Orthology groups listed as participating in pathways not found in bacteria or otherwise not reflective of true function. This is simply due to bacteria containing homologs of enzymes showing important roles. Therefore, it is worth carefully checking KEGG pathway annotations to ensure that they are reasonable for any system studied. This result together with higher chl a and cBOD content detected in August, potentially implying higher carbon-fixation activities.

The KOs for N, P, and some carbohydrate-related pathways were further

investigated. As for nitrogen cycles, the predicted functions were mainly divided into three groups, namely N-fixation genes (e.g., *nif*), N reduction-related genes (e.g., *nap*, *nir*, *nor*), and N regulatory proteins (e.g., PII) (Figure 4.4). The most abundant counts were nitrate/nitrite reductase subunits, followed by the *nif* related genes and some specific N transporter or reductase proteins. It is noted that some *nif* or N-regulatory proteins were elevated at the end of June, early July, and August (e.g., *nifX*, *nifH*, *nifV*), while some are more abundant in May (e.g., *nifH*, N regulatory protein). By contrast, the main nitrate and nitrite reductase subunits were relatively higher in May and early June and less detectable in August and September, suggesting the availability of inorganic N at the beginning of the summer months.

As for P and carbohydrate-related functions, the KOs in response to assimilation

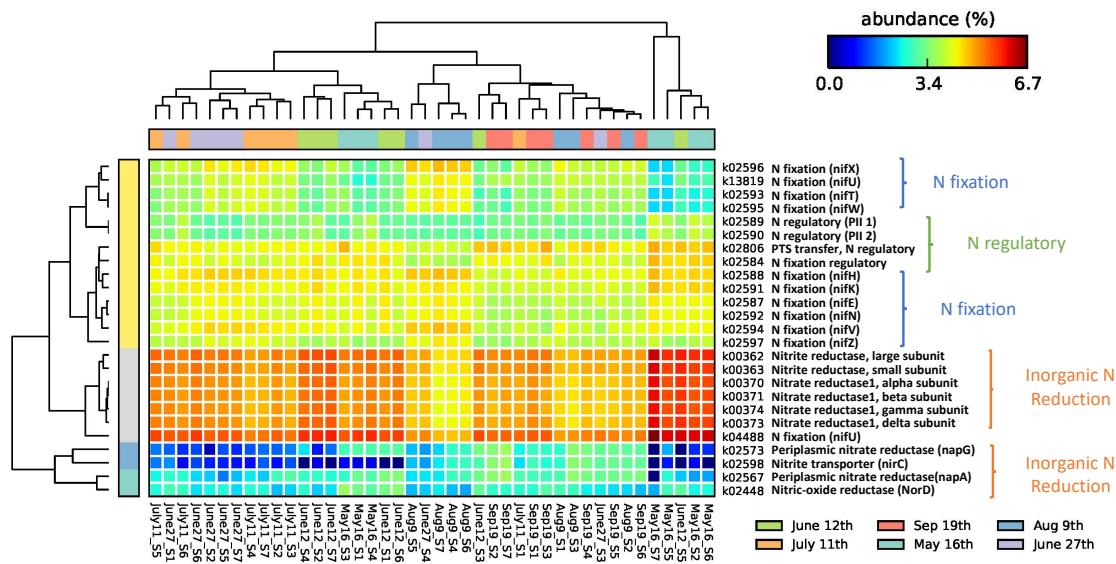


Figure 4.4 KOs correlated with nitrogen metabolisms at log scale (samples were clustered by date).

or coregulated as part of the *Pho* regulon were mostly studied (Figure 4.5). Specifically, polyphosphate kinase (PPK), inorganic pyrophosphatase (PPA), pyrophosphatase (PpaX), guanosine 3'-diphosphate 5'-diphosphate ((p)ppGpp), the inorganic phosphorus transporter (PiT) family, polyphosphate glucokinase (PPGK), and glucose-6-phosphate dehydrogenase (G6PD) were more abundant in May. It is suggested that phytoplankton responded to early summer nutrient stress and started to assimilate P and maybe created storage from the early summer. The exopolyphosphatase (PPX) and fructose-6-phosphate phosphoketolase (F6PPK) counts were elevated in August, indicating the possibility of utilizing stored nutrients during the peak bloom and extreme nutrient limitation conditions. The phosphonate and phosphinate-related proteins were increased in May (*phnB*, *phnP*) or August (*phnM*, *phnJ*), which could be most active in heterotrophic bacteria and some cyanobacteria for organic P utilization (Dyhrman et al., 2009).

Similarly, PTS-related functions were highly abundant, mostly during May or August, for

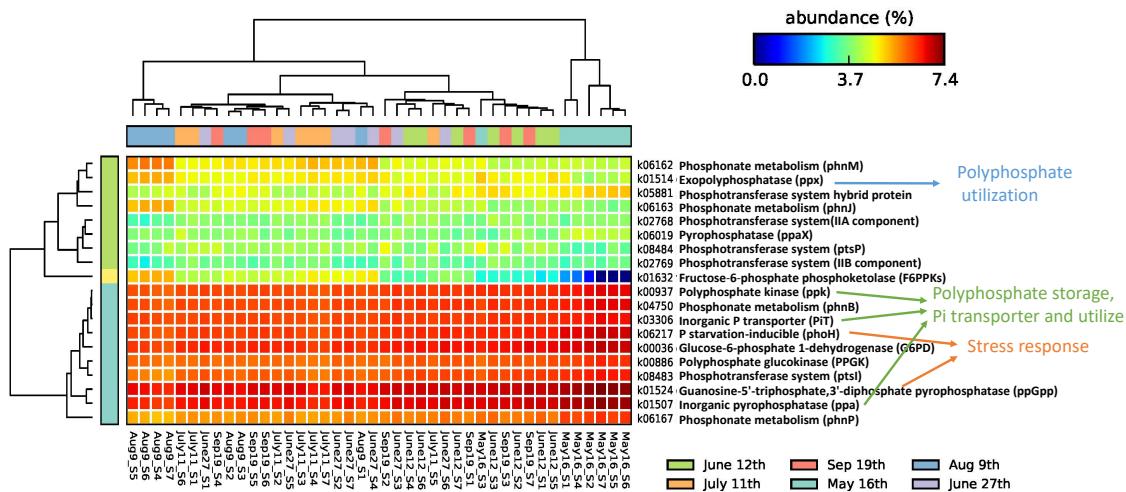


Figure 4.5 KOs correlated with phosphorus and carbohydrate at log scale (samples were clustered by date).

the transportation and phosphorylation of a variety of sugars and sugar derivatives (Deutscher et al., 2014).

4.5.5 Genomic DNA-Based Gene Quantification and mRNA-Based Gene Expressions for Nutrient Starvation and MC Related Genes

The Sanger sequencing of cloned plasmids for the *mcy* gene suggested the presence of *Microcystis panniformis* FACHB-1757 (14 out of 24 cloned) and *Microcystis aeruginosa* strain UV027 (7 out of 24 cloned) in the lake. Nevertheless, no direct evidence was found for common *mcy* gene possessors, such as *Planktothrix* (Christiansen et al., 2003), *Anabaena* (Halinen et al., 2007), or *Dolichospermum* (Teikari et al., 2019). The change of functional genes (e.g., their absolute presence) and their corresponding expressions (based on mRNA) for *Microcystis* 16S (*Mic16S*), microcystin-producing (*mcyA* and *mcyG* in *Microcystis*), N-fixing (*nif*), and high-affinity Pi transport genes (*pst*) along with time are plotted in Figures 4.6 and 4.7. All standards were amplified at efficiencies of nearly 80%. As for the absolute gene copies based on qPCR of genomic DNA, most sites had increased copies for all five genes with time and peaked in either early June or Aug/Sep (Figure 4.6). The trend of the *nif* gene was consistent with the *pst* gene, while *Mic16S*, *mcyA*, and *mcyG* could be considered as a group. The copies of *nif* were mostly close to *pst*, except for the sites Mouth of Goshen Bay and Bird Island Buoy, where *pst* gene copies were 1–2 folds more than *nif* copies. Apart from that, the *nif/pst* gene copies were significantly higher than *Mic16S/mcyA/mcyG* at the Entrance to Provo Bay on June 12th (beginning of bloom) and June 27th (peak of bloom). Overall, the copies for *nif/pst* mostly peaked in the period of June 12th to Aug 9th; however, the

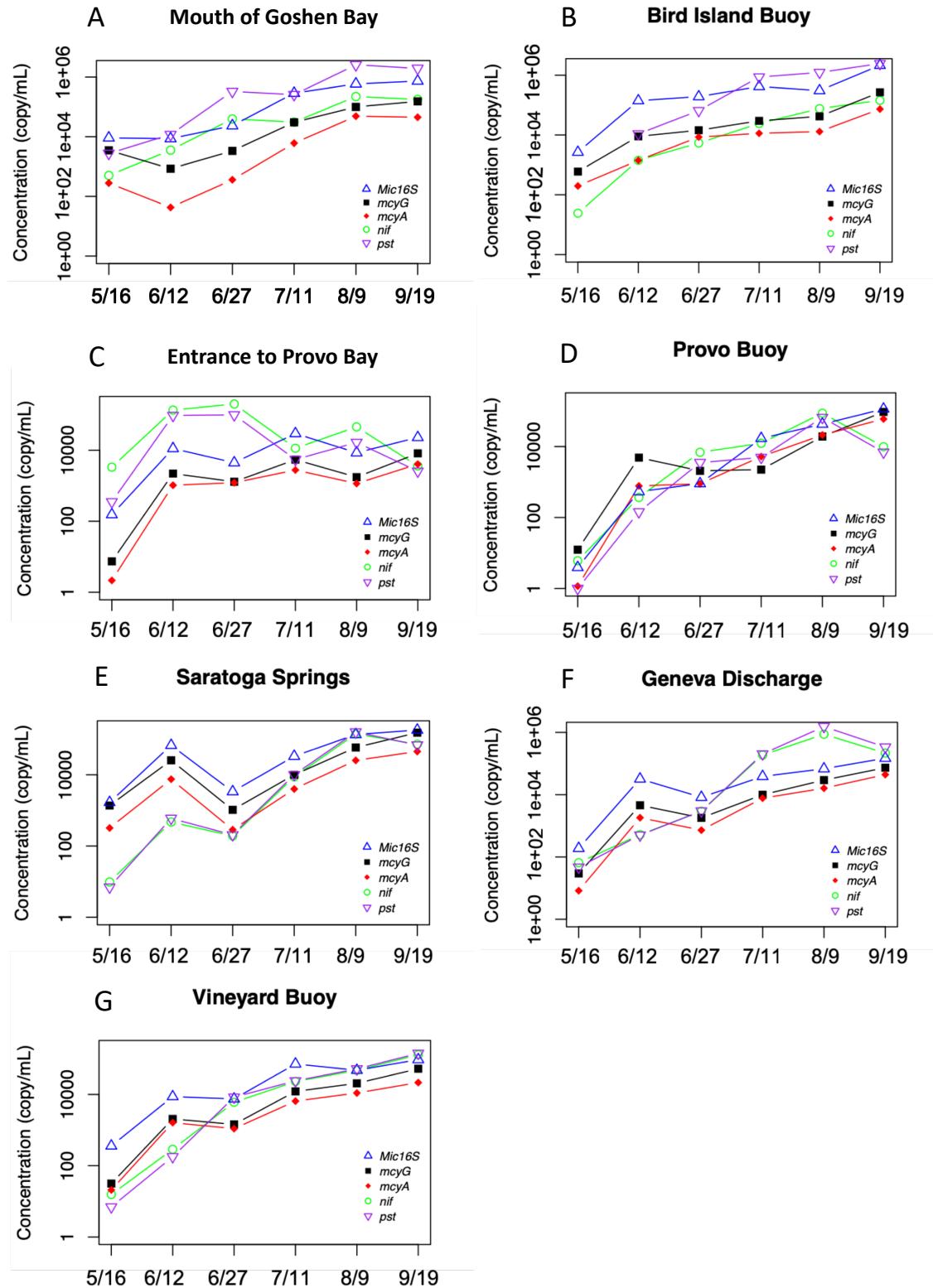


Figure 4.6 qPCR for the quantification of gene distributions.

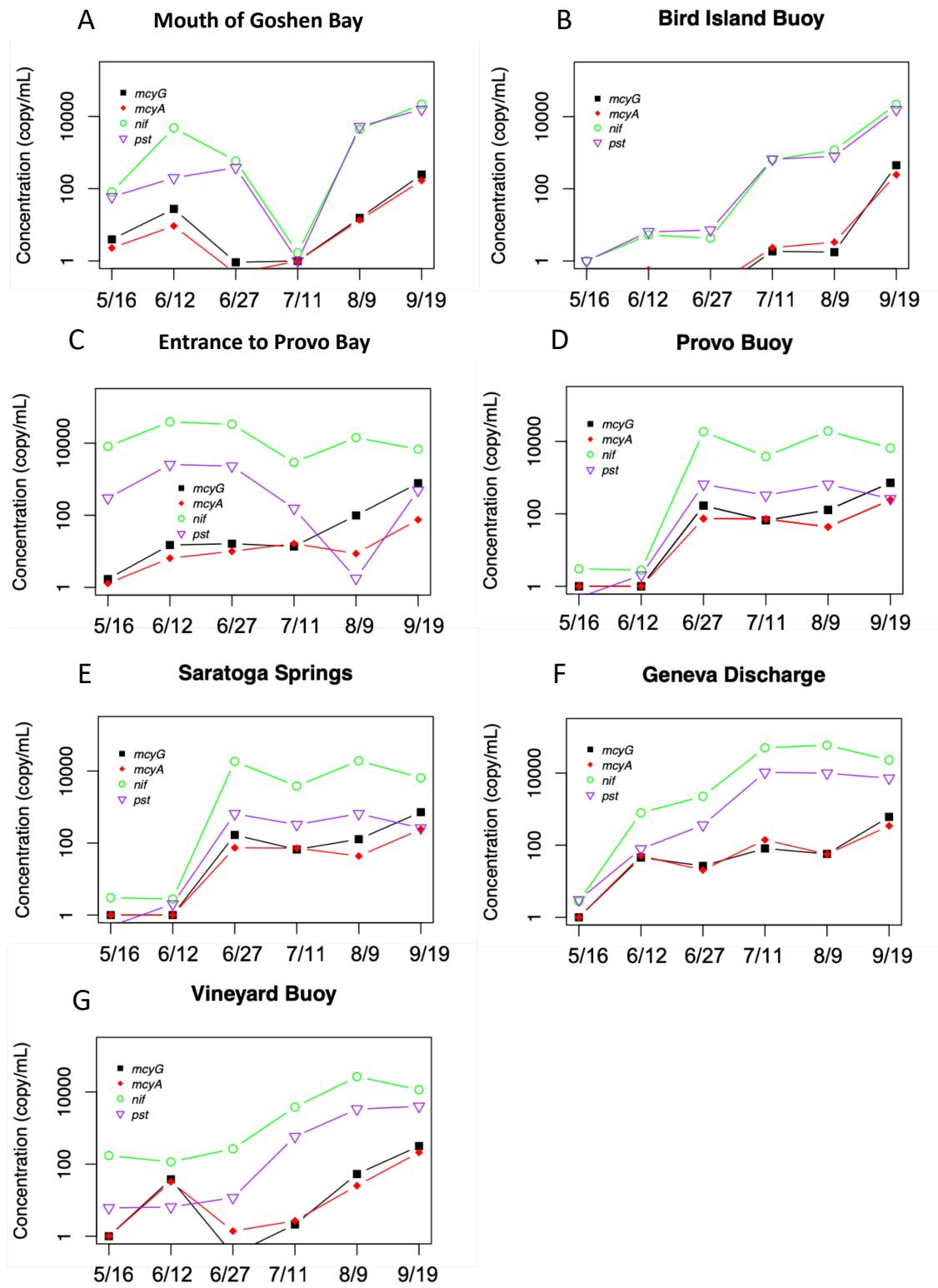


Figure 4.7 RT-qPCR for the quantification of gene expressions.

highest copy numbers for *Mic16S/mcyA/mcyG* were found in September.

