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Ecophysiology of toxic bloom-forming cyanobacteria and their symbionts

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Ecophysiology of Toxic Bloom-Forming Cyanobacteria and their Symbionts

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Abstract

Currently, there is a pernicious microbe in the genus *Microcystis* that is putting the world's freshwater resources at risk. A member of the phylum Cyanobacteria, otherwise referred to as blue-green algae, *Microcystis* forms massive algal blooms in lakes and has the potential to produce a toxin, which does not only harm humans, but also pets, livestock, and aquatic life. As recently as 2016, in the city of Toledo, Ohio, nearly 1 million residents went without clean drinking water for an entire weekend due to one of these *Microcystis* blooms. However, *Microcystis* does not only bloom in Ohio. In fact, it has been reported on every continent in the world with the exception of Antarctica. Despite tremendous efforts invested into research, the exact factors leading to *Microcystis* blooms remain unsolved. Most studies have examined the role of abiotic, or nonliving, factors such as warm temperatures or nutrients from fertilizer runoff as being important drivers of these blooms. However, recent attention has shifted to the potential role of biotic factors. *Microcystis* blooms should not be thought of as being caused by one single species, but rather a complex assemblage of a variety of different types of bacteria and other microbes that interact with the algae in a variety of different ways. It is this perspective that has driven the hypotheses, methodology, and analyses of this thesis. A comprehensive literature review in chapter one puts my work in a broader context. I then characterize and describe the community structure associated with *Microcystis* in established cultures and compare that to the microbiomes of other cyanobacteria and eukaryotic algae in my second chapter. Finally, in chapter three, I analyzed the effects of heterotrophic bacteria isolated from natural *Microcystis* blooms in Lake Erie (USA) and Lake Tai (China) on the growth and physiology of *Microcystis aeruginosa* through

detailed co-cultures. The data and analyses contained herein are a critical next-step in attempting to understand the drivers behind *Microcystis* blooms as we work toward better protecting our freshwater resources.

Chapter I

Literature Review

1. Introduction

Microcystis is one of the most common bloom-forming species of cyanobacteria (Visser *et al,* 2005). Blooms of *Microcystis* are expanding worldwide mainly due to the eutrophication of freshwater environments combined with other biotic and abiotic factors. The toxins produced during these blooms have a major impact on fishing, recreation, and tourism, as well as livestock, aquatic life, and public health.

2. Cyanobacterial Harmful Algal Blooms

Formerly referred to as blue-green algae, cyanobacteria are a phylum of photosynthetic bacteria that are responsible for converting Earth's reducing atmosphere to an oxidizing one approximately 2.7 Ga based on fossil records (Van Kranendonk *et al,* 2012). They are a type of algae that form the base of many marine and freshwater food webs (Paerl and Paul 2012). Cyanobacterial harmful algal blooms (cHABs) are formed when cyanobacteria grow to high cell densities and have the potential to negatively impact water quality by producing harmful toxins (Huisman *et al,* 2018). Upon decomposition, these blooms can adversely affect aquatic life by creating zones of hypoxia and anoxia in the water (Rabalais *et al,* 2010). As global temperatures continue to increase, these blooms are predicted to become even more prolific both in terms of biomass and frequency (Lurling *et al,* 2017).

Over the past several decades, non-native cyanobacteria have begun to invade both marine and freshwater systems, which may pose a threat to global biodiversity (Sukenik *et al,* 2012). The successful invasion of new environments can often be attributed to their unique physiological characteristics like the ability to take a dormant

resting stage (Sukenik *et al,* 2012). Some species are thermal tolerant which is critical for survival during cold winter months (Briand *et al,* 2004). Another attribute that allows some species of cyanobacteria to proliferate is the capacity for ammonium uptake or fixation of atmospheric nitrogen, allowing them to avoid nitrogen limitation (Briand *et al,* 2004). Diazotrophic species are defined as those that can fix atmospheric nitrogen into a biologically available form (Dyhrman *et al,* 2006). Species that have gas vesicles (allowing for buoyancy) can create a thick scum on the surface of the water, effectively blocking light penetration and gaining a competitive advantage over co-occurring phototrophs (Calandrino and Paerl 2011).

The North American Great Lakes comprise approximately 18% of the world's available fresh water. Lake Erie is the shallowest of the five Great Lakes and is plagued by frequent cHAB blooms (Huisman *et al,* 2018). Many of these bloom events are caused by the *Microcystis* genus of cyanobacteria, which proliferate under conditions such as global warming and nutrient enrichment (Paerl and Paul 2012).

2.1 Toxins

The first well-documented case of a cyanobacterial bloom affecting drinking water resources was in November of 1979, on Palm Island, Australia, where a bloom of *Cylindrospermopsis* (*Raphidiopsis*) *raciborskii* led to human hepatoenteritis in ten adults and 138 children (Byth 1980). Cyanobacteria have continued to make headlines because of their toxicity not only to humans, but also for aquatic life and animal husbandry. Some cHAB species are also capable of producing toxic secondary metabolites (cyanotoxins) that include cyclic peptides and alkaloids. Cyclic peptides include hepatotoxins, such as

microcystins and nodularins, and the alkaloids include dermatoxins, such as lungbyatoxins and aplaysiatoxins; hepatotoxins such as cylindrospermopsins; and neurotoxins such as anatoxin-A, anatoxin-A(S), and saxitoxins. (Van Apeldoorn *et al*, 2006 and Chorus and Welker 1999). There are approximately forty genera of cyanobacteria that are responsible for cyanobacteria toxin poisonings with the main ones being *Anabaena* (*Dolichospermum*), *Aphanizomenon, Cylindrospermopsis, Lyngbya, Microcystis, Nostoc,* and *Oscillatoria* (*Planktothrix*) (Carmichael 2012). Standard filtrations and treatment processes remove toxic cells and toxins from drinking water below an acceptable level for acute lethal effects, however, in the case of large blooms, or inadequate water treatment, toxins have been present in municipal drinking water supplies in the U.S and Australia and have been implicated in human illness (Carmichael 2012). Poisonings among terrestrial animals are mostly attributed to drinking infested freshwater and vary based on animal size and species sensitivity (Watanabe *et al,* 1995).

2.2 Global Distribution

Cyanobacteria are able to occupy a vast and diverse geographic area ranging from sandstone in the Antarctic to pavement in the hot deserts of Mexico, from outdoor ponds in California to shallow lagoons in India, from ultraoligotrophic oceans to hypersaline intertidal flats (Whitton and Potts 2000 and Wynn-Williams 2000). Evolutionarily, these organisms have been successful, which allow them to bloom frequently in aquatic environments (Paerl 2014). Numerous freshwater environments have experienced recurring cyanobacterial blooms including: Green Bay (Wisconsin, USA), Saginaw Bay (Michigan, USA), Lakes Erie and Michigan (US-Canada), Lake Ponchartrain (Louisiana,

USA), Lake Okeechobee (Florida, USA), Lake Tai (Wuxi, China), Lake Victoria (Tanzania, Africa), and Lake Manyara (Tanzania, Africa) (Paerl 2014, Huisman *et al*, 2018, Kimambo *et al*, 2019). The extensiveness of their geographic area indicates broad physiological adaptations including heat resistance, photoprotective pigments, buoyancy regulation, and the formation of symbiotic relationships with heterotrophic bacteria (Paerl 2014). This ability to adapt has resulted in a disproportionate global increase in abundance of cyanobacteria relative to other phytoplankton (Huisman *et al*, 2018).