Figure 4.7 shows the actual expressions of four functional genes based on mRNA and RT-qPCR. The expression of *nif/pst* genes (0 to 10^5 copies/mL) were relatively higher than *Microcystis*'s *mcyA/mcyG* (0 to 10^3 copies/mL) groups at all the sites. Apart from the sites Mouth of Goshen Bay and Entrance to Provo Bay that had early significant expressions of *nif/pst* on May 16th and June 12th, most other sites experienced an increased expression after June 27th. The expressions of toxin-related genes kept increasing until September. Additionally, there was a small peak on June 12th at sites Mouth of Goshen Bay, Entrance to Provo Bay, Geneva Discharge, and Vineyard Buoy. The gene expressions were at the plateau from the end of June to September for the two deeper sites (Provo Buoy and Saratoga Springs). The *Mic16S* was presented at 4–8 folds and not shown in the graphs.

4.6 Discussion

4.6.1 Community Successions in the Microbial Community

During CyanoHABs

The analysis of bacterioplankton at the phylum level demonstrated the dominance of Actinobacteria, Bacteroidetes, and Proteobacteria in the summer season (panel A in Figure 4.2). The most significant change at the phylum level was the increased relative abundance of Cyanobacteria during the bloom period, which is similar to the findings in 2017 and some other eutrophic lakes (Parulekar et al., 2017; Scherer et al., 2017; Li et al., 2019). Algal blooms generally had a greater effect on community evenness rather than richness, according to the alpha diversity analysis (Berry et al., 2017), which was also

detected by the pielou_e test in this study. For the phylum interactions, Cyanobacteria was significantly negatively correlated with Actinobacteria ($r = -0.84, p < 0.001$), Bacteroidetes ($r = -0.67, p < 0.001$), and Proteobacteria ($r = -0.68, p < 0.001$), based on the Spearman correlations.

In terms of the cyanobacterial community, the summer CyanoHABs in the shallow alkaline lake experienced three distinct stages based on cyanobacterial composition (Panel B in Figure 4.2). Initially, in May, the lake was mainly composed of the genera *Cyanobium* belonging to the phylum Cyanobacteria and to the order *Synechoccales*. *Cyanobium* morphotypes are among the most abundant cyanobacteria in marine environments. As a consequence, their ability to produce toxins can represent a health risk worldwide (Das and Dash, 2019). Among all cyanobacteria, *Cyanobium* had a negative correlation with all the other genera, and the most significant correlation was with *Planktothrix* ($r = -0.81, p < 0.001$). Compared with larger phytoplanktonic cells, picocyanobacteria have a relatively smaller volume and larger surface-to-volume ratios, which enables faster nutrient uptake and growth rates (Suttle and Harrison, 1986). However, special N-fixation capabilities were not reported in either *Cyanobium* or *Synechococcus* (Zehr, 2011), showing them to be outcompeted by other, larger phytoplankton under N starvation.

The second stage was detected in early June when *Aphanizomenon* and *Dolichospermum* appeared in Provo Bay and gradually accounted for a higher percentage of the cyanobacterial community (Figure 4.2). Toxin-producing *Microcystis* also appeared at this stage. The active presence *Microcystis* was further confirmed by absolute *mcy* gene quantification (Figure 4.6) and *mcy* gene expression (Figure 4.7). The bloom of

Aphanizomenon and *Dolichospermum* in Provo Bay continued until the end of June when it gradually spread to the south and north parts of the lake. The early formation of *Aphanizomenon* and *Dolichospermum* community in Provo Bay could be attributed to the richer nutrients, which were rapidly consumed by earlier-stage algae and picocyanobacteria *Cyanobium* and further triggered nitrogen-limitation conditions promoting the dominance of *Aphanizomenon* and *Dolichospermum*. Similar to other eutrophic lakes, this stage could be highly correlated with the N- fixation nature of diazotrophs (Beversdorf et al., 2013; Harke et al., 2015; Lu et al., 2019), which helped dominate the nutrient- limitation conditions. However, different from the lakes that observed cyanobacteria shift to toxic non-diazotrophs soon after the bloom of N-fixers, the relative abundance of *Cyanobium* again increased at some sites. As a result, the co-occurrence of *Cyanobium* and *Aphanizomenon/Dolichospermum* on the entire lake scale lasted for 2–3 months until *Aphanizomenon/Dolichospermum* became the dominant genus at most sites in August. The success of *Aphanizomenon* and *Dolichospermum* could be attributed to their having *nif/pst* gene clusters (Figures 4.6 and 4.7) when available nitrate and ortho-P were extremely scarce in the lake (Beversdorf et al., 2013; Komárek, 2013; Lu et al., 2019). This was confirmed by our water quality sampling, where we found nitrate-N and ortho-P were non-detectable in August's and September's samplings. Further, the coexist of these two genera for a long period of time could be attributed to their similar genomic features, nutrient acquisition systems, and environmental niche (Driscoll et al., 2018).

In the third stage, *Planktothrix*, a genus typically without heterocyst, appeared in August's sampling and became the dominant group in September. A significant

correlation was found between *Planktothrix* and *Aphanizomenon* ($r = -0.14$, $P < 0.01$), because *Planktothrix* dominated after *Aphanizomenon* in the lake. Along with them, the MCs-producing population was also enlarged and more MCs/MCs-producing genes were detected at this stage (Figures 4.6 and 4.7). The previous sampling generally neglected the late summer *Planktothrix* blooms (Li et al., 2019) and attributed the presence of MCs mostly to the lysis of cell debris. The successional stages of CyanoHABs were also identified by alpha diversity. The unique nature of Provo Bay and its relatively higher nutrient conditions made it an easy target for blooms.

4.6.2 Bacterial Dynamics in Relation to Different Environmental Factors

At the bacterial community level, Proteobacteria, Actinobacteria, and Bacteroidetes were observed to be negatively correlated with the bloom indicators (e.g., pH, DO, Chla, and cBDO), but mostly positively correlated with nutrients ($r = 0.03$ to 0.55). The varying r values indicated differences in nutrient requirements among organisms (Schauer et al., 2005; Allgaier et al., 2007; Jezbera et al., 2011), but could generally result in N or P deprivation conditions. Cyanobacteria was significantly negatively correlated with Actinobacteria ($r = -0.84$, $p < 0.001$), Bacteroidetes ($r = -0.67$, $p < 0.001$), and Proteobacteria ($r = -0.68$, $p < 0.001$), based on the Spearman correlations. By contrast, Cyanobacteria responded less to the surrounding nutrient conditions. Some cyanobacteria are affected less by the surrounding nutrient conditions and can trap into nutrient pools that are not typically accessible to other phytoplankton (Cottingham et al., 2015). It could have resulted from their possession of high-phosphorus system that activated under low P conditions (Dignum et al., 2005), as well

as hydrolysis enzymes (e.g., PPA and PPX) that can release ortho-P from pyro- or polyphosphates (Gómez-García et al., 2003). The N-fixation strategy is another tool for cyanobacteria to bring new “N” source into the ecosystem and fuel the phytoplankton community (Schindler et al., 2008; Beversdorf et al., 2013; Scott and Grantz, 2013). Among all cyanobacteria, *Cyanobium* had a negative correlation with all the other genera and the most significant negative correlation was with *Planktothrix* ($r = -0.81, p < 0.001$). *Cyanobium* and *Microcystis* were positively correlated with nitrate-N ($r = 0.23 - 0.39, p < 0.05$) and ortho-P ($r = 0.09 - 0.36, p < 0.05$). Compared with larger phytoplanktonic cells, picocyanobacteria have a relatively smaller volume and larger surface-to-volume ratios, which enables faster nutrient uptake and growth rates (Suttle and Harrison, 1986). However, only a few of them have special nutrient management strategies (e.g., N-fixation), causing them to be outcompeted by other, larger phytoplankton under nutrient starving conditions.

Another significant correlation was found between *Planktothrix* and *Aphanizomenon* ($r = -0.14, P < 0.01$), because *Planktothrix* dominated after *Aphanizomenon/Dolichospermum* in the lake. As for their response to nutrients, *Dolichospermum* and *Planktothrix* were negatively related to all the nutrients measured ($r = -0.1$ to 0.44). The increased temperature effect was positive for *Aphanizomenon* ($r = 0.53, p < 0.001$) but negative for *Planktothrix* ($r = -0.14, p < 0.01$). It is reported that *Aphanizomenon flos-aquae* could grow above 8°C with an optimum temperature ranging from 23 to 29°C (Tsujimura et al., 2001). Although *Planktothrix* favored higher temperatures ($> 25^{\circ}\text{C}$) (Lürling et al., 2013; Gomes et al., 2015), our study did observe a dominance of *Planktothrix* when the temperature fell to around 20°C .

4.6.3 The N Metabolisms of Bacterial Community and Successions

The N metabolisms mostly correlated the bacterial activities with the N-fixation, nitrite/nitrate reduction, and nitrogen genes regulation during different periods of bloom (Figures 4.4, 4.6, and 4.7). Cyanobacteria bring new “N” sources into the ecosystem with strategies such as N-fixation under N-limiting conditions (Schindler et al., 2008; Beversdorf et al., 2013; Scott and Grantz, 2013). Although the lake was not N-limited most of the time, except for some occasions in September based on the TDN:TDP ratios ($> 16:1$), potential N-fixers (e.g., *Aphanizomenon* and *Dolichospermum*) were some of the most dominant genera found in nitrate and ortho-P depleted conditions in August. Interestingly, *Aphanizomenon* was positively related to all the *nif* genes (*nifZ*, *nifW*, *nifV*, *nifT*, *nifK*, *nifN*, *nifX*, *nifE*, and *nifU*) reported in this study ($r = 0.60$ to 0.79 , $p < 0.001$). *Aphanizomenon* is long found as a genus dominating summer assemblages and tends to enhance the growth of non-fixing cyanobacteria afterward (Elser et al., 2000; Beversdorf et al., 2013; Lu et al., 2019). Additionally, *Dolichospermum* is positively linked with *nif/nif* expressions ($r = 0.79$ to 0.89 , $p < 0.001$), as it is known for the possession of heterocysts at regular intervals across the fil-ament (Komárek, 2013) and blooms with/without N limitation (Yema et al., 2016; Scherer et al., 2017).

Different from the N-fixation period, nitrate and nitrite reductase subunits were relatively higher in the early summer (Figure 4.4). The correlation analysis found that heterotrophic bacterioplankton (Actinobacteria, Bacteroidetes, and Proteobacteria) were positively linked with many nitrate reductase subunits ($r = 0.27$ to 0.84 , $p < 0.05$) but negatively linked with *nif* gene and gene expressions ($r = -0.21$ to -0.73 , $p < 0.05$). Among cyanobacteria, *Cyanobium* was negatively linked with *nif* gene/gene expressions

($r = -0.65$ to -0.70 , $p < 0.01$), but positively correlated with the presence of some nitrate and nitrite reductase subunits ($r = 0.34$ to 0.49 , $p < 0.05$) (Figure 4.4). These cyanobacteria are known for not having heterocysts for N-fixation. This phenomenon could result from the significant composition of nitrate reducers in Proteobacteria, Actinobacteria, Firmicutes, and Cyanobacteria groups (Bru et al., 2007; Palmer and Horn, 2012; Zhao et al., 2015). In fact, except for a few N-fixers, most cyanobacteria rely on ammonia assimilation followed by glutamine synthesis and photosynthetic nitrate assimilations for biosynthesis in CyanoHABs (Andriesse et al., 1990; Flores et al., 2005; Muro-Pastor et al., 2005). Apart from that, cyanobacteria can act as a carbon source for predominant denitrifiers to remove N from the lake (Chen et al., 2012), creating an N-limitation environment. This condition may also favor the long-term stay of N-fixers in the lake.

As for N gene regulation systems, the N regulatory system PII has a higher abundance in May or early June, while the *nif*-specific regulation protein was also detected to be relatively high during other periods (Figure 4.4). The N regulatory system PII did not significantly affect or correlate with any communities, which is surprising as it contains a broad group of signal transduction proteins present in Bacteria, Archaea, chloroplasts in Algae and plants (Herrero et al., 2001; Huergo et al., 2013). By contrast, the *nif*- specific regulatory protein was negatively correlated with *Aphanizomenon* and *nif* gene ($r = -0.34$, $p < 0.05$), which is contrary to the finding that detected upregulation of *nif*- specific proteins under N-fixation conditions (Yan et al., 2010).