3. *Microcystis*

Microcystis is one of the most common genera of cyanobacteria that forms blooms in freshwater systems and has the potential to cause major problems with drinking water, fisheries, and recreation for humans (Harke *et al*, 2016). *Microcystis* can also produce a potent hepatotoxin, microcystin, that can harm and even kill aquatic life and livestock (Harke *et al*, 2016). Aside from producing toxins, the high biomass of *Microcystis* blooms may lead to hypoxia or anoxia in the water column upon bloom termination, killing both plants and aquatic animals (Watson *et al*, 2016). Given its negative impact on the environment, *Microcystis* blooms are classified as cHABs. These blooms have been extensively studied, and the current paradigm suggests that the severity and toxicity of *Microcystis* blooms are often enhanced when levels of nitrogen and phosphorus are elevated (Paerl and Huisman 2008). Alarmingly, it is expected that these blooms will be further perpetuated by rising global temperatures (Paerl and Huisman 2008); a projection that is even more distressing given *Microcystis* blooms have already been reported in 108 countries, on every continent except for Antarctica; seventy-nine of

which had confirmed microcystin toxins (Harke *et al* 2016). Previous work regarding bloom formation and geographic spread of *Microcystis* has focused on the study of abiotic factors such as the aforementioned nutrients and temperature. An understudied, and potentially important aspect to the promotion of toxic blooms are biotic factors (e.g. microbial interactions).

3.1 Ecological Consequences of *Microcystis* Blooms

The accumulation and breakdown of algal biomass leads to changes in concentrations of dissolved oxygen, dissolved organic carbon, and pH (Li *et al,* 2011). This can lead to hypoxia (low levels of dissolved oxygen) or anoxia (depletion of dissolved oxygen) causing "dead zones" in the water; destroying the balance of the food web (Li *et al,* 2012). Rapid changes in oxygen concentrations can trap fish in hypoxic zones or lead them to avoid bottom waters where their primary diet is located (Scavia *et al,* 2014). *Microcystis* also has the ability to alter the pH of the ecosystem to well above 9, allowing it to outcompete other phytoplankton (Wilhelm *et al,* 2020). For example, by raising pH conditions above 9.26, the silica cell wall of diatoms becomes soluble (Wilhelm *et al,* 2020).

3.2 Public Health and Economic Impact

Microcystis has notoriety due to the harmful effects of its production of the cyanotoxin, microcystin. The etymology of the name itself – *mikros* meaning "small" and *kystis* meaning "bladder" – is indicative of this peptide being a potent liver toxin (Falconer *et al,* 1983). The presence of this toxin in drinking water is rightful to cause

concern. Unlike other toxigenic planktonic cyanobacteria, *Microcystis* may be toxic up to 95% of the time (Carmichael *et al,* 1988). The effects of microcystin have been linked to various types of illness including colorectal and liver cancer due to drinking water in China (Yu *et al,* 2001 and Zhou *et al,* 2002). The toxin promotes the growth of liver tumors in mice and rat experiments (Nishiwaki-Matsushima *et al,* 1992). There have been multiple occurrences of bloom events affecting potable water. In 2014, nearly 500,000 residents of Toledo, Ohio were left without clean drinking water for an entire weekend due to microcystin levels in Lake Erie being above the WHO limit (Steffen *et al,* 2017). In May of 2007, over 2 million residents of Wuxi, Jiangsu Province, China were without clean drinking water for a week due to a *Microcystis* bloom in Taihu (Qin *et al,* 2010). Unsuccessful efforts were made to divert water from the Yangtze River which exacerbated the contamination by moving the bloom into the intake (Qin *et al,* 2010).

Fishing industries have been heavily impacted by blooms of *Microcystis*. Lake Erie is one of the most profitable natural resources in the United States and also experiences some of the worst cHABs (Wolf *et al,* 2017). By preventing light penetration to lower depths, algal scum can lead to the death of fish and other aquatic life. Additionally, the presence of algae can also cause shutdowns preventing access to the water and severely impacting local angling economies (Wolf *et al,* 2017). Freshwater eutrophication also poses an issue for tourism because of algal taste and odor, water clarity, and aesthetic perception. People are less likely to swim, fish, and boat during heavy algal blooms. Recreational usages of freshwater and lakefront property values comprise $0.67-3.96 billion in economic losses in the United States (Dodds *et al,* 2009). This nationwide problem is likely reflected globally.

3.3 Geographic Distribution of *Microcystis*

Blooms of *Microcystis* have been recorded in 108 countries covering every continent in the world, with the exception of Antarctica (Harke *et al,* 2016). Lakes across the Midwest (Missouri, Iowa, Kansas, and Minnesota) show a direct correlation between blooms of *Microcystis* and nutrient enrichment due to agriculture (Graham *et al,* 2004). Not wholly a freshwater problem, the San Francisco Bay Estuary is also subject to annual blooms of *Microcystis* (Lehman *et al,* 2005).

One of the most problematic occurrences of *Microcystis* blooms is in Lake Tai (Taihu), in China. Taihu is the third largest lake in China, and is situated in a heavily populated area that serves as both a drinking water and irrigation source (Chen *et al,* 2003). The lake is shallow (less than 10 m) and faces extreme eutrophication due to being downstream from over 200 rivers, brooks, and canals as well as receiving effluents from the cities of Wuxi and Changzhou (Chen *et al,* 2003). *Microcystis* has dominated in both temperate and tropical climates spanning the globe (Harke *et al,* 2016). It appears as though *M. aeruginosa* has this cosmopolitan distribution due to its high diversity and lack of phylogeographic structure (Van Gremberghe *et al,* 2011).

4. Abiotic Drivers of Blooms

cHABs are influenced by abiotic factors such as light levels, water temperature, pH changes, and nutrient availability (Anderson et al, 2002). These all serve as targets in efforts to mitigate blooms.

4.1 Nutrients

Anthropogenic nutrient loading is one of the major contributors in the formation of blooms of *Microcystis* (Paerl 2014). Excessive inputs of both nitrogen and phosphorus promote eutrophic conditions in lakes; often resulting in cHAB blooms like those caused by *Microcystis* (Elser *et al,* 2007). This is even more evident in shallow lakes, such as Lake Erie, where phosphorus accumulates in the sediment (Paerl 2018). Human activity can be blamed for much of the increased nitrogen and phosphorus inputs into freshwater systems; especially agriculture (Huisman *et al,* 2018). Inorganic phosphorus is an important limiting factor for growth and has typically been the focus of controlling cyanobacterial blooms based on the logic that nitrogen can be reduced via nitrogen fixation by the cyanobacteria (Nalewajko *et al,* 2001 and Schindler *et al,* 2008). However, *Microcystis* are one of the few genera of cyanobacteria that do not have the ability to fix nitrogen and often dominate hyper-eutrophic lakes where there is an abundance of both nitrogen and phosphorus (Cottingham *et al,* 2015). Recent mitigation strategies have shifted gears and now target both nitrogen and phosphorus control (Kim *et al,* 2020).

There has been a global increase of greater than 35% in the usage of urea as a nitrogen fertilizer in the past four decades (Glibert *et al,* 2006). The input of urea into freshwater systems, via fertilizer runoff, not only promotes growth of non-diazotrophs, but also inhibits the growth of diazotrophs (Finlay *et al,* 2010). *Microcystis* has the ability to use urea as a nitrogen source which gives it yet another advantage over other phytoplankton (Wilhelm *et al,* 2020).

4.2 Temperature and Global Climate Change

Cyanobacteria grow at slightly higher temperatures (above 25°C) than other phytoplankton allowing them to outcompete other eukaryotic algal taxa when faced with global climate change (Paerl and Paul 2012). Additionally, cyanobacteria have faster growth rates with warmer temperature than their eukaryotic counterparts (Huisman *et al,* 2018). Warm temperatures also increase vertical stratification; however gas vesicles allow *Microcystis* to travel to the top of the water column and accumulate on the surface preventing light from reaching species below (Paerl and Paul 2012). The nonlinear pattern of temperature is yet another benefit to *Microcystis,* as temperature fluctuates, toxin production rapidly increases (Wilhelm *et al,* 2020). While *Microcystis* is a freshwater cyanobacteria, some strains are able to tolerate up to 30% salinity, which is relevant with increased summer droughts and sea level rises (Paerl 2014). Effects of climate change, such as overall temperature warming coupled with changes in precipitation may allow *Microcystis* to infiltrate previously unsuitable waters and may also allow them to bloom longer into the fall and winter months.

5. Microbial Interactions in the Phycosphere

The phycosphere is the immediate area around algal cells in which nutrient concentrations are substantially higher than in the surrounding water column. Often, bacteria thrive in this environment, feeding off the organic carbon and other cellular exudates produced by the algae (Seymour *et al,* 2017). In the case of *Microcystis,* the algal cells produce and secrete a substance composed of polysaccharides and organic

matter that holds cells together in a colony. Within this extracellular polymeric substance (EPS), an assemblage of heterotrophic bacteria is embedded. This intimate association allows for interactions to occur between the *Microcystis* and heterotrophic bacteria that allow for the potential exchange of nutrients (Yuan *et al*, 2009). For example, it has been suggested that *Microcystis* may be aided by associated nitrogen-fixing bacteria which can provide the alga with a source of reduced nitrogen (Beversdorf *et al,* 2013). Much of what we know about interactions within the phycosphere come from marine systems; for example, massive blooms of *Emiliania huxleyi* have been observed in correlation with a species of *Roseobacter* that may promote growth by secreting plant hormones known as auxins (Seyedsayamdost *et al*, 2011). Microbial interactions can also be algicidal, whereby the presence of bacteria may lead to the death of the algal cell, releasing organic material that contributes to the bacterial food chain (Bell *et al*, 1972). Given that previous studies have demonstrated strong effects of bacteria on marine algae, it is critical to understand the role bacteria are playing in promoting or antagonizing blooms of *Microcystis*.

6. Conclusions

Toxic blooms of *Microcystis* are a global concern. The occurrence of cHABs have risen on a global scale and are likely to continue to do so when being promoted by global warming, raises in CO_2 concentrations, and continued eutrophication due to agriculture (Huisman *et al,* 2018). Cyanobacteria are evolutionarily resilient and have demonstrated remarkable ability to adapt throughout 2.7 billion years. While several long-term

management strategies have been identified, there is still much left to understand about why these blooms occur.

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Chapter II

Bacterial Community Structure of Non-Axenic Cyanobacterial Cultures

1. Abstract

Microbial interactions within the phycosphere have been recognized as being a major component in influencing bloom dynamics. The core microbiome of *Microcystis* remains largely unstudied. In order to determine community structure, we performed 16S sequence analysis in Qiime2 from non-axenic cultures. We found a clustering pattern in which the individual ASVs (amplicon sequence variants) represented were most similar between two strains (NIES 843 and CCMP 3462) of *M. aeruginosa* than they were to another cyanobacterium or to a marine eukaryote. The NIES 843 strain contained ASVs with a high percent abundance from the Sphingomonadaceae family. The CCMP 3462 strain contained ASVs with a high percent abundance from the Xanthomonadaceae and Chitinophagaceae families. The overlap between the two *Microcystis* strains contains multiple *Microcystis* ASVs. The *Raphidiopsis* also has a high percent abundance of *Cylindrospermopsis* (*Raphidiopsis*). This indicates that some of the similarity comes from the identity of the alga the culture is comprised of. There is some overlap between the three cyanobacteria with Sphingomonadales ASVs. These data suggests that the host drives the microbiome.

2. Introduction

Cyanobacterial harmful algal blooms (cHABS) are of growing concern due to the threat that they pose on water quality globally. Once thought to be homogenous populations, it is now known that the perpetrators behind these blooms are accompanied by a suite of heterotrophic bacteria (Eiler and Bertilsson 2004). In fact, the accumulation and subsequent breakdown of bloom-forming cyanobacteria have a strong impact on

aquatic bacterial community composition (Li *et al,* 2011). Algal cells secrete organic molecules, such as amino acids and monosaccharides, that are energetically costly to produce (Seymour *et al,* 2017). The justification for this process is the establishment of a bacterial community within the phycosphere (Seymour *et al,* 2017). These organic molecules serve as chemoattractants and are often secreted by the algal cell during early phases of cell growth (Seymour *et al,* 2017). Another potential benefit to bacteria in the phycosphere is a reduced carbon source. Phytoplankton play a large role in global carbon cycling and bacteria (both free-living and particle-attached) utilize up to 50% of the dissolved organic carbon that is fixed by photosynthesis (Azam *et al,* 1983 and Cole *et al,* 1982). Another benefit is that the phycosphere may physically protect bacteria from grazing (Brunberg 1999).

The phycosphere does not solely exist to provide a habitat for bacteria, however, as a growing body of evidence suggests that *Microcystis* may be provided with essential nutrients and other metabolites from their heterotrophic partners. For example, it has been recognized that the ability for *Microcystis* to proliferate in freshwater environments is largely dependent on nitrogen availability (Gobler *et al,* 2016 and Qian *et al,* 2017). *Microcystis* is non-diazotrophic (it does not fix N_2), but N_2-fixing bacteria may be present in the phycosphere and could provide *Microcystis* with a source of nitrogen (Yang *et al,* 2017). *Microcystis* may also be provided with a source of dissolved inorganic carbon (Dziallas *et al,* 2012), which can be limiting during high-biomass blooms.