4.6.4 The P and Some Carbohydrate Metabolisms of Bacterial Community and Successions

The P and carbohydrate metabolisms detected a trend to report and respond to nutrient starvation (PPK, (p)ppGpp, PiT, Pho, and G6PD) during early summer, while utilizing the stored polyphosphate (PPX and F6PPK) during the extreme ortho-P scarce period, mostly in August or September (Figure 4.5). The dominance of cyanobacteria during blooms could have resulted from their possession of a high-affinity Pi transport system that activated under low Pi conditions (Dignum et al., 2005), as well as hydrolysis enzymes (e.g., PPA and PPX) that can release ortho- P from pyro- or poly-phosphates (Gómez-García' et al., 2003). Specifically, the PPX gene is activated at P starvation conditions to degrade poly-P (Adams et al., 2008), which is highly correlated with the presence of *Aphanizomenon* ($r = 0.77, p < 0.001$). Similar to PPX, PPA and PPaX are commonly upregulated under Pi-limitation conditions that convert pyrophosphates into two phosphate ions (Gómez-García' et al., 2003; Harke and Gobler, 2013). In this study, the PPA and PPaX were activated early in May, when *Cyanobium*/heterotrophic bacterioplankton were the dominant groups and more pyrophosphate could be present (Fuszard et al., 2013). Apart from that, F6PPK was also highly elevated in August and significantly positively correlated with the presence of *Aphanizomenon* ($r = 0.79, p < 0.001$), which was a crucial enzyme involved in the central carbohydrate metabolism in heterofermentative bacteria and recently characterized in *Anabaena* sp. PCC 7120 (Moriyama et al., 2015). Cyanobacteria (especially *Aphanizomenon*) was also found positively correlated with “Phosphonate and phosphinate metabolism” related KOs ($r = 0.82, p < 0.001$), such as PhnM and PhnJ proteins, which are responsible for the

hydrolysis of the C-P bond (Metcalf and Wanner, et al., 1993). It is not surprising that cyanobacteria may utilize organic matter (e.g., phosphonates), as it may give them more competition over autotrophs at Pi-limiting conditions (Gilbert et al., 2004; Vahtera et al., 2007; Harke et al., 2012; Harke and Gobler, 2013; Teikari et al., 2018).

By contrast, more functions related to nutrient storage and acquisition into cells were elevated during the early summer, including some stress or starvation-induction functions (Figure 4.5). For example, the presence of PPK was slightly correlated with *Aphanizomenon* ($r = 0.08$) but highly correlated with heterotrophic bacterioplankton ($r = 0.28$ to 0.61 , $p < 0.001$), which is a highly conserved region in prokaryotes and responsible for the reversible polymerization of ATP to make polyphosphate for Pi storage in cells (Brown and Kornberg, 2004). PPGK is a polyphosphate-dependent glucokinase found in many organisms, such as diazotrophic cyanobacteria (Klemke et al., 2014; Albi Rodríguez and Serrano, 2015), and was positively correlated with *Aphanizomenon* ($r = 0.33$, $p < 0.01$). Moreover, the G6PD was activated in May, not significantly correlated with *Aphanizomenon* or *Dolichospermum*, but it was reported to be reluctant for N-fixation in heterocysts and respiration for vegetative cells under dark conditions (Summers et al., 1995). Furthermore, Pi transporters represented by *pst* clusters were upregulated starting in May (Dyhrman and Haley, 2006; Pitt et al., 2010). Specifically, the PiT family was only positively correlated with *Aphanizomenon* ($r = 0.33$, $p < 0.01$), while the *pst* gene/gene expression quantified by qPCR and RT-qPCR were positively correlated with *Aphanizomenon*, *Dolichospermum*, and *Planktothrix*. It is reported that *Nostocales* (e.g., *Aphanizomenon* and *Dolichospermum*) are selectively promoted by high P/low N conditions (Suikkanen et al., 2013; Andersson et al., 2015).

Their associations with *pst* clusters may provide new thoughts into the P assimilation and explain their dominance in the lake's summer nutrient-limiting conditions (Lu et al., 2019).

The stress and starvation-inducible functions were induced in May (Figure 4.5). Specifically, the phosphate starvation-inducible protein PhoH was upregulated during May and faded slightly after (Santos-Beneit, 2015). Although it's not clear evidence in the study, the activation of *pho*-like genes would cause an increase in both Pi uptake and polyphosphate accumulation rates (Morohoshi et al., 2002). Moreover, stringent response signaling (p)ppGpp, a stress response alarmone in response to amino acid starvations and mediate polyphosphate accumulation under nutritional stress (Kuroda, 2006; Abranches et al., 2009), was found activated in early May. It is commonly accumulated at the initial stage of heterocyst formation and triggered by darkness in cyanobacteria (Zhang et al., 2013; Hood et al., 2016).

4.6.5 The Potential MCs- Producing Cyanobacteria and Associations with Nostocales

Similar to other studies that observed an interaction of N-fixers and toxin-producing strains (Elser et al., 2000; Beversdorf et al., 2013; Chia et al., 2018; Lu et al., 2019), the lake also experienced a pre-toxic period in June and a post-toxic bloom in September, together with the bloom of *Nostocales*. The microcystin concentrations increased near the Provo Bay area in June, together with the observation of the first significant evidence of N-fixing genes/gene expressions or N-fixers. It was also correlated with the time when *Microcystis* initially appeared in the lake. However, the

relative abundance of *Microcystis* decreased during June and again increased in September with enhanced total MCs concentrations at the sites Provo Buoy, Saratoga Springs, Geneva Discharge, and Vineyard Buoy. The post-toxic bloom was observed in September when CyanoHABs were considered to retreat due to temperature effects. The UDWQ also reported the detection of microcystins above 0.1 µg/L in the open water area until November (UDWQ, 2018). Before September, the entire lake experienced 2–3 months' dominance of *Aphanizomenon/Dolichospermum* from June to August. Above all, two peaks of *Microcystis* (up to 10^6 copies/mL 16S rRNA gene) were observed on June 12th (at some sites) and Sep 9th (the entire lake), which is right after or coexistent with the presence of filamentous cyanobacteria. Since *Microcystis* coexists or blooms after N-fixing cyanobacteria, it is safe to hypothesize that N-fixation would be one of the main N-providing pathways (Beversdorf et al., 2013).

The correlation analysis showed a significant negative correlation between toxin-producing genes/gene expressions (Figures 4.6 and 4.7) and *Cyanobium* ($r = -0.61$ to -0.78 , $p < 0.005$), while showing positive correlations with *Planktothrix* ($r = 0.62$ to 0.73 , $p < 0.05$) and *Microcystis* ($r = 0.39$ to 0.62 , $p < 0.1$). Moreover, the presence of MC-LR and MC-RR are significantly correlated with the *16SMic/mcyA/mcyG* gene and gene expressions ($r = 0.33$ to 0.8 , $p < 0.05$). As for the detected MCs producers, *Microcystis aeruginosa* is commonly presented in many eutrophic lakes, while *Microcystis panniformis* is a species originated from tropical lakes (Bittencourt-Oliveira et al., 2007). *Microcystis panniformis* was first reported in Lake Taihu, a temperate lake, implying global warming has driven its distribution from tropical zones to subtropical zones (Zhang et al., 2012). Typically, the proliferation of *Microcystis* is a phenomenon in many

eutrophic lakes and was affected by N availability in the lake (Xu et al., 2010). Under P deficient conditions, the increases of MCs production were observed due to the activation of *Pho* regulon (Oh et al., 2000; Harke and Gobler, 2013). However, the N-limitation typically decreased the net microcystin- production by decreasing the specific cell division rate (Orr and Jones, 1998; Harke and Gobler, 2013). *Microcystis* and *Planktothrix* have distinct morphologies and functions (Guellati et al., 2017), however, they are both bloom-forming, potential MCs-producing cyanobacteria, and their co-habitation was seen in some freshwater lakes (Nixdorf et al., 2003; Paerl et al., 2011; Davis et al., 2014; Francy et al., 2016). Typically, *Planktothrix* dominated lakes with high TDP and low light conditions (Bonnilla et al., 2012). Their proliferation may be complemented with N sources produced by N-fixers. In this study, lower temperatures in late summer could be one of the main factors favoring *Planktothrix* (27.5 °C, Lürling et al., 2013) rather than *Microcystis* or *Aphanizomenon*, which require relatively higher growth temperatures (Reynolds, 2006).

4.7 Conclusions

By screening bacterial communities, specific functions, and monitoring water quality changes, we successfully found linkages among these parameters. Our data suggested long-term dominance of N-fixing cyanobacteria in the eutrophic Utah Lake. The shift of the cyanobacterial community was driven by both environmental factors and metabolism dynamics, especially N and P metabolisms in the lake. The results suggested that the long- term dominance of *Aphanizomenon* and *Dolichospermum* in the lake could have been attributed to their activation of the nitrogen fixation (*nif*) and P-affinity (*pst*)

genes under nutrient stress conditions. Additionally, the cop-presence of *Aphanizomenon* and *Dolichospermum* in the lake at different sampling events depicted the functional redundancy of in CyanoHABs. The results suggested how genomic contents can influence cyanobacterial diversity and richness, along with environmental factors such as nutrients. Additionally, cyanobacteria may function in early summer to initiate a starvation alert and store nutrients, while utilizing the stored nutrients or turn on N-fixation during heavy CyanoHABs and nutrient-limiting conditions. Excess N, fixed by diazotrophic filamentous cyanobacteria, could also be the food supplying the succeeding growth of *Microcystis* and *Planktothrix*. The detection of *Microcystis panniformis*, an MCs-producing species originated from the tropical zone, may imply potential climate change in subtropical areas. The correlations with different functions (e.g., organic matter utilization) suggested that cyanobacteria may develop varieties of metabolites to acquire energy. For future suggestions, sampling frequencies could be increased to closely monitor the community successions during CyanoHABs. More attention needs to be paid to late summer community succession and the post-toxic period (August and September). It is suggested that the reduction of nutrient input into the lake alone may not prevent CyanoHABs, but could reduce the toxicity levels by supplying fewer nutrients to non-fixing communities, thus preventing possible community shifting from nontoxic to toxic species in the future. Results acquired from this study could be helpful in identifying mechanisms in freshwater lakes that observed a co-existence or succession of various members of a cyanobacterial community with distinct functions.

4.8 References

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CHAPTER 5

THE NITROGEN AND PHOSPHATE RELATED STRINGENT RESPONSE OF CYANOBACTERIA AND BACTERIOPLANKTON COMMUNITY AT DIFFERENT STAGES OF A CYANOBACTERIAL BLOOM

5.1 Abstract

The bloom of cyanobacteria in freshwater lakes is harmful and has become a persistent issue in the ecosystem. The diverse responses to abiotic and biotic traits enhanced the complexity of the system, making it difficult to resolve the issues. The biotic factors were less noted than the abiotic factors, although many factors such as interspecific interactions and functional diversity greatly influenced cyanobacterial blooms. To further look into the biotic variations, metagenomics and metatranscriptomics analysis were conducted for samples collected from different stages (i.e., before, during and after the peak bloom) of two sampling sites. Specifically, the metagenome-assembled genomes (MAGs) were recovered, and the genes involved in N and P cycling, as well as the environmental stringent response, were predicted. Transcriptomics profiling was conducted by normalizing samples and mapping transcripts back to the generated gene files. As a result, 527 and 251 refined MAGs were generated for the sites Provo and Geneva, respectively. Cyanobacteria has activated

several functions to tackle the relative nutrient starvation conditions. From the study of the dominant cyanobacterial and bacterioplankton MAGs, it is noted that the ammonium-N is the central component of the N cycle, as the filamentous cyanobacteria (e.g., *Aphanizomenon flos-aquae* and *Dolicheopsernum circinale*) dominated the N cycling by conducting N₂-fixation and assimilatory nitrate reduction. In contrast, the transcript counts for bacterioplankton-dominated pathways (e.g., denitrification) were negligible. Besides, the *pst* transporters and Pho regulon were activated to enhance the utilization for P. The obtaining of alkaline phosphatase genes in cyanobacteria (e.g., *PhoA* in *Dolicheopsernum*, *PhoX* in *Planktotrix* and *Microcystis*) suggested that they can actively synthesize/release alkaline phosphatase and decompose the ambient organic P into bioavailable forms. Similarly, genes related to the poly-P storage and stringent response alarmone (p)ppGpp synthesis/hydrolysis were also activated during the bloom. The N-Pi-stringent response associations may function to resist the starvation conditions, outcompete the bacterioplankton and thus sustain the harmful blooms. Inconsistency was found between physicochemical parameters and some transcripts. Remarkably, the heterocyst control and glutamine synthesis kept increasing even in the “after-bloom” period. These findings suggest that resolving cyanobacterial blooms probably takes more than just the reduction of external nutrient loadings.

5.2 Keywords

Cyanobacterial blooms, nutrient starvation, N cycling, Pho regulon, stringent response.

5.3 Introduction

Harmful cyanobacterial blooms have been a global environmental issue that threatens the sustainability of freshwater ecosystems (Paerl, 2018). It results in surface water quality deterioration, high economic losses and health-threatening (Dodds et al., 2009; Brooks et al., 2016; Carmichael & Boyer, 2016). Despite the ongoing research and budget applied, the frequency and intensity of these blooms are increasing over time (Posch et al., 2012; Xiao et al., 2019). The recovery effort is likely hampered by climate change and the systems' complexity that responds to varieties of abiotic and biotic traits (Wells et al., 2015; Visser et al., 2016).

Freshwater lake systems have varieties of microbial communities. Some groups are more “important” than others by actively involved in primary production and nutrient cycling. As most phytoplankton well-managed carbon sources by carbon-fixation and photosynthesis, nitrogen (N) and phosphorus (P) become the two most limiting nutrients in the aquatic environment (Elser et al., 2007). Traditionally, primary production in freshwater systems have been viewed as P limited, and measures to restrict P input in lakes were successfully taken in 1970s (Stump et al., 2012; Schindler et al., 2016). N was more noted as a limiting nutrient in the last decades due to the cooccurrence and dominance of non-diazotrophs (e.g., *Microcystis* and *Planktothrix*) in harmful algal blooms with the enhancement of N loadings (Paerl et al., 2011; Harke et al., 2016a). The dominance of these taxonomies also impacts bloom toxicity by producing the N-rich cyanotoxins-microcystins, which is a hepatotoxin for humans and prevails in many harmful cyanobacterial blooms worldwide (Carvalho et al., 2008; Backer et al., 2010; Francy et al., 2016; Hu et al., 2016). Even if the importance of restricting N is noted,

long-term lake studies demonstrated that reducing N input also favored bloom towards N-fixing cyanobacteria (e.g., *Dolichospermum* and *Aphanizomenon*) and therefore could not effectively control cyanobacterial blooms (Schindler et al., 2008; Higgins et al., 2018). Even worse, the bloom of diazotrophs not only bring new “N” source into the ecosystem to meet their demands but may fuel the phytoplankton community and sustain the growth of toxin-producing and non-diazotroph taxonomy (Beversdorf et al., 2013; Lu et al., 2019; Li et al., 2020). Therefore, there remains a debate regarding the most effective and cost-efficient measures to predict and curb harmful cyanobacterial blooms. Climate changes may worsen the current situation as the lake eutrophication is likely to intensify with the rising surface water temperature (Whitehead et al., 2009; Rigosi et al., 2014), suggesting the urgent need to understand more about the complex systems.

The cyanobacteria and bacterioplankton have evolved different ways to cope with nutrient starvation and environmental stress. Although some may utilize organic or other complex forms of nutrient (e.g., urea, dissolved free amino acid and organic P), the inorganic N and P are the primary and limiting nutrient sources for most phytoplankton (Markou et al., 2014; Moschonas et al., 2017; Schoffelen et al., 2018). For example, cyanobacteria that cannot fix N will form dormant-like cells to survive prolonged periods of nitrogen starvation, which can resist the stringent conditions better than the vegetative cells (Klotz et al., 2016). Additionally, toxic *Micocystis* in poor N conditions tend to have slower growth and toxin production than the nontoxic strains in the replete N conditions (Long et al., 2001; Vézie et al., 2002; Harke & Gobler, 2013). Not to mention the N-fixers that potentially dominated the lakes with low N:P ratios (Smith, 1983; Havens et al., 2003). Similar starvation responses were also found for P starvation. The inorganic

phosphate (Pi) transport is essential for all organisms to live. With the excess Pi in the ambient environment, the low-affinity phosphate transporter system (PiT) is the primary channel for Pi assimilation, whereas the high-affinity Pi transporter (*pstSABC*) is repressed (Santos-Benito et al., 2008; de Almeida et al., 2015). Under Pi limitation, the *pstSABC* transporter is activated with ATP consumption to cope with the starvation condition. The *pstSABC* transporter belongs to phosphate (Pho) regulon, which is a global regulatory mechanism involved in bacterial Pi management and was first discovered in *E.coli* (Wanner & Chang, 1987). In addition to Pi transport, Pho regulon also capable of obtaining Pi from organic phosphates by excreting extracellular alkaline phosphatase (APase), and store energy through synthesis and forming of polyphosphate (poly-P) (Monds et al., 2006; Gomez-Garcia et al., 2013). Recently, the activation of Pho regulon was more detected in cyanobacteria and related blooms (Su et al., 2007; Harke et al., 2016b; Lu et al., 2019; Li & Dittrich, 2019). However, discussion of the complete Pho regulon, the central phosphorus assimilation pathway in a cyanobacterial bloom, is hitherto lacking.

In addition to the activation of the above metabolism, bacteria can also activate stringent response regulatory systems, changing the cell conditions from fast growth to growth arrest (Masuda 2012). The alarmones involved in stringent response are guanosine tetraphosphate and guanosine pentaphosphate (collectively referred to as (p)ppGpp) (Hsieh & Wanner, 2010). The(p)ppGpp has been proved to be sensitive to many types of stress, including but not limited to light-dark (Puszynska & O’Shea, 2017), nutrition deprivation (Jain et al., 2006), heat shock (Schäfer et al., 2002), and oxidative stress (Fritsch et al., 2020). Recently, more works have brought to light links between the

production of (p)ppGpp and N/P starvation response. For instance, close relationships were found between the activation of Pho regulon and (p)ppGpp (Lamarche et al., 2008; de Almeida et al., 2015). Additionally, it seems that stringent response is widely distributed in cyanobacteria and responded to N limitation (Atkinson et al., 2011; Jin et al., 2020). For non-N fixers, *Microcystis* upregulated stress-related genes and exerted protective effects by promoting colony formation and reducing oxidative damage (Jin et al., 2020). For N-fixers, the ppGpp commonly accumulated at the initial stage of heterocyst formation (Zhang et al., 2013). Regardless, studies are still lacking to study the larger scale's stringent response during harmful cyanobacterial blooms. Whether the ppGpp production is activated during a harmful cyanobacterial bloom and its associations with the N and Pi starvation metabolisms is still questioned.

To tackle the question, metagenomic and metatranscriptomic analysis was conducted to examine the draft genomes of cyanobacteria and bacterioplankton, and their gene expression at different stages of a cyanobacterial bloom. The metagenomic binning was used to construct the metagenome-assembled genomes (MAGs) of the microbial community inhabiting the surface water of a freshwater lake. The near-complete MAGs were annotated for N, Pi and stringent response-related pathways, and served as reference genomes for the gene expression profiling of the community. A similar pipeline was well applied for the community metabolic analysis of anammox granules (Lawson et al., 2017). Utah Lake is the lake sample and was severely hampered by harmful cyanobacterial blooms in this decade. It has diverse cyanobacterial taxonomy and experienced community successions from N-fixers in early and mid-summer to the non-fixers in the late summer (Li et al., 2020). The recovery of dominant bacterial genome

and their metabolic work will contribute to understanding the mechanisms of cyanobacteria dominance in the ecosystem, their successions and correlations with bacterioplankton.

5.4 Methods

5.4.1 Sample Collection

Six samples were collected from two independent sites from Utah Lake in 2018. Site Provo is a shallow Bay and site Geneva represents more about the open water area. Samples were collected from different periods, aiming for before heavy bloom, during- and after- the bloom. Provo Bay (the shallow site) has an early blooming period than the rest of the lake. Samples selected for Provo were from early May, mid-June and July, while the samples for Geneva were selected from mid-June, August and September.

5.4.2 Water Quality Parameter Analysis

Temperature, pH, dissolved oxygen, conductivity, and depth were measured in situ. Water samples were directly filtered through 0.22 µm filters (HPLV 4700, Fisher Scientific) before measuring ambient soluble nutrients. Filters with planktonic biomass were kept at -20 °C before metagenomic analysis and in Invitrogen® RNALater stabilization solution before metatranscriptomic analysis. Dissolved nutrient anions (nitrate-N, nitrite-N, and orthophosphate-P) in the filtrate were analyzed using Ion Chromatography (IC) (Metrohm 883 Basic IC plus) following EPA method 300 (Pfaff, 1993). The ammonium-N, total nitrogen (TN), and total phosphorus (TP) were measured using Low Range Ammonia TNTplus Vial Test (TNT830, Hach, USA), Total

Nitrogen TNT Reagent Set (LR, Hach), and Total Phosphorus TNT Regent Set (LR, Hach), respectively. The chlorophyll a was analyzed as an indicator for eutrophication status following the standard methods of water and wastewater (APHA, 1999). The dissolved organic carbon was measured by the standard carbonaceous biochemical oxygen demand (cBOD₅) bottle test following EPA 450. The microcystin concentrations (MCs) were measured starting in June according to EPA method 544.

5.4.3 DNA, RNA Extraction, and Illumina NovaSeq Sequencing

The DNA and RNA were extracted separately from stored filters using PowerWater ®DNA isolation kit (Qiagen Inc, Valencia, California) and PureLink® RNA Mini Kit (Thermal Fisher, USA), respectively. Following RNA extraction, residual genomic DNA was removed from total RNA using an on-column PureLink® DNase set (Life Technologies, NY, USA). Extracted DNA and RNA were kept in -80 °C until further analysis. Quality check and concentration confirmation were conducted using an Invitrogen® Qubit 4 Fluorometer before the library prep. The DNA and RNA library were constructed using the Illumina® TruSeq DNA PCR-Free Library Prep Kit and Illumina® TruSeq Stranded Total RNA Library Prep Kit. Sequencing was performed on an Illumina® Novaseq 6000 platform, its fastest production-scale sequencing instrument, through the High-Throughput Genomics (HTG) at the Huntsman Cancer Institute (HCI), University of Utah. A hundred million paired reads were equally shared by three samples, and the length of the reads is 150 bp.

5.4.4 Metagenomics and Metatranscriptomics Analysis

FastQ files with quality parameters were obtained after sequencing. The quality of raw paired-end reads was checked using FastQC v0.11.7 (Andrews, 2010). Following this, the quality trimming and filtering of poor-quality reads were achieved by Trimmomatic v0.38 (Bolger et al., 2014) at the criteria of minimum leading and trailing score of 3, slidingwindow at 4:15, and the minimum length of 36, and adapter removal. After quality filtering, the metagenomic pipeline follows assembly, binning, bin refine and annotation. Specifically, samples from sites Geneva and Provo were co-assembled separately using metaSPAdes v3.13.0 (Bankevich et al., 2012) with a range of kmer values (21, 33, 55, 77, 99, 127). The contigs longer than 1 kbp were retained. To prepare binning, assembled contigs were indexed and aligned to raw reads using BWA v1.1.4 (Li & Durbin, 2009). The generated SAM file was subsequently converted to a bam file, sorted, and organized using samtools v1.9 (Li et al., 2009). The depth of the converted bam file was calculated by *jgi_summarize_bam_contig_depths* in MetaBAT v2.12 (Kang et al., 2019). The contigs larger than 1500 bp were binned into draft metagenome-assembled genomes (MAGs) by MetaBAT v2.12 using their default parameters. The recovered MAGs were profiled and checked for quality using the *lineage_wf* function of CheckM v1.0.7 (Parks et al., 2015). MAGs with completeness higher than 10% and contamination greater than 5% were manually refined using Anvi'o following their metagenomic pipeline (Eren et al., 2015). The bins were finally refined to completeness higher than 10% and contamination less than 5%. The taxonomy and metabolic functions of refined MAGs were further annotated using Prokka v1.14.6 (Seemann, 2014), GTDB-Tk v 0.3.2 (Chaumeil et al., 2020) and MicrobeAnnotator v.3.9.8 (Ruiz-Perez et al.,

2021) using the default functions. After all, the refined MAGs were mapped back to the quality-filtered reads to obtain the relative abundance of the entire community. The relative abundance of MAGs was estimated based on the proportion of a bin relative to the number of reads, which was adjusted by the size of bins.

For metatranscriptomics analysis, rRNA reads were removed by SortMeRNA v2.1 (Kopylova & Touzet, 2012) by mapping to their default rRNA database. Similar to the metagenomics analysis, raw non-rRNA reads were quality filtered before subsequential analysis using Trimmomatic v0.38. The quality controlled non-rRNA reads from six samples were normalized individually by in-silico normalization in Trinity v.2.8.5 (Haas et al., 2013) with maximum coverage of 50X. The normalized reads were then mapped to the predicted genes of refined MAGs for the transcript abundance estimation. It used the Kallisto alignment-free abundance estimation methods in Trinity v.2.8.5 (Haas et al., 2013; Bray et al., 2016). The parameters of transcripts length, effective length, estimate counts and transcript per million (TPM) were obtained from the abundance estimation.

5.4.5 Statistical Analysis

In addition to the bioinformatic analysis, analysis and figures were also conducted using R version 4.0.3 (R Development Core Team, 2013). Especially, the heatmaps with annotations were created using package ‘heatmap3’. The Pearson correlation analysis between genome relative abundance and transcript counts (TPM) was analyzed using the package ‘corrplot’ at a 95% confidence interval. The chord diagram displaying the inter-relationships between different transcripts was created using the in package ‘circlize’.

The bar plots were created by the OriginPro 2021b release (OriginLab, USA).

5.5 Results

5.5.1 Water Quality Parameters

The water quality parameters were analyzed from both Provo and Geneva sites before, during and after the bloom. Provo Bay (the shallow site) has an early blooming period than the rest of the lake, including the Geneva site. The heavy bloom was indicated by the higher chlorophyll a concentrations during the bloom period for Provo (98.21 µg/L) and Geneva (131.72 µg/L) than other bloom periods. Specifically, the chlorophyll a concentrations increased by 347% and 640% from before- to during- the bloom for the sites Provo and Geneva, respectively. Other than chlorophyll a, the DO concentration, pH and TN:TP ratio were also detected higher for both sites during the bloom period. The inorganic nutrients were deprived during the entire growing seasons of algal bloom. The ammonium-N concentration was measured below 0.03 mg N/L. The nitrate-N and ortho-P were mostly non-detectable except for the before-bloom period of the site Geneva. Moreover, the TP and TN were in the ranges of 0.11 – 0.16 mg P/L and 0.43 – 4.80 mg N/L. According to the *USEPA Nutrient Criteria Technical Guidance Manual: Lakes and Reservoirs*, essentially an ambient TP concentration of greater than about 0.01 mg/L and or a TN of about 0.15 mg/L is likely to predict blue-green algal bloom problems during the growing season. For both sites, TP concentrations were relatively lower during the heavy bloom period. Although the inorganic nutrients were deprived, high amounts of complexed organic forms of nitrogen and phosphorus were presented in the lake. Different variations of microcystins (MCs) were detected for the

two sites. The MC concentrations were detected highest during-bloom for Provo and after-bloom for Geneva.

5.5.2 The Relative Abundance of the Draft Genome and Phylogenetic Analysis

Quality controls were made for metagenomics and metatranscriptomics analysis. For metagenomics, 97.4% to 98.3% raw reads were retained for each sample to have an average per base Phred Quality Score of about 36. The binning using Metabat2 generated 499 and 251 raw bins for sites Provo and Geneva. As a stagnant eutrophic shallow bay, Provo Bay was known for heavy nutrient pollution and higher species abundance. With higher than 10% completeness, 58% of total raw bins for Provo and 51% for Geneva proceeded for further refinement. The Anvi'o refine finally generated 527 and 251 bins with higher than 10% completeness and less than 5% contamination. The cyanobacterial MAGs account for 7.8% (Provo) and 7.6% (Geneva) of total refined bins. Bacterioplankton MAGs with N metabolism module were mainly discussed to understand nitrogen cycling during the bloom. The bin statistics for part of these cyanobacterial and bacterioplankton bins (all bins with N metabolism module and >30% completeness) were listed in Table 5.1 and discussed throughout the study. The MAGs belong to the same taxonomy have similar GC% content.