The bacteria in the phycosphere may be different than the bacteria found in the surrounding water column. Individual colonies of *Microcystis* possess a different taxonomic composition within their phycosphere compared to whole water bacterial

communities (Smith *et al,* 2021). Unique phycosphere communities are hypothesized to be a result of strain-specific interactions and changes in the physiochemical environment over time (Smith *et al,* 2021). Much about the phycosphere microbiome remains a mystery. For example, some recent studies have gone so far as to suggest that *Microcystis* lacks a core microbiome and that clustering patterns could be explained by antibiotics that *Microcystis* produces that inhibit the growth of various bacterial taxa differently (Smith *et al,* 2021). Other studies have suggested that bacteria associated with *Microcystis* have highly similar community structures that are based upon selection pressure by *Microcystis* to facilitate the growth of bacteria with specific function rather than taxa (Yang *et al,* 2017; Jankowiak and Gobler 2020). To help resolve these disparities and further explore the interactions within the phycosphere, I characterized the bacterial community structure associated with two different strains of *Microcystis aeruginosa* that have long been maintained in culture. For comparison, I also characterized the bacterial community structure of *Raphidiopsis*, another high-biomass cHAB genus. A marine, eukaryotic HAB was also analyzed as an outgroup.

3. Methods

M. aeruginosa (NIES 843), *M. aeruginosa* (CCMP 3462), and *Cylindrospermopsis (Raphidiopsis)* (CS-505 and CS-1102) cultures were maintained in CT media (Ichimura, 1971) at 26 °C on a 12:12 light:dark cycle. *Aureococcus anophagefferens* (CCMP 1853) was maintained in L1 media (Guillard and Hargraves 1993) at 19 °C on a 14:10 light:dark cycle. Cells from all cultures were harvested by filtering onto Sterivex filters and stored at -20 °C. DNA was extracted by breaking open

the Sterivex, removing the inner filter, and using the PowerWater DNA (QIAGEN) extraction kit according to manufacturer's instructions. The two *Microcystis* cultures were prepared in duplicate. The *Aureococcus* was also prepared in duplicate. The *Raphidiopsis* was prepared with one copy of the CS-505 strain (toxic) and one copy of the CS-1102 strain (non-toxic). DNA was then sent to GeneWiz (South Plainfield, NJ) for 16S library preparation and sequencing.

Sequences in .fastq format were analyzed using the QIIME2 (Quantitative Insights Into Microbial Ecology) software package (version 2020.11.0). Paired-end demultiplexed fastq sequence files were prepared for import into QIIME2. A manifest file was created in order to import the sequences. A visualization file was created and the imported sequences were demultiplexed using demux. The sequences were trimmed based on the demultiplexed visualization output and an ASV (amplicon sequence variant) table was created. A file defining the metadata was created. Both Deblur and DADA2 were used for sequence quality control. The filtered, denoised data was summarized to produce two visualization files that explore how rarefaction depths impact the data. Silva was used to generate taxonomy because it generated fewer unclassified results. The QIIME2 Moving Pictures tutorial was followed to generate alpha and beta diversity plots, taxonomic bar plots, ASV tables, and rarefaction curves. Alpha diversity (Shannon Diversity, Pielou's Evenness, and Faith's Diversity) was used to explore differences within the samples and a Kruskal-Wallis pairwise analysis of variance was used to determine if the differences in alpha diversity between the samples were significant. Beta diversity (Bray-Curtis Distance, Jaccard Distance, and Weighted and Unweighted

UniFrac Distance) were used to explore differences between the samples. $P < 0.05$ was considered statistically significant. An ASV heatmap was also generated.

4. Results and Discussion

4.1 Alpha and Beta Diversity

α-diversity is a measure of community richness or diversity within a system. The differences in α-diversity metrics (Shannon diversity, Faith's diversity, and Pielou's evenness) were insignificant between the four cultures (P-value > 0.1). After DADA2 filtering, there were a total of 603,401 and 424,475 filtered reads in the *M. aeruginosa* NIES 843 samples, replicates 1 and 2 respectively. There were 887,510 and 580,636 filtered reads from replicates 3 and 4 of the *M. aeruginosa* CCMP 3462 strain. The filtered reads totaled 804,507 for replicate 5 and 744,214 for replicate 6 from the *R. raciborskii*. There were a total of 664,010 and 631,678 filtered reads from replicate 7 and 8 of the *A. anophagefferens* culture. The α-rarefaction curves show that the level of diversity between the four cultures is similar (**Figure 1**). Additionally, at this sequencing depth, the total α-diversity was captured (**Figure 1)**. Low α-diversity may be an indicator for high β-diversity (Koleff *et al,* 2003) and that does in fact appear to be the case here.

β-diversity is a measure of community dissimilarity or a quantification of different communities within a region. High β-diversity indicates a low level of similarity. Across three metrics of β-diversity, there were similar clustering patterns between individual replicates from the same culture as well as amongst the cyanobacteria (**Figure 2**). An analysis of the individual ASVs driving this clustering pattern will be discussed below.

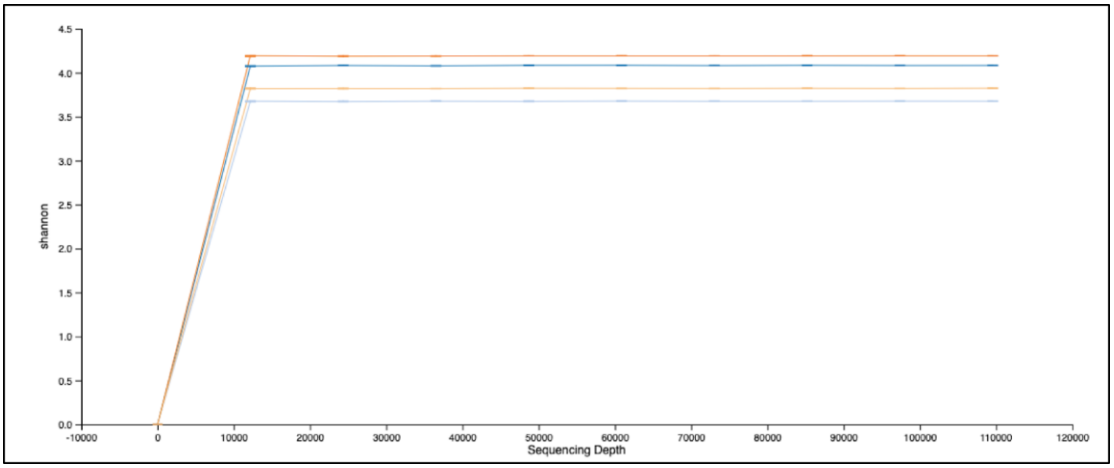

Figure 1. Alpha rarefaction curve using Shannon diversity metric. *Aureococcus* dark blue, *Cylindrospermopsis* light blue, CCMP *Microcystis* dark orange, and UTK *Microcystis* light orange.