The relative abundance of the draft genomes before, during, and after the bloom was shown in Figure 5.1. As a complex natural freshwater system, the refined and recovered bins account for 26.8% (Provo) and 21.6% (Geneva) of the total assembled reads. About 10% - 12.84% of reads belonging to refined bins were assigned to

Table 5.1 Good Bins

ID	Taxonomy	Completeness (%)	Contamination (%)	Predicted genes
G_PLANK	s_Planktothrix agardhii	95.74	0.66	3778
P_PLANK	s_Planktothrix agardhii	95.2	2.73	4152
	s_UKL13-PB			
P_APHA	sp001593825	80.22	1.78	3118
	s_UKL13-PB			
G_APHA	sp001593825	43.1	1.72	1719
	s_Dolichospermum			
P_DOLI	circinale	75.67	2	2533
	s_Dolichospermum			
G_DOLI	circinale	73.89	1.56	3205
	s_Microcystis			
P_MICR	aeruginosa_A	71.96	1.83	3477
	s_Microcystis			
G_MICR	aeruginosa_A	29.48	0	1233
G_VULC1	g_Vulcanococcus	39.11	1.72	1194
G_VULC2	g_Vulcanococcus	32.76	3.45	871
P_VULC	g_Vulcanococcus	51.91	1.9	1260
P_PSEU	g_Pseudanabaena	38.22	1.72	2043
P_CYAN1	f_Cyanobiaceae	37.09	4.48	1076
P_CYAN2	f_Cyanobiaceae	48.28	1.72	2041
P_CYAN3	g_PCC7001	54.01	2.31	1434
P_ANAE	c_Anaerolineae	90	1.09	4940
P_AQUA	g_Aquabacterium	46.88	3.08	1722
P_BALN	o_Balneolales	30.17	0	1491
P_ELST	g_Elstera	41.93	0	2459
G_FLAV	g_Flavobacterium	79.1	1.7	2009
P_GEMM1	o_Gemmatimonadales	44.09	4.4	1553
P_GEMM2	o_Gemmatimonadales	40.96	2.2	2037
P_HYLE	g_Hylemonella	79.77	0	2446
G_PEDO	o_Pedosphaerales	79.96	3.28	3218
P_PEDO	o_Pedosphaerales	91.18	4.49	3577
P_RUBR	g_Rubrivivax	59.48	0	2643
G_RUBR	g_Rubrivivax	30	3.45	1116
P_SPHI	g_Sphingopyxis	86.13	3.58	3675
P_TERR	f_Terrimicrobiaceae	35.09	0.68	1746
P_SOLI	f_Solirubrobacteraceae	97.01	0	3878
G_SOLI1	f_Solirubrobacteraceae	93.59	0	4142
G_SOLI2	f_Solirubrobacteraceae	77.08	1.21	2487
G_SOLI3	f_Solirubrobacteraceae	71.46	3.87	1622

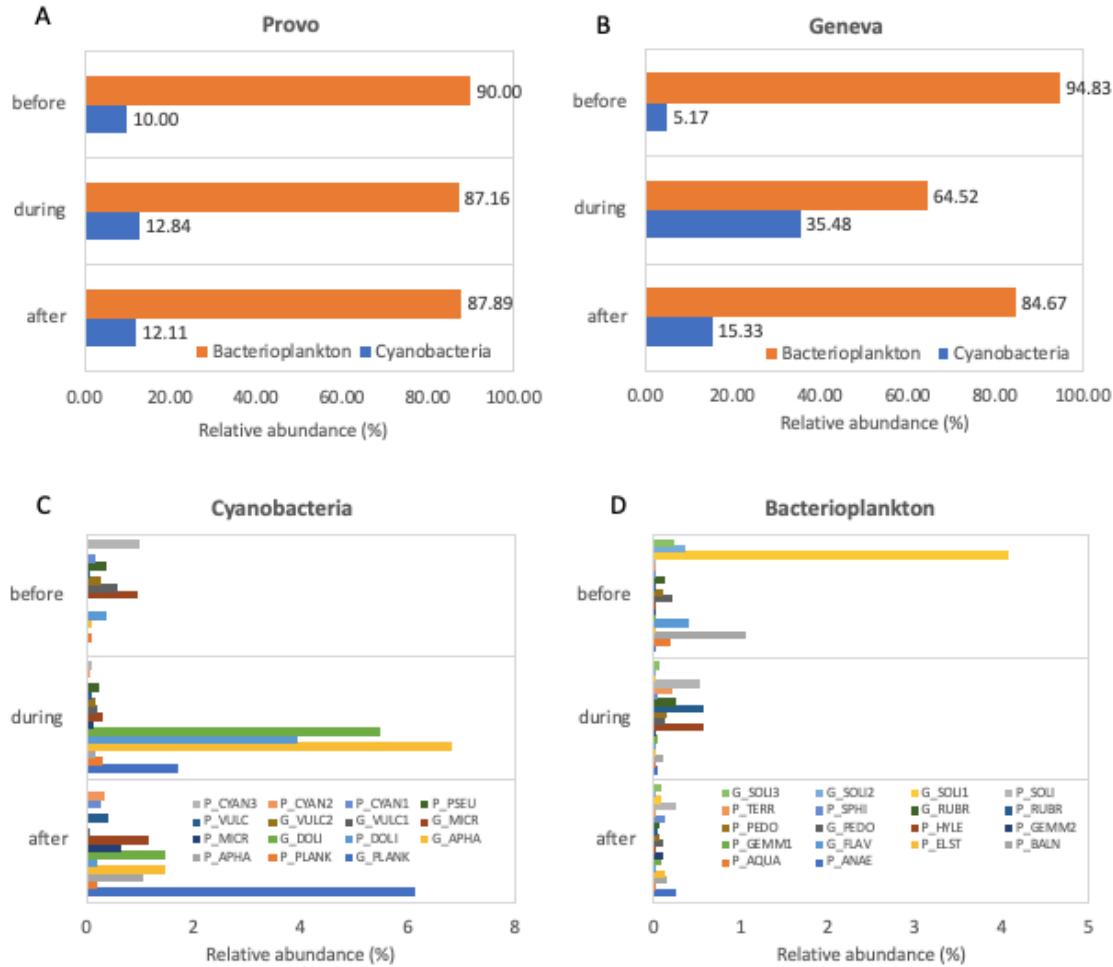


Figure 5.1 Genome relative abundance.

cyanobacteria at the sites of Provo (Figure 5.1A) and 5.17% - 35.48% at the sites of percentage during the bloom period for Geneva than the Provo site. Figures 5.1C and D detailed the relative abundance variations of individual MAGs (all bins with N metabolism module and >30% completeness) for cyanobacteria and bacterioplankton. Specifically, the cyanobacteria MAGs observed the highest abundance during the bloom (19.72%), followed by the after-bloom period (13.44%). In contrast, bacterioplankton MAGs found the highest abundance before the bloom (6.86%). Among the

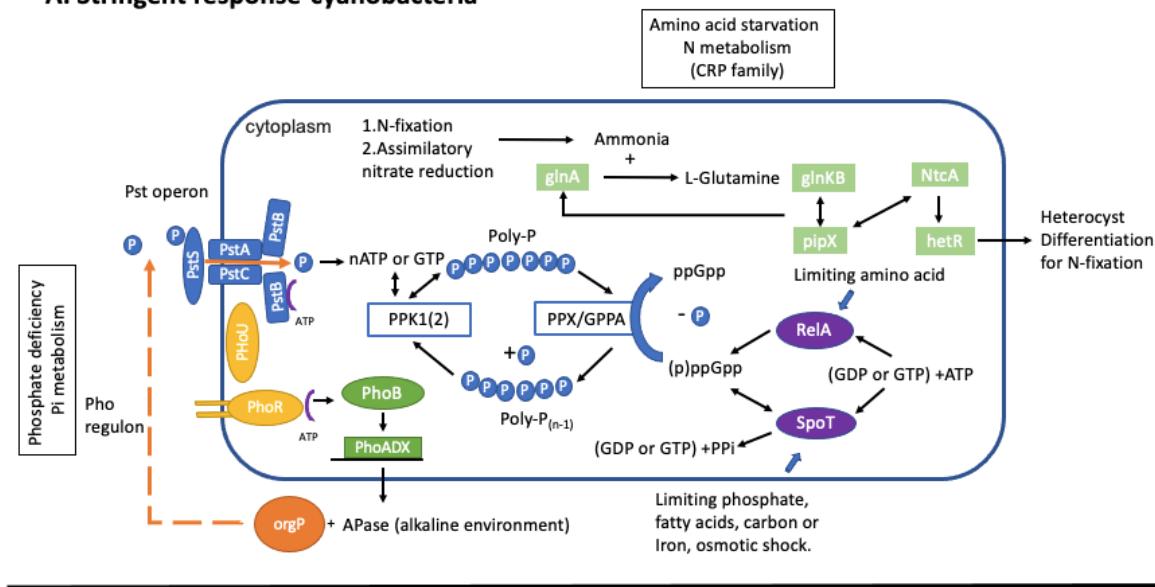
cyanobacterial MAGs, *Dolichospermum circinale* was the dominant species during the bloom for both sites (3.92% for P_DOLI and 5.50% for G_DOLI). *Aphanizomenon flos-aquae* was also the primary MAG during Geneva's bloom (6.84% for G_APHA). *Planktothrix agardhii* dominated the after-bloom period of the site Geneva. Compared with them, *Microcystis aeruginosa* was in relatively lower relative abundance for both sites (<1.2%). Similar community successions were discussed in our previous 16S sequencing analysis (Li et al., 2020). As for bacterioplankton, the dominated MAGs belong to the Solirubrobacteraceae (4.08% for G_SOLI1) and Balneolales (1.06% for P_BALN) before the algal bloom. Other dominant bacterioplankton belong to Pelagibacterales, Nanopelagicales, Kapabacterials, Burkholderiales, Verrucomicrobiales, Chloroflexi and others, not in the main discussion of this paper.

5.5.3 Variations of Transcriptomics at Different Stages of Bloom

5.5.3.1 Model of Stringent Response Mediated Nutrient-Scavenging during a Cyanobacterial Bloom

According to the current literature and our transcripts quantification, the model of stringent response of cyanobacteria and bacterioplankton for nutrient-scavenging may work closely, as described in Figure 5.2. Specifically, both tend to activate the stringent response system during a cyanobacterial bloom. The transcripts of polyphosphate kinase *PPK* (*PPK1* and *PPK2*) (Zhang et al., 2002) and exopolyphosphatase/guanosine pentaphosphate phosphohydrolase (*PPX/GPPA*) were detected, which suggested bacteria actively store polyP and release Pi as an energy source and P_i reservoir (Rao et al., 2009). Additionally, the synthetase/hydrolase of the stringent alarmone molecules (p)ppGpp

A. Stringent response-cyanobacteria



B. Stringent response-bacterioplankton

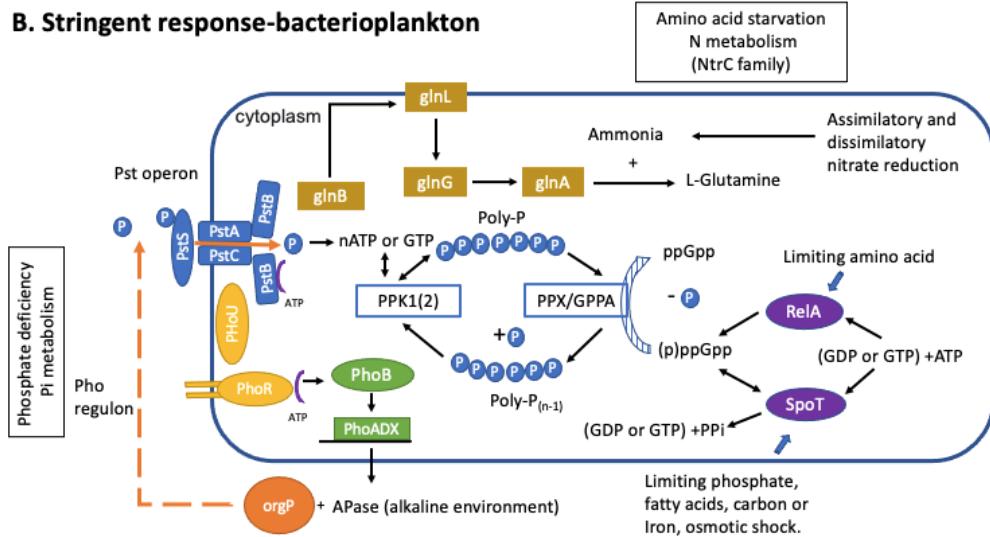


Figure 5.2 Models for stringent response for cyanobacteria and bacterioplankton.

were activated. It is generated by the reaction of GDP or GTP and ATP, mediated by enzyme *relA* in response to amino acid starvation and bifunctional *spoT* for all other forms of stress (e.g., carbon and Pi starvation) (de Almeida et al., 2015). With the enhanced expression of the (p)ppGpp, bacteria strongly inhibited all macromolecule synthesis and led to growth arrest. Although both forms are subjected to stringent response, the ppGpp usually is more active than pppGpp (Mechold et al., 2013). The conversion of pppGpp to ppGpp is conducted by the enzyme GPP (pppGpp phosphohydrolase). However, recent studies also underscored that exopolyphosphatase /guanosine pentaphosphate phosphohydrolase (*PPX/GPPA*) may play essential roles in poly-P and ppGpp homeostasis (Malde et al., 2014; Rohlfing et al., 2018). The *pstSABC* Pi transporter was found activated to cope with orthophosphate (Pi) shortage (Uluşeker et al., 2019). It destabilized *PhoU* and further phosphorylated *PhoR- PhoB*, a two-component system most studied in *E.coli* with the Pi starvation (Tommassen et al., 1982). The activated *PhoR-PhoB* operon initiated the transcription of alkaline phosphatase (e.g., encoded by *PhoA*, *PhoX* and *PhoD* in prokaryotes), which plays an important role in the mineralization of dissolved organic phosphorus (DOP) into bioavailable Pi to promote cyanobacterial uptake (Kageyama et al., 2011; Ma et al., 2019; Zhang et al., 2020). Among alkaline phosphatase, the *PhoA* was long found to respond to P starvation depending on the induction of *PhoR-PhoB* operon, while the *phoX* and *phoD* genes were later found to belong to Pho regulon, as well as by functional assessment of putative *PhoB* binding sites (Hulett et al., 1994; Monds et al., 2006).