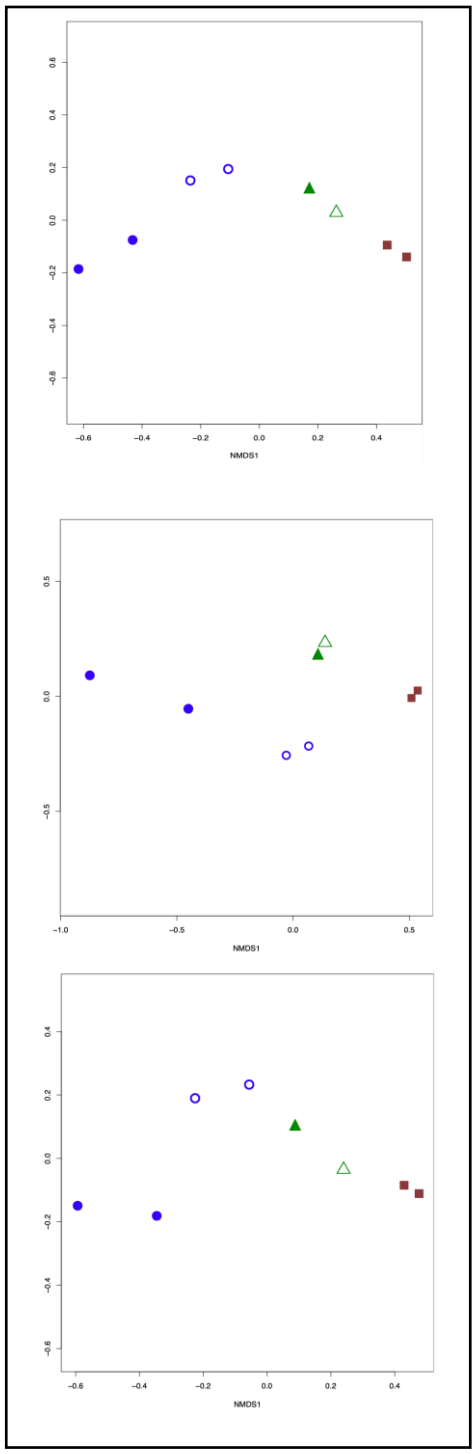

Figure 2. Non-metric Multi-dimensional Scaling (NMDS) plots of (a) Bray-Curtis distance, (b) weighted UniFrac distance, and (c) unweighted UniFrac distance. Blue circles represent *M. aeruginosa* NIES 843 (closed) and *M. aeruginosa* CCMP 3462 (open). Green triangles represent *R. raciborskii* CS-505 (closed) and *R. raciborskii* CS-1102 (open). Brown square represents *A. anophagefferens* (closed).

4.2 Taxonomy

The taxonomic classification provides a broad overview of community structure of the microbiomes. Across all four cultures, the predominant phyla represented are Proteobacteria, Cyanobacteria, and Bacteroidetes (**Figure 3**). It was unsurprising to see Cyanobacteria be highly represented in the *Microcystis* and *Raphidiopsis* cultures because the algae in those established cultures are members of the phylum Cyanobacteria. *Aureococcus* is a eukaryotic member of the class Pelagophyceae, so the Cyanobacteria ASVs that appear in the *Aureococcus* sample are most likely chloroplast signatures. Proteobacteria and Bacteroidetes are dominant phyla among the microbial consortia of other harmful algal blooms (Hattenrath-Lehmann and Gobler 2017). It is interesting to note that the phylum Actinobacteria was not represented at a level above 1% in either of the *Microcystis* strains because Actinobacteria are abundant in lakes where *Microcystis* blooms (Lew *et al*, 2015). This absence could suggest that while Actinobacteria are present during blooms, they are not associated with *Microcystis* colonies in culture. At the class level, Alphaproteobacteria was highly represented across all samples tested (**Figure 4**). Both of the duplicates of the *Microcystis* cultures had high frequencies of Oscillatoriophycideae (27.734%, 27.143%, 38.605%, and 46.817%), which is the class that *Microcystis* belongs to. The *Raphidiopsis* cultures had high frequencies of their respective class as well, Nostocophycideae (51.041% and 49.721%). There were some notable differences between the two strains of *Microcystis* at the class level including the presence of Saprospirae and Sphingobacteriia in the CCMP strain that were not present above 1% in the NIES strain. There were members of the Bacteroidetes family that were present in the *Aureococcus*, including Flavobacteriia that were totally

absent from any of the cyanobacterial cultures. The taxonomic breakdown at the order level highlighted some differences between the two *Microcystis* strains and even differences between duplicates from single strains (**Figure 5**). The NIES strain was comprised of a large amount of Sphingomonadales (55.302% and 68.447% respectively) and the CCMP strain had much less (3.272% and 4.681% respectively). The order that the algal strain belongs to was once again highly frequent among the cyanobacteria; Chroccocales (27.734%, 27.143%, 38.605%, and 46.817%) for *Microcystis* and Nostocales (51.041% and 49.721%) for *Raphidopsis*. At the order level, the outgroup, *Aureococcus*, had a much different composition, unsurprisingly. The makeup of *Aureococcus* has primarily Rhodobacterales (51.113% and 49.555%) and Flavobacteriales (22.490% and 22.458%). While taxonomy does provide insight into community structure, it is still only a broad overview.

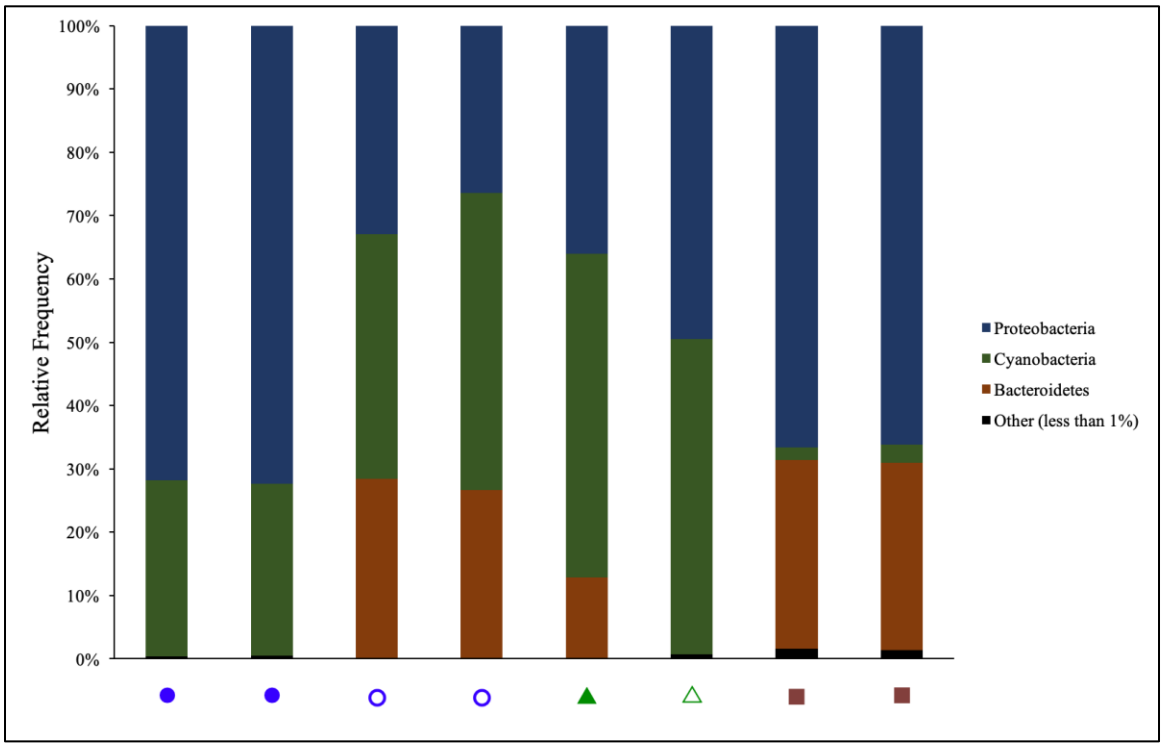

Figure 3. Relative abundance of bacterial ASVs at the phylum level. All phyla represented at a level less than 1% were grouped into the Other category. Blue circles represent *M. aeruginosa* NIES 843 (closed) and *M. aeruginosa* CCMP 3462 (open). Green triangles represent *R. raciborskii* CS-505 (closed) and *R. raciborskii* CS-1102 (open). Brown square represents *A. anophagefferens* (closed).

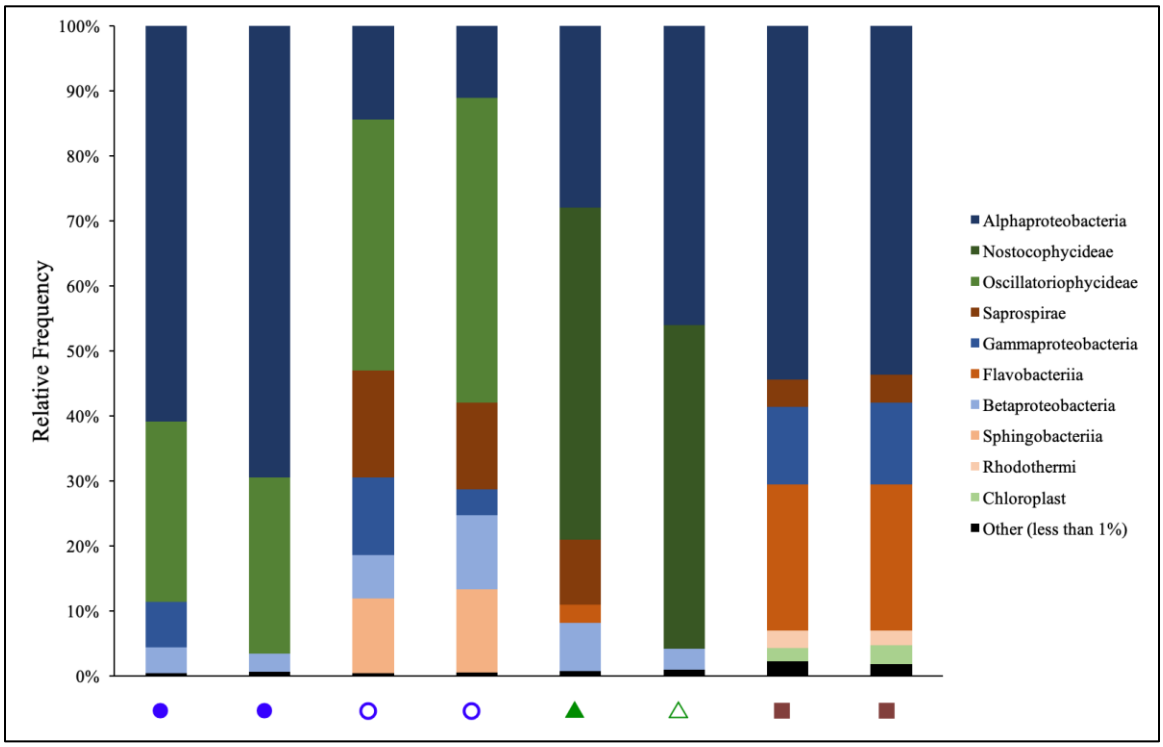

Figure 4. Relative abundance of bacterial ASVs at the class level. All classes represented at a level less than 1% were grouped into the Other category. Blue circles represent *M. aeruginosa* NIES 843 (closed) and *M. aeruginosa* CCMP 3462 (open). Green triangles represent *R. raciborskii* CS-505 (closed) and *R. raciborskii* CS-1102 (open). Brown square represents *A. anophagefferens* (closed).

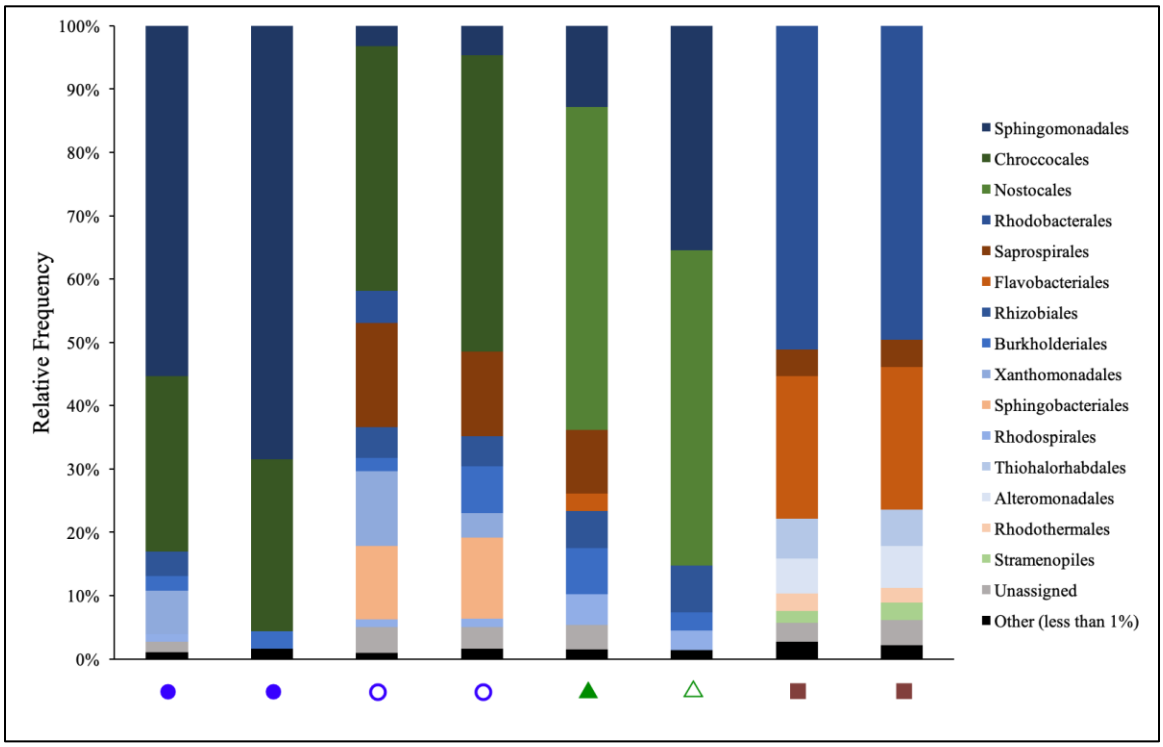

Figure 5. Relative abundance of bacterial ASVs at the order level. All orders represented at a level less than 1% were grouped into the Other category. Blue circles represent *M. aeruginosa* NIES 843 (closed) and *M. aeruginosa* CCMP 3462 (open). Green triangles represent *R. raciborskii* CS-505 (closed) and *R. raciborskii* CS-1102 (open). Brown square represents *A. anophagefferens* (closed).