In addition to the above stringent response, cyanobacteria and bacterioplankton also detected to response to N starvation, but with slightly different N pathway and

regulatory systems. Notably, cyanobacteria acquired inorganic N mainly through N₂-fixation (some filamentous cyanobacteria) and assimilatory nitrate reduction assimilation (ANRA). At the same time, the bacterioplankton incorporated the dissimilatory nitrate reduction assimilation (DNRA), ANRA, denitrification or the combined. Similarly, quite different N regulations were found. The N regulatory P_{II} protein (*glnB*) is a regulator of glutamine synthesis and N metabolisms in response to N availability in many bacteria, archaea and eukaryotes (Son & Rhee, 1987; Arcondéguy et al., 2001; Muro-Pastor et al., 2005). NtrC is an N-limitation regulator, and its operon *glnALG* activated under N starvation in heterotrophs like *E.coli* in mediating glutamine synthesis (Pahel et al., 1982; Brown et al., 2014). It was found in some of the bacterioplankton (Figure 5.2 B). It also found that NtrC can activate the *relA* gene during nitrogen starvation in *E.coli* (Brown et al., 2014). For cyanobacteria, the global N regulator *ntcA* which belongs to the CRP family of bacterial transcriptional effectors, is widely spread and highly conserved (Frías et al., 1993; Lee et al., 1999). It also corresponds with the signal transduction protein P_{II} and the coactivator PipX (a global controller in cyanobacteria) in response to the 2-oxoglutarate (2-OG) accumulation during the N starvation, as well as being an activator for heterocyst differentiation (*hetR*) (Frías et al., 1994; Espinosa et al., 2014). Additionally, the stringent response metabolites ((p)ppGpp) was involved in the N starvation mechanisms of heterocystous and non- heterocystous cyanobacteria, whereas the direct correlation between *relA* and *ntcA* was not yet cleared (Calderón-Flores et al., 2005; Zhang et al., 2013; Jin et al., 2020).

5.5.3.2 The N Pathways of Dominant Cyanobacteria and Bacterioplankton

To further investigate the phytoplankton N utilization during bloom and confirm the hypothesis, the N metabolism and photosynthesis KEGG modules for the well-refined MAGs (all bins with N metabolism module, >30% completeness and <5% contamination) are shown in Figure 5.3. Most cyanobacteria MAGs (left heatmap) have assimilatory nitrate reduction modules, while more bacterioplankton MAGs (right heatmap) contain dissimilatory nitrate reduction and denitrification gene sets. Some bacterioplankton MAGs also have assimilatory nitrate reduction modules or combined N pathways (mixed assimilatory/dissimilatory nitrate reduction and denitrification). No nitrification module was found. The existence of complete ammonia oxidation pathway (i.e., comammox) was in doubt, as only a small portion of the module (the nitrate reduction gene portion) was completed. The only MAGs that contain nitrogen fixation

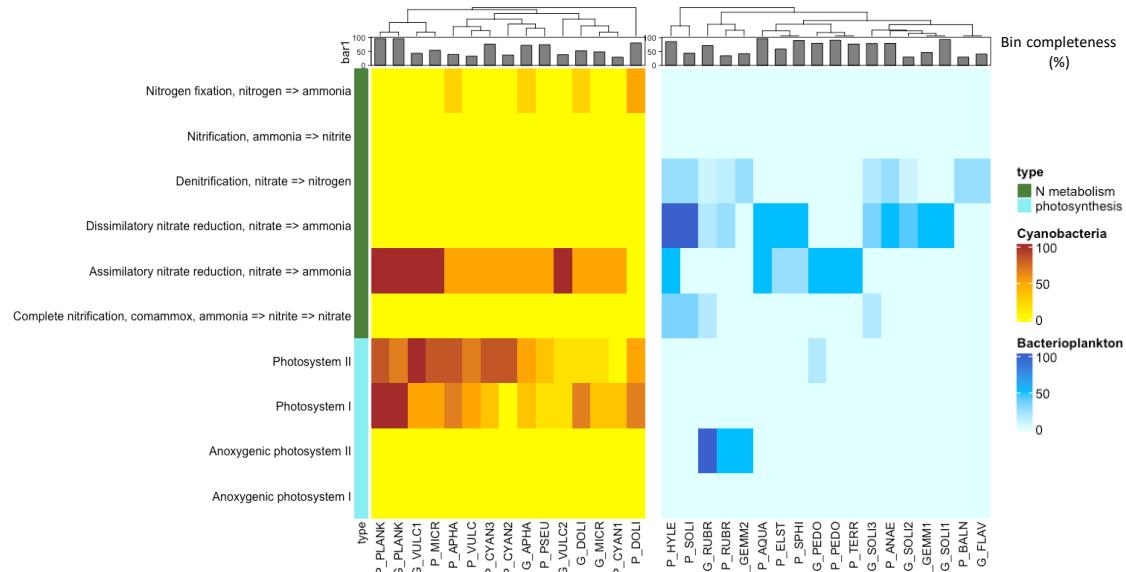


Figure 5.3 The nitrogen and photosystem module for the cyanobacteria and bacterioplankton MAGs.

modules are assigned to the taxonomy of *Aphanizomenon flos-aquae* and *Dolichospermum circinale*, which has the *nifDHK* gene clusters. The cyanobacteria and bacterioplankton community can also be well distinguished by photosynthesis systems. Like algae and plants, cyanobacteria contain oxygenic Photosystem I and II, while some bacterioplankton (e.g., purple non-sulfur genus *Rubrivivax*, ID: P/G_RUBR) undergoes anoxygenic photosynthesis in anaerobic conditions (Nagashima et al., 2012). These organisms do not produce oxygen through photosynthesis but utilize other compounds (e.g., sulfur and hydrogen) as reducing agents.

KEGG module contains functional units of gene sets in metabolic pathways, including molecular complexes. To further investigate the expression of nitrogen pathways and regulatory systems, the transcripts variants of MAGs at different stages of the algal bloom were shown in Figure 5.4. The transcript counts (TPM) summed the metabolites of cyanobacteria and bacterioplankton involved in N cycling (Figure 5.4 A top) and N regulatory mediation (Figure 5.4 B top).

It can be seen that ammonium-N is the central component in the N cycle of the bloom (Figure 5.4 A). The N₂-fixation module (*nifDHK*) was quantified to have the highest expression among all (540 – 907 TPM) and a gradually increasing trend throughout the after-bloom period, even though only two species were found as N₂-fixers. In addition, cyanobacteria also dominated the assimilatory nitrate reduction activities (ANRA) (95.6 -99.9%). The most active cyanobacterial involved in this process included *Aphanizomenon flos-aquae*, *Dolichospermum circinale*, *Planktothrix agardhii* and *Microcystis aeruginosa*, which correlated well with their predominant status in the bloom period. Similarly, the ANRA activities observed a gradual increased trend in gene



Figure 5.4 The inorganic N metabolism and regulatory involved genes and gene expressions.

expression (73-191 TPM). As for the inorganic nutrient assimilations, the ammonium-N transporters (*amt*) generally had higher activities than the nitrate/nitrite assimilations (*nrt*) for both cyanobacteria and bacterioplankton. Moreover, the denitrification (0 – 6.40 TPM) and dissimilatory nitrate reduction (0.13-1.38 TPM) related transcripts for the bacterioplankton were found but negligible in the bloom season-the after-bloom period. Further, the transcript counts were relatively more abundant in Provo than Geneva. The results were similar to the findings of the denitrification genes in the bloom of the Baltic Sea (Tuomainen et al., 2003).

The N assimilation and cycling were further mediated by the N regulatory systems, especially under the N starvation conditions (Figure 5.4 B). In this study, *glnG*-

glnL operon was only found in bacterioplankton (e.g., P_HYLE) with low transcripts (0.23-2.91 TPM) (Figure 5.4 B). By contrast, the *pipX-ntcA* operons (57-376 TPM) were globally found in cyanobacteria. Moreover, the heterocyst differentiation control (*hetR*) transcripts were largely detected in the MAGs of *Aphanizomenon* and *Dolichospermum* (112 -391 TPM). A trend of enhancement was detected for *hetR* from the before- to after-bloom periods, corresponding with the N₂-fixation modules. Similarly, the N regulatory PII protein (*glnB*) and *glnA* glutamine synthesis's expressions were commonly found and abundant in cyanobacteria community (209 – 809 TPM) and bacterioplankton (17 to 155 TPM). Generally, for most cyanobacterial N regulatory complexes, the gene expression continued, regardless of the decrease in genome relative abundance. An exception was the *ntcA* global N control, which had a peak expression during the bloom for Geneva's samples. Nevertheless, the transcripts for N regulatory for bacterioplankton were negligible or decreased, mainly during the bloom. It can be inferred that the “after-bloom” period was not yet returned to the initial non-bloom status of the lake, although the chlorophyll a concentrations were significantly reduced than the peaking time.

The transcript variations of gene sets and corresponding MAGs' ID were further detailed in the heatmaps below. Many cyanobacteria-dominated gene sets were highly expressed during or after the bloom, except for some non-bloom species (e.g., *Pseudanabaena* and *Synechococcus*-like strain *Vulcanococcus*). In contrast, bacterioplankton transcript sets for denitrification, N assimilation, dissimilatory nitrate reduction and *glnG-glnL* operon mostly decreased along with the bloom, except for a few (e.g., Elstera, ID:P_ELST) that presented after the bloom. Other than the dominated gene sets found in pathways of high complete bins, unique genes were also found in some

MAGs with low completeness. For instance, *nifK* gene was found in a MAG of *Aphonizomenon flos-aquae* (G_APHA4, completeness 10.34%) and this transcript peaked after the bloom (127 TPM). Additionally, the nitrate assimilation gene *nrtD* was found in *Cyanobium sp. PCC 7001*(P_CYAN29, completeness 12.07 %) and the respiratory nitrate reductase *nariI/V* was found in *Vampirovibrionia* (P_VAMP, completeness 25.36%). *Vampirovibrionia* are a sister group to all cyanobacteria and may disclose the traits of the evolution of oxygenic photosynthesis (Grettenberger et al., 2020). However, no further information was revealed due to the high incompleteness of the bin.

5.5.3.3 The Activation of Phosphorus Transport and Pho Regulon

The Pi uptake-related metabolic activities of the well-refined bins were further studied. Both cyanobacteria and bacterioplankton activated the high-affinity transport system (*pst*) and other parts of the Pho regulon during the cyanobacterial blooms under low Pi conditions (Figure 5.5). Part A detailed the sum of transcripts of different gene categories for cyanobacteria (top left) and bacterioplankton (top right). Different from N gene clusters, phosphorus-related domains were commonly found to have multiple copies with slight variations. The transcripts for cyanobacteria were significantly enhanced at the during- and after-bloom periods, while the bacterioplankton observed a decreased trend along with the bloom. Among cyanobacteria, the MAGs collected from Provo Site (May, June and July) followed a soaring trend even in the after-bloom period. While Geneva site, which represents more extensive bloom in open water (June, August and September), found peak transcripts counts in August. It was pretty different from the N regulatory that maintained or even enhanced transcripts levels for both sites in the after-

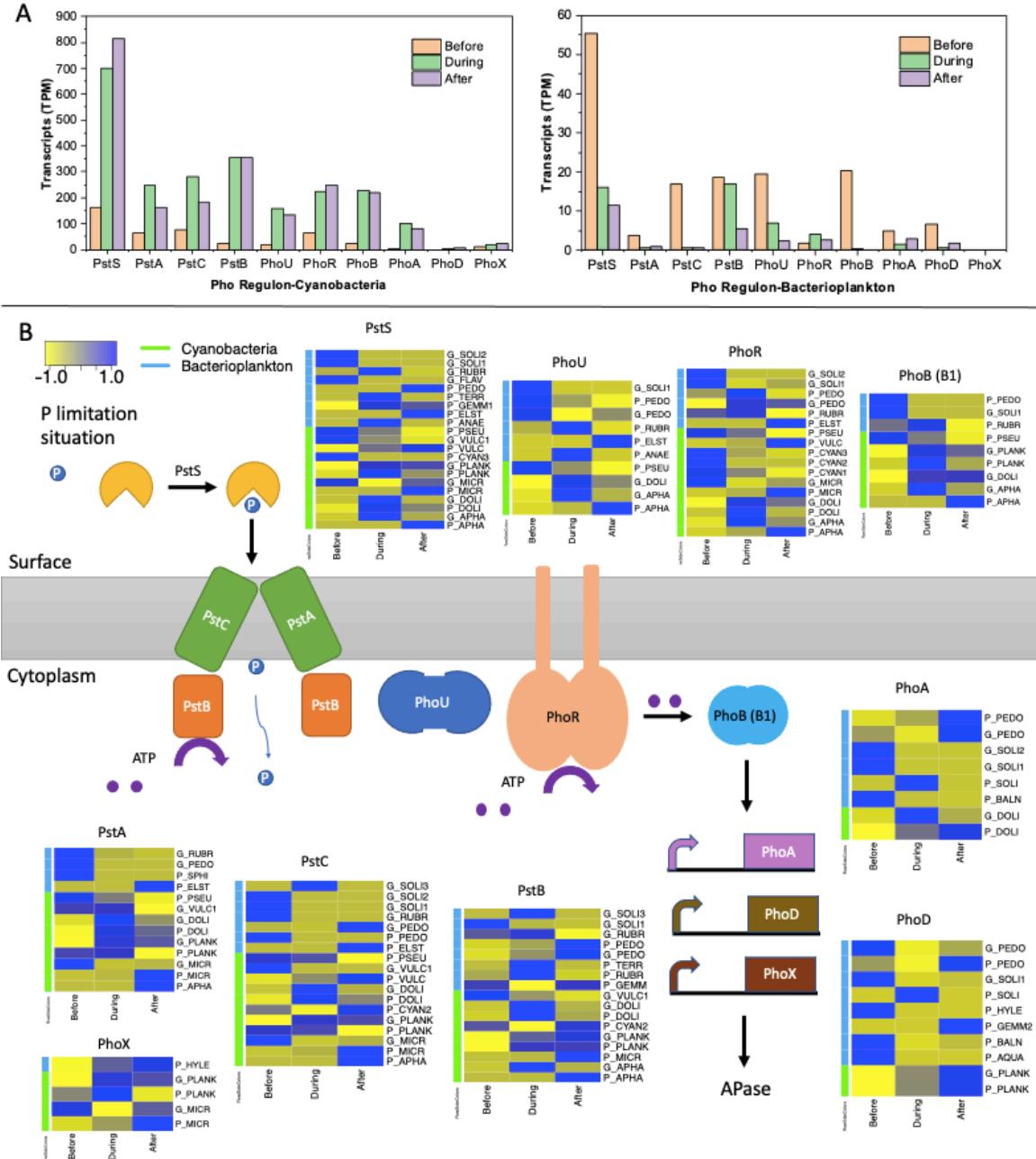


Figure 5.5 The inorganic P transport and assimilation involved gene and gene expression.

bloom periods. For both cyanobacteria and bacterioplankton, the highest expression was found for *pstS* domain, where the filamentous cyanobacterial genome (e.g., *Aphanizomenon* and *Dolichospermum*) can have up to 4-6 to bind with extracellular Pi. It was followed by *pstB* (23-357 TPM) for cyanobacteria. The expression of these two proteins even retained relatively high after the peak bloom. By contrast, overall cyanobacterial *pstA* (64 – 248 TPM) and *pstC* (77 – 283 TPM) expressions peaked during the bloom. While the essential functions of *pst* complex for cyanobacteria was revealed from previous studies (Harke et al., 2006b; Lu et al., 2019), the contribution of Pho regulon, the core phosphate assimilation pathways during a cyanobacterial bloom were rarely discussed (Su et al., 2007). The transcript counts for the *PhoURB* (19 – 249 TPM) were lower than the *pstS* and *pstB* domain, but around the same level of *pstA* and *pstC*. A similar trend was found for these two complexes, implying their tight associations in mediating Pi transport. The functions of *PhoU* were still elusive. In some bacteria, it is a negative modulator of expression of the Pho regulon and a feedback control system, as the *PhoU* expression is strictly dependent on *PhoP* activation (Muda et al., 1992; Martín-Martín et al., 2018). However, another study also found it does not regulate *PhoB* activity but may affect intracellular phosphate accumulation through the *pst* transporter (Lubin et al., 2016). With the *PhoB* phosphorylated, the dimer forms transcriptional factors for the alkaline phosphatase (APase) operons (*PhoA*, *PhoX* and *PhoD*). Relatively lower transcripts of APase were found for both cyanobacteria (13-124 TPM) and bacterioplankton (2.2- 11.6 TPM). The *PhoA* was expressed in *Dolichospermum circinale* (2.5 – 101.2 TPM), whereas *PhoD* was expressed in *Planktothrix agardhii* (0.18 – 5.53 TPM) with lower abundance (Figure 5.5 part B). Moreover, both *Planktothrix* and

Microcystis' MAGs contain *PhoX*, which is a recently described phosphatase that is more widely distributed in marine bacteria (Sebastian & Ammerman, 2009). The previous study also noticed that the upregulation of the *PhoX* gene by *Microcystis* helped them dominate cyanobacterial communities (Harke et al., 2016 b).

It is identified that the Pho regulon played important functions in acquiring Pi for cyanobacteria that the sum of transcripts belongs to cyanobacteria accounts for 55.1%-99.8% of the total *pstSABC* and 49.8% - 100% of the *PhoURB* complex. The percentages increased along with the bloom and cyanobacteria dominating the phytoplankton community. The heatmaps of Pi assimilation-related transcripts and corresponding MAGs' ID at different stages of bloom were further detailed in Part B. The overall trend found the activation of bacterioplankton before the bloom and cyanobacteria community during or after the bloom, which highly corresponded with the relative genome abundance and N metabolisms genes. Exceptions were some cyanobacteria that present relatively higher genome abundance before the bloom (e.g., G_MICR, P_CYAN3), and bacterioplankton that present after the bloom (e.g., P_ELST, G_G_PEDO).

5.5.3.4 The Activation of Stringent Alarmone during

Cyanobacterial Bloom Nutrient Starvation

Overall, the stringent response system was significantly activated for cyanobacteria during the cyanobacterial bloom, and the transcripts for bacterioplankton were also measurable. The poly-P and (p)ppGpp molecules were not directly measured; however, the gene sets responsible for their synthesis and hydrolysis were highly expressed. For example, the *PPK* (31-184 TPM) and *PPX/GPPA* (23 - 423TPM) genes

were highly activated for cyanobacteria along with the bloom (Figure 5.6). The study of well-refined bins found that many MAGs had *PPK1* or *PPK2* (or combined) expression, including all dominated cyanobacterial species during the bloom. Additionally, *PPX/GPPA* expression for cyanobacteria was found and also peaked during-bloom. The poly-P storage and hydrolysis can be estimated from the variations between PPK and *PPX/GPPA*. Specifically, the Provo site (initial bloom of the lake) found higher cyanobacterial counts of *PPK* than *PPX*, indicating the poly-P tends to be stored at this point. At the same time, MAGs collected from Geneva (later bloom of the lake) tended to break up poly-P. The bacterioplankton community has an opposite trend. The highest *PPK* expression was detected in *Aphanizomenon flos-aquae* (P_APNA, 73 TPM) after

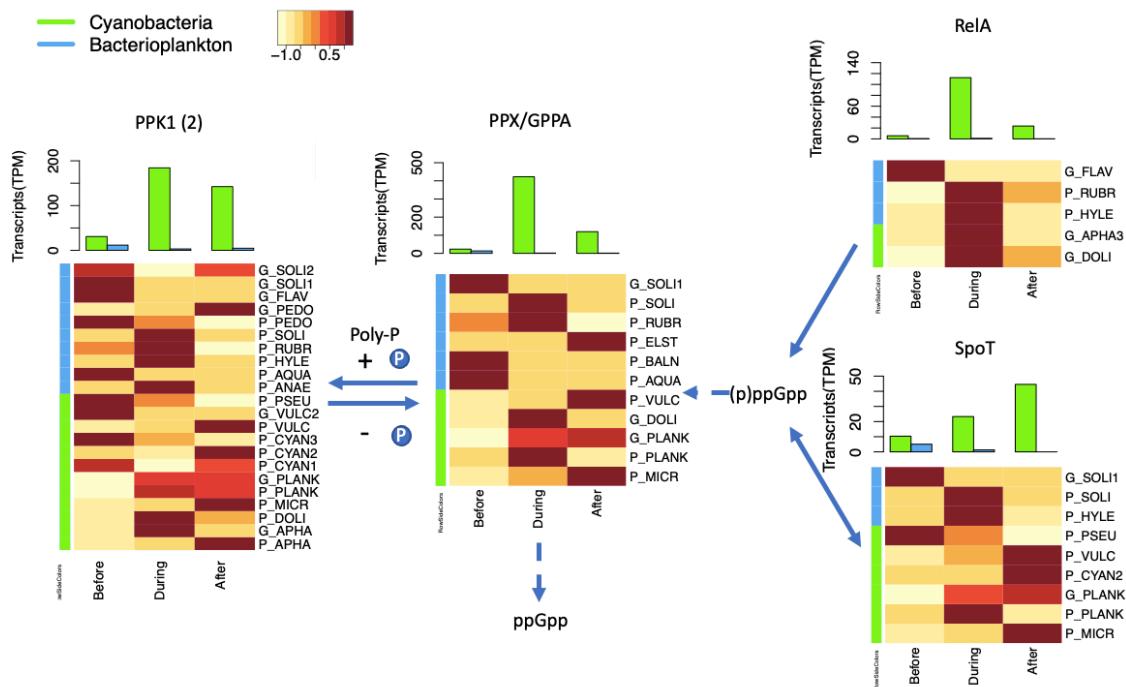


Figure 5.6 The changes of stringent response alarmone transcripts in challenging environment.

the bloom, while the highest *PPX/GPPA* expression was found in *Dolichospermum circinale* (G_DOLI, 400 TPM) during the bloom. It is quite contradicting that (p)ppGpp influenced the poly-P levels by inhibiting PPX activities (Rohlfing et al., 2018). The bacterioplankton community also found the enhancement of *PPK* transcripts for Pedosphaerales (G_PEDO) and Solirubrobacteraceae (G_SOLI2), as well as *PPX/GPPA* transcripts for Elstera (P_ELST) after the bloom. Both genes (*relA* and *spoT*) responsible for stringent response alarmone production and reverse reaction were expressed. Specifically, amino acid starvation response gene *relA* was highly enhanced in *Dolichospermum circinale* (G_DOLI, 48 TPM) and *Aphanizomenon flos-aquae* (G_APHA3, 64 TPM) during- the bloom, correlating with their peaked presence and *nifDHK* expressions. Similarly, the during-bloom period also discerned the peak *relA* expression for several bacterioplankton MAGs in response to N starvation. Nevertheless, carbon and Pi stringent response synthesis *spoT* was continuously expressed with the proceeding of cyanobacterial bloom. The dominant cyanobacterial MAGs, *Planktothrix agardhii* (P_MICR, 25 TPM) and *Microcystis aeruginosa* (G_PLANK, 18 TPM), were enhanced after the peak bloom, correlating well with their relative genome abundance changes in the lake. It may also emphasize that the cyanobacterial communities were still under environmental stress by producing the (p)ppGpp alarmone, even during the considered “after-bloom” period.

5.6 Discussion

5.6.1 The Lake's Blooming Conditions Viewing

from the Abiotic and Biotic Factors

Characterizing and even predicting a cyanobacterial bloom is considered difficult due to the large varieties of cyanobacteria genera, associated with the bacterioplankton community and responses to different environmental traits (Woodhouse et al., 2016; Tromas et al., 2017). Therefore, different methods were created to monitor the occurrence of cyanobacteria bloom at short- and long-term, such as remote sensing by satellite, microscopic counting, estimation of pigment (e.g., chlorophyll a and phycocyanin), molecular methods (e.g., DNA or RNA based), and biochemical and physicochemical methods (e.g., nutrients and other parameters) (Srivastava et al., 2013). In our study, the chlorophyll a concentrations, environmental parameters and metagenomics /metatranscriptomics were indicators of the cyanobacterial bloom proceedings. The chlorophyll a directly measured the pigment content of algal biomass at different cycles of the bloom. The trend highly corresponded with the presence of the dominant cyanobacteria species (i.e., *Aphanizomenon flos-aquae* and *Dolichospermum circinale*) that occupied high relative abundance of the bacterial community (Figure 5.1). Blooms can have a significant impact on the microbial community through direct (for example, microbe-microbe interactions) and indirect effects (for example, changes to lake chemistry). For instance, the enhanced pH generally implied an ongoing bloom when carbon dioxide was up-taken for carbon-fixation (Tromas et al., 2017). Similarly, the water chemistry could be impacted by the structure and diversity of the microbial community with bacterial assimilation and decomposition (Li et al., 2015; Woodhouse et

al., 2016). Yet, the chemical parameters in this study did not well-indicate the cycles of bloom, as many of them were non-detectable with nutrient deprivation. One important finding is that some gene expressions tend to be over-activated at certain intervals of bloom (e.g., N regulatory and *nifHDK* in after-bloom, Figure 5.4) and not correlated well with other biochemical and physicochemical parameters. Likely, any disturbance at this point could trigger another cycle of bloom, since bacteria were still under stringent conditions or working on substrate synthesis. The study of cyanobacteria blooms still focused more on abiotic parameters than biotic interactions (Guedes et al., 2018). Nevertheless, measures to predict and reduce cyanobacterial blooms would be compromised if these biotic factors (e.g., transcript estimation and function analysis) were ignored from the study (Oh et al., 2007; Taranu et al., 2012).

5.6.2 The Effects of Cyanobacterial Bloom on Nutrient Cycling

The switch between cyanobacteria and bacterioplankton at different stages of bloom affected the surface water bacterial community, and also altered the nutrient cycling and contributed to functional perturbation of the freshwater system (Woodhouse et al., 2016; Guedes et al, 2018). From the transcript point of view, the main N pathways changed from denitrification and DNRA to N₂-fixation and ANRA with the community composition shift towards N₂-fixation cyanobacteria (Figure 5.3 and 5.4). Even in the initial sampling time-May, the transcripts of *nif* were noticeable high, which means the cyanobacteria may start functioning earlier. It is evident that ammonium-N was the central N molecule for the N cycles during the heavy cyanobacterial boom, similar to the marine system (Wan et al., 2018). The evidence of enhanced N₂-fixation, ANRA,

ammonium assimilation and decreased denitrification efficiency all contributed to the ammonium production and utilization. Apart from these, the glutamine synthesis activity (*glnA*) was largely enhanced during the bloom, which is the primary catalyst for ammonium assimilation (Bolay et al., 2018). Besides, bacteria could also be nitrite or nitrite-N starved viewing from water quality parameters and N pathways (Beversdorf et al., 2013). By conducting N₂-fixation, cyanobacteria may be able to fuel the lake system with new “N” through leaking ammonium or organic N (e.g., glutamine and glutamate) into the ambient environment (Glibert & Bronk, 1994) and mineralization after cell death or lysis. It has been suggested the released N source can well-feed the surrounded phytoplankton community or even induce the subsequent bloom of non-heterocystous cyanobacteria and toxin-producers (Agawin et al., 2007; Lu et al., 2019; Li et al., 2020).