4.3 Individual OTUs

A Bray-Curtis similarity analysis demonstrated that the replicates for each strain clustered together, indicating that the bacterial community associated with each individual strain were more similar to each other than to different strains being examined **(Figure 6)**. Further, the two strains of *Microcystis* (NIES and CCMP) were more similar to each other than to the other cyanobacteria, *Raphidiopsis*. The least similar to those cyanobacteria was the outgroup, *Aureococcus*. An analysis of the abundance of individual ASVs from each sample indicated what was driving these clustering patterns (**Figure 7**). The two *Microcystis* strains were more similar to each other than they were to the other cyanobacteria and the overlap that does exist could suggest generalist species. Some of the most abundant ASVs were identified as the alga from which the culture was derived. ASVs 2 and 5 were *Microcystis*. ASV 3 is *Cylindrospermopsis* (renamed *Raphidiopsis*). In the NIES strain, there are multiple ASVs that are from the Sphingomonadaceae family (ASVs 4, 15, and 21). Members from this family have previously been isolated from the *Microcystis* phycosphere (Zhang *et al*, 2021). The most abundant ASV across any of the 8 samples was a Saprospirales (ASV 1) in the NIES strain which also has been linked with a *Microcystis* bloom (Guedes *et al*, 2018). Some of the most abundant ASVs in the CCMP strain were from the Chitinophagaceae and Xanthomonadaceae families (ASVs 6 and 10). Members of the Chitinophagaceae family have been isolated from freshwater reservoirs (Jin *et al*, 2014). Members of the Xanthomonadaceae family have been previously isolated from *Microcystis* laboratory cultures (Chun *et al*, 2017). The most abundant ASV in the *Aureococcus* cultures was a seawater bacterium from the *Marivita* genus (ASV 9) (Zhong *et al*, 2015).

The phycosphere, and specifically the composition of the heterotrophs within, plays a vital role in the formation of *Microcystis* blooms (Cook *et al,* 2019). While some variation is inevitable, there are general patterns; the two strains of *Microcystis* more closely resemble each other than they do the other cyanobacterium. This suggests that despite geographic distance, the host dictates the microbiome. This logic would coincide with the gene loss hypothesis based on the Black Queen Hypothesis (Morris *et al,* 2012). Due to the loss of function (by the algal cell), specific needs are met based on the bacterial ability; which often correlates with taxonomy. There could be active selection by the *Microcystis* cell for bacteria that are able to complete a certain function (Jackrel *et al,* 2019). The overlap with *Raphidiopsis* could indicate a generalist cyanobacteria – or freshwater species – microbiome. These species of heterotrophs are likely involved in a type of vitamin biosynthesis that most phytoplankton are auxotrophic for (Cook *et al,* 2019).

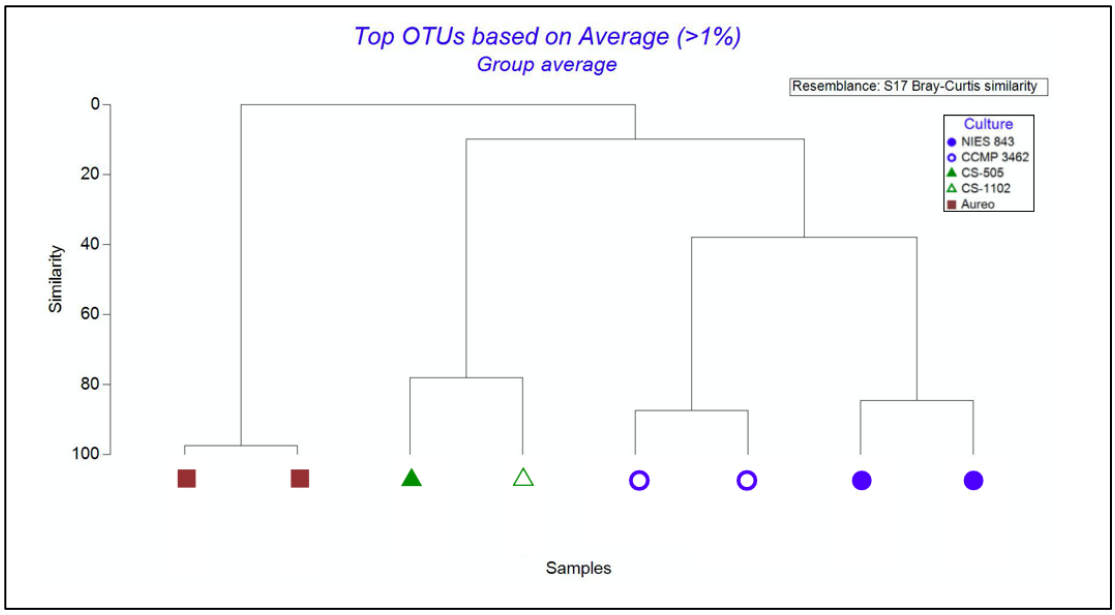

Figure 6. Percent similarity of top 26 individual ASVs based on Bray-Curtis similarity. Blue circles represent *M. aeruginosa* NIES 843 (closed) and *M. aeruginosa* CCMP 3462 (open). Green triangles represent *R. raciborskii* CS-505 (closed) and *R. raciborskii* CS-1102 (open). Brown square represents *A. anophagefferens* (closed).