Cyanobacteria may drive lake P cycling by accessing pools of P that are not easily acquired or generally available for eukaryotic groups (Cottingham et al., 2015). For example, cyanobacteria could access P stored in lake sediment, migrate vertically through stratified water column by buoyancy, and activate the high affinity-phosphate uptake system at low P concentrations (Head et al., 1999; Xie, 2006). The Phosphate (Pho) regulon is a global regulatory mechanism involved in bacterial Pi management that was first characterized in *Escherichia coli*, and later in many other bacterial species (Wanner and Chang, 1987). The Pho regulon consists of *pst* operon that can be activated when phosphate concentration is low (Figure 5.5). It is different from the general phosphate transport system (*PiT*) that functions when phosphate is higher than certain thresholds (Mackey et al., 2019). Further, the alkaline phosphatase (*PhoA*, *PhoD* and *PhoX*) has long been identified in many bloom microbiotas, especially when the lake is phosphorus-

deficiency (Berman 1970; Valdespino-Castillo et al., Ma et al., 2019). Lab-scale studies also confirmed its expression in the bloom-forming cyanobacterium (e.g., the classical alkaline phosphatase (*PhoA*) in *Anabaena sp. pcc 7120* and putative alkaline phosphatase (*PhoX*) in *Microcystis aeruginosa*) to overcome phosphorus-limitation by exploiting organic sources of P (Harke et al., 2012; Muñoz-Martín et al., 2011). Among three alkaline phosphatase genes, *PhoA* transcript was the most abundant in our samples due to the presence of numerous filamentous cyanobacteria (e.g., *Dolichospermum*) during the bloom. Above all, the activation of Pho regulon is a sign that cyanobacteria and bacterioplankton were well-adapted to the low ambient Pi concentrations and influenced the P cycling during a cyanobacteria bloom. In addition to the nutrient assimilation, phosphorus (e.g., TP and ortho-P) may also be released from the bloom-cyanobacteria during the decline of the bloom in a eutrophic lake (Zhang et al. 2018). The large amount of bioavailable ortho-P could support the growth of newborn cyanobacteria or be absorbed by sediment, creating a microbial-mediated internal P cycling among the sediment, water column, benthic and phytoplanktonic communities within the lake.

5.6.3 The Stress Response in the Cyanobacterial Bloom

The study of the metatranscriptomics profiling suggested the lake community was under starvation or other stringent conditions from three aspects: N starvation regulation and the activation of N₂-fixation, the activation of high affinity P transporter and Pho regulon, and the activation of *relA-spoT* Homologue (RSH) family that are the key players in synthesizing and degrading the stringent alarmone (p)ppGpp. The N₂-fixation was heavily regulated as an energy-intensive process. Studies found it was activated only

under severe N source starvation (e.g., ammonium and nitrate) especially when the 2-OG levels exceed a critical threshold in cells (Li et al., 2003; Valladares et al., 2008). By activating the N₂-fixation, the 2-OG compounds initiated a chain of N regulatory genes in cyanobacteria, including many global N control genes (e.g., *ntcA*, *pipX*, PII) and heterocyst formation (*hetR*). Those transcripts were detected to increase with the bloom in the study. The transcriptional regulator NtcA has long been found as a requirement to trigger heterocyst differentiation in cyanobacteria (Herrero et al., 2004). Moreover, a study found it can bind to the promoter of the toxin-producing genes of the *Microcystis* and be upregulated under N-limiting starvations (Ginn et al., 2010). Remarkably, the *nifHDK* and *hetR* transcripts were overly expressed for both sites in the after-bloom period, indicating the lake was still under N stress when the bloom was considered fading (Figure 5.4). As for bacterioplankton, the overall *glnA* and PII transcripts decreased in the mid-bloom, while the additivities of *glnG-glnL* had an opposite trend (Figure 5.4). It suggested that the bifunctional *glnG-glnL* operon may down-regulate the glutamine synthesis during- the heavy bloom (MacNeil et al., 1982). Additionally, the N or amino acid starvation stress will likely induce the expression of *relA* (Figure 5.6), which could upregulate *hetR* and other stringent response behaviors (e.g., salt and oxidative resistance) for cyanobacteria and ensure the survival of them (Zhang et al., 2013; Jin et al., 2020). A corresponding trend was found between *relA* and *ntcA* at the Geneva site.

Moreover, studies found that Pho regulon is an integrated component of bacterial stringent response. Instead of a simple regulatory circuit for controlling phosphate homeostasis, Pho regulon is essential for bacterial virulence and stress response (Lamarche et al., 2008). For example, the *pst* operon is encoded together with *PhoU* in

the polycistronic *pstSCAB–PhoU* operon. The mutation or activation of *pstSCAB* will induce the expression of *PhoU*, and sequentially the turned-on of *PhoR/PhoB* operon (Wanner, 1996). The *PhoR/PhoB* two-component regulatory system affects poly-P formation by importing more ortho-P into the cell and controls the poly-P kinase gene *PPK* in some bacterial species (Ghorbel et al., 2006). The imbalance in poly-P reserves can benefit bacteria in reserving phosphate, providing energy sources in different biological processes, and resisting adverse environmental conditions (Brown & Kornberg, 2004). Additionally, phosphate-limitation will activate the *spoT*-dependent (p)ppGpp accumulation, which will initiate changes that globally handle nutritional stress. The tight correlations between (p)ppGpp and Pho regulon were confirmed by testing the (p)ppGpp synthesis when *pst* or other parts of the Pho box were muted (Spira & Yagil, 1998). Above all, the activation of Pho regulon, poly-P synthesis/hydrolysis genes (*PPK/PPX*), and *spoT* genes suggested that cyanobacterial and bacterioplankton communities were under phosphorus-starvation stress during the bloom (Figure 5.5 and 5.6). However, in contrast to the N regulatory system that kept increasing throughout the bloom event, most P-related transcripts were the highest during the peak bloom (for site Geneva) and decreased when the bloom of the entire lake starts fading (Figure 5.5). It may also indicate that the capacity to acquire phosphorus is one of the limiting factors for the bloom to continue.

5.6.4 The Implications of Nutrient Controlling on Cyanobacterial Bloom

With the stress response, cyanobacteria may be well suited to the nutrient-limiting conditions during cyanobacterial blooms. Despite the measures to restrict P input in lakes

taken to curb the cyanobacterial blooms (Stump et al., 2012; Schindler et al., 2016), the sediment of the freshwater lake was commonly rich in phosphorus (Holtan et al., 1988). In general, sediment acts as a net P sink. Still, it may serve as a P source depending on the physicochemical properties of the sediment and the overlying water column, especially in shallow lakes such as Utah Lake (Hogsett et al., 2019; Randall et al., 2019). Ultimately reducing the effects of P would be difficult unless geo- or chemical-engineering methods are taken to immobilize sediment P (Egemose et al., 2009; Copetti et al., 2016). Besides, researchers also found that curbing N cannot effectively control the cyanobacterial blooms; rather, it induces the bloom of N₂-fixing cyanobacteria (Conley et al., 2009; Paerl et al., 2011).

This evidence was observed on a macroscale, but the micro-level mechanism underlying the ecological effects is still poorly studied (Jin et al., 2020). The N-Pi-stringent response associations could contribute to the resistance of cyanobacteria to environmental changes and the dominance of the microbial community in our study. However, the direct interactions between cyanobacteria and bacterioplankton (e.g., exchange of nutrients and oxygen, the suppression or termination of bloom) were still lacking interpretation, apart from the relative abundance changes of the community composition (Bagatini et al., 2014; Osman et al., 2017). Further, the bloom events at two sites can be viewed as a portion of the whole, representing the initial (Provo) and following (Geneva) stages of the entire lake bloom. Both stages of bloom were quite resilient. For instance, the N and Pi related transcripts increased throughout the initial stage, while some N metabolism (e. g., glutamine synthesis) still kept their consistency at the following stage. Apart from the Pho regulon, it is reported that stringent response is

widely distributed in cyanobacteria since bioinformatics analysis found that many cyanobacteria possess RSH analogs (Atkinson et al., 2011). It globally diverted the bacterial cells from rapid growth to survival and responded to multiple stress simultaneously, such as upregulating N₂-fixation for N₂-fixers (Zhang et al., 2013), forming chlorosis for non-fixers (Klotz et al., 2016), and controlling the Pho regulon for Pi uptake and organic-P decomposition in many bacteria (Spira & Yagil, 1998). All these methodologies will enhance the occurrence, resilience and sustention of the cyanobacterial blooms. In our study, the *relA* and *spoT* also corresponded well with the upregulation of several gene operons and the overall presence of the genome. On the other hand, the stringent responses may enervate cyanobacteria or some bacteria as some of the functions (e.g., fixation N₂ from the atmosphere) were energy-intensive endeavors (Gutschick, 1978).

Above all, the diminish of some physicochemical parameters (e.g., pH and chlorophyll a) may not be enough to detect the stages of cyanobacterial blooms and determine whether the cyanobacterial blooms still sustain. To better understand the Cyanoblooms, molecular methods are needed to target specific functions or transcriptomics profiling. The enhancement of specific transcripts at the after-bloom period suggests it is very likely the system is still under stress and tends to activate more genes. With the strong resilience of the bacterial communities during the cyanobacterial blooms, any disturbance or the runoff increase would possibly incur another peak of bloom. Therefore, in addition to remediating nutrients input, other methods may be taken to tackle the blooms of cyanobacteria effectively.

5.7 Conclusions

N and P are two primary factors triggering cyanobacterial blooms and the nutrient reduction methods were taken as effective measures to curb the blooms. However, studies found that reducing nutrients input did not always effectively lessen the cyanobacterial blooms. For example, reducing inorganic N in the lake may favor the community composition shift towards N₂-fixers at a larger-scale observation. By looking into the metagenomics and transcriptomics profiling, varieties of cyanobacteria were present during the cyanobacterial blooms. In addition to the activation of the heterocyst by some filamentous cyanobacteria, many cyanobacteria and bacterioplankton had also activated Pho regulon and stringent response systems. The Pho regulon allows bacteria to enhance affinity for Pi assimilation and decompose organic P to facilitate Pi uptake. The utilization of organic P emphasizes the importance of controlling TP in addition to the inorganic portion. The stress response may associate with the N and P regulatory systems in bacteria when the ambient environment was nutrient starvation. These methodologies may help cyanobacteria resist the low-nutrient conditions, outcompete the bacterioplankton (lower transcripts) and thus sustain the harmful blooms. In the long-term, abiotic factors were commonly taken into consideration, while the biotic factors were less noted. Some of the inconsistency between the abiotic and biotic factors, especially at the after-bloom stage, also highlights the importance to consider the activities of microbial communities besides measuring the physicochemical parameters. The lake was still at risk of bloom if any disturbance or runoffs were incurred at this time. These findings suggest that resolving cyanobacterial blooms probably took more than just the reduction of external nutrient loadings.

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CHAPTER 6

CONCLUSIONS

Cyanobacteria are the primary organisms in a lake that causes toxic algal blooms. Conventionally, microscopic techniques were mainly used for the detection of cyanobacterial communities. For the first time, high-throughput sequencing was applied for the study of bacterial communities at Utah Lake. We successfully demonstrated the presence (and in some cases the dominance) of filamentous cyanobacteria and picocyanobacteria *Synechococcus* in the lake. The lake was initially dominated by *Cyanobium/Synechococcus* during May and later primarily by *Aphanizomenon* and *Dolichospermum* depicting N₂-fixation functions. The lake experienced a more extended N₂-fixing period (2–3 months) before non-fixing cyanobacteria (*Planktothrix*) dominated the entire lake in late summer. Excess N, fixed by diazotrophic filamentous cyanobacteria, could also be the food supplying the succeeding growth of *Microcystis* and *Planktothrix*. *Microcystis* was found as the toxin-producer. Actinobacteria, Bacteroidetes, and Proteobacteria were dominant phyla of heterotrophic bacterioplankton in the summer season. Their relative abundance was negatively correlated with cyanobacteria but responded to higher nitrate concentration.

The transcriptomic profiling of cyanobacteria and associated bacterioplankton revealed functional changes at different stages of bloom. It is noted that the ammonium-N

is the central component of the N cycle, as the filamentous cyanobacteria (e.g., *Aphanizomenon flos-aquae* and *Dolicheopsernum circinale*) dominated the N cycling by conducting N₂-fixation and assimilatory nitrate reduction. In contrast, bacterioplankton-dominated N pathways (e.g., denitrification and dissimilatory nitrate reduction) were negligible during the bloom. Additionally, the Pho regulon was activated to enhance inorganic P uptake, with cyanobacterial community dominated the process and enhanced expressions during the bloom. The obtaining of alkaline phosphatase genes in cyanobacteria (e.g., *PhoA* in *Dolicheopsernum*, *PhoX* in *Planktotrix* and *Microcystis*) suggested that they can actively synthesize/release alkaline phosphatase and decompose the ambient organic P into bioavailable forms. Moreover, genes related to the poly-P storage and stringent response alarmone (p)ppGpp synthesis/hydrolysis were also activated during the bloom, which may be tightly correlated with the activation of particular N and P functions.

To sum up, the N-Pi-stringent response associations may function to resist the starvation conditions, outcompete the bacterioplankton and thus sustain the harmful blooms. Inconsistency was found between physicochemical parameters and some functions at the after-bloom period, meaning that the bloom was not ended as supposed to. Especially for the N₂-fixation cyanobacteria, the overactivation of the N₂-fixation activity, the energy-intensive endeavor at later bloom stages may lead to their faster decay. It also implies that molecular tools are very handy to determine the bloom stages other than the traditional monitoring strategies. Even with lower chlorophyll a concentrations, the high gene expressions of cyanobacteria was detected, suggesting that any additional input of nutrients at this moment can potentially trigger another cycle of

bloom.

These findings further suggest that resolving cyanobacterial blooms probably takes more than just reducing external nutrient loadings. N-limitation conditions may trigger the bloom of diazotrophs and bring “new” N into the ecosystem. Similarly, the inorganic P can also be released from the sediment, affiliating the growth of diazotrophs. With the P-rich sediment and alkaline conditions, the Utah Lake was pretty unique compared with other freshwater lakes worldwide. With the current lake conditions, the N₂-fixation taxas (e.g., *Aphanizomenon* and *Dolichospermum*) may continue to be the dominant genera in the summer bloom seasons. However, controlling nutrients, especially the TN and TP, is still essential for controlling Utah Lake’s cyanobacterial blooms. First of all, the reduction of N source input may minimize the dominance of N-fixers in the long-term as the overall eutrophication status of the lake was reduced. Secondly, controlling TN may prevent the community shift to *Microcystis*, a toxic strain capable of producing N-enrich cyanotoxins. The input of TP is also a significant concern as the decomposition of organic P into bioavailable P by bacteria-released alkaline phosphatase during the cyanobacterial blooms may also accelerate the P cycling. Future research may increase the sampling frequency and further explore the relationship among nutrient input, cyanobacterial activities and the ecological tipping point. Other biotic (e.g., cyanophages) and abiotic factors affecting cyanobloom may be further studied and measures to tackle it may be more investigated.

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