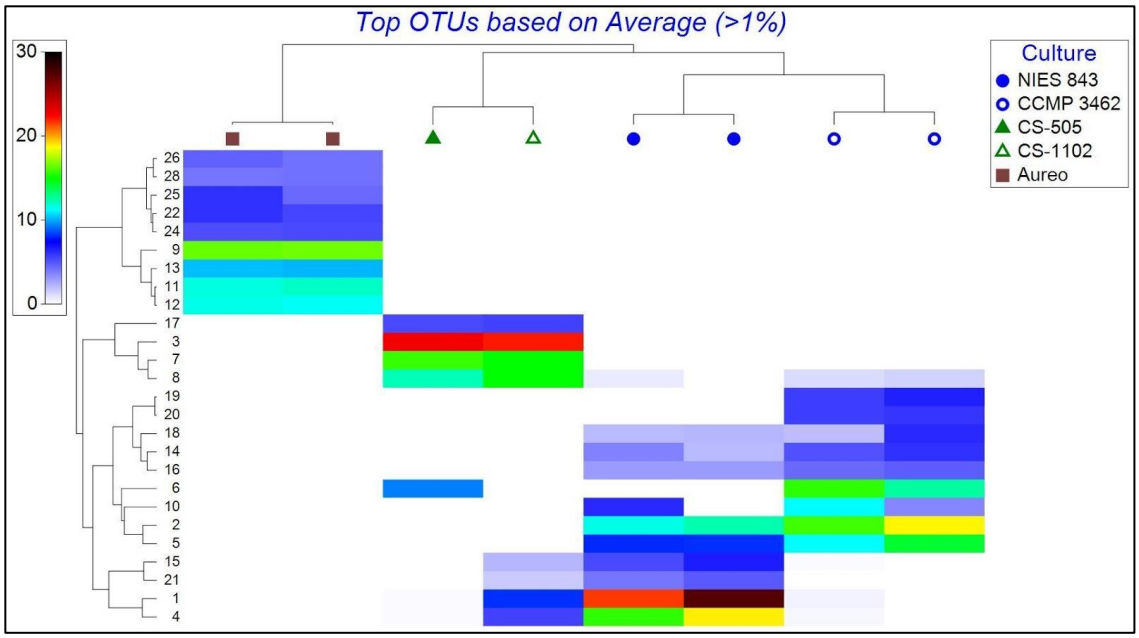

Figure 7. Percent relative abundance of the top 26 individual ASVs in each culture. The warmer the color indicates the more abundant the ASV. Blue circles represent *M. aeruginosa* NIES 843 (closed) and *M. aeruginosa* CCMP 3462 (open). Green triangles represent *R. raciborskii* CS-505 (closed) and *R. raciborskii* CS-1102 (open). Brown square represents *A. anophagefferens* (closed).

5. Conclusions

The clustering patterns seen in the β-diversity analysis and the ASV heatmap suggest that the host drives the microbiome. Two *M. aeruginosa* strains (NIES and CCMP) that were isolated from opposite sides of the globe, have bacterial communities that are more similar to each other than to a filamentous cyanobacterium (*R. raciborskii*), or than to a marine eukaryote (*A. anophagefferens*). Additionally, Actinobacteria was absent from the cultures. This suggests that the phylum Actinobacteria, while abundant in lakes where *Microcystis* blooms, is not associated with the colonies.

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Chapter III

Response to Heterotrophic Bacteria in Co-Culture

1. Abstract

Heterotrophic bacteria were collected and isolated from natural *Microcystis* blooms occurring in Lake Erie (USA) and Lake Tai (China). Detailed co-culture growth experiments were conducted with two isolates from Lake Erie (a putative *Exiguobacterium* and putative *Acidovorax*) and one isolate from Lake Tai (a putative *Stenotrophomonas*) in order to examine their effects on *Microcystis* growth and physiology. *Exiguobacterium* (Lake Erie) and *Stenotrophomonas* (Lake Tai) had strong growth promoting effects on *M. aeruginosa* while *Acidovorax* (Lake Erie) had positive effects on the photosynthetic efficiency of *Microcystis*

2. Introduction

Microcystis forms toxic blooms globally and poses a threat to freshwater environments (Harke *et al,* 2016). Although abiotic factors such as rising temperatures and increased anthropogenic nutrient loading are thought to be large contributors to the formation of these blooms (Paerl 2014), a growing body of evidence suggests that heterotrophic bacteria may be playing an important (albeit unknown) role in *Microcystis* bloom dynamics (Shen *et al,* 2011). *Microcystis* cells are held together in colonies, surrounded by a carbohydrate-rich mucilage in which heterotrophic bacteria are embedded (Shirai *et al,* 1989). This region is referred to as the phycosphere; akin to the rhizosphere in plants (Bell *et al,* 1972). The bacteria are provided with primary metabolites such as sugars or amino acids while the algae receive nutrients and vitamins (Seymour *et al,* 2017). The tight coupling of heterotrophic bacteria and *Microcystis* colonies, combined with the potential for nutrient/metabolite exchange in the

phycosphere, indicates that these toxic bloom events must be viewed as a system rather than a single species being the causative agent.

The exact effects that different species of heterotrophic bacteria have on the growth and physiology of *Microcystis* is largely unknown. However, previous work has demonstrated that there are three basic categories of ecological relationships that can form between phytoplankton and heterotrophic bacteria. First, the relationship can be a true mutualism in which both partners benefit, with phytoplankton cells gaining nutrients or other metabolites from the products of the bacteria, while bacteria gain access to reduced carbon sources from the phytoplankton extracellular polymeric secretions (Seymour *et al*, 2017). Second, the relationship could be antagonistic with the phytoplankton and bacterioplankton in competition for resources and/or through the direct synthesis and secretion of inhibitory allelopathic compounds (Seymour *et al*, 2017). A third category could be commensalism, with the bacterioplankton benefiting from the photosynthetic exudates while leaving the phytoplankton cells largely unaffected (Seymour *et al*, 2017).

This study further investigates the potential effects of heterotrophic bacteria on *Microcystis*. Hundreds of bacterial strains were isolated from natural *Microcystis* blooms in Lake Erie (USA) and Lake Tai (China). From these, three strains were chosen for detailed co-culture experiments assessing the impact of the individual strains on the growth and physiology of *M. aeruginosa* NIES 843. Samples were also taken for RNAseq analysis, but those data are not included herein.

3. Methods

3.1 Sample Collection

Samples were collected from the Western Basin of Lake Erie, Ohio, USA during an active bloom of *Microcystis* during the summer of 2017. Both 20 μm and 80 μm mesh plankton nets were used to collect water samples to ensure that only bacteria that were tightly coupled with the *Microcystis* were collected. The water collected was then filtered through 0.2 μm Sterivex filters and stored on ice while being transported back to the lab where they were then stored at -20 °C. This process was repeated in the summer of 2018 at the Taihu Laboratory for Lake Ecosystem Research in Lake Tai, China. 20 μm and 80 μm plankton nets were used to collect tightly coupled bacteria and the samples were filtered on 0.2 μm polycarbonate filters before being transported back to the United States with ice packs. Samples were then stored at -20 °C until extraction.

3.2 Isolation and Identification

Bacteria were isolated using Luria broth (LB) and CT (Ichimura, 1971) agar plates. 500 μL of water samples from Lake Erie were pipetted onto LB and CT agar plates, spread with sterile cell spreaders, and incubated at 26 °C. Filter samples from Lake Tai were resuspended in liquid media before being spread onto LB and CT agar plates. LB plates were incubated for 48 hours before single colonies were streaked onto new plates and CT plates were incubated for seven days. Re-streaking of single colonies was repeated until pure cultures were obtained. Purity was further confirmed via Gram staining.

In order to obtain a putative identity of the strains, pure single colonies were inoculated in 5 mL of broth and incubated in a shaking incubator at 26 °C and 200 rpm

for 24 hours. 1500 µL of turbid broth was transferred to 1.5 mL microcentrifuge tubes and centrifuged at 13000 rpm. The supernatant was discarded. The pellet was resuspended in 500 µL of sterile water and heated for 15 minutes in a 95 °C dry bath. The tubes were then centrifuged at 13000 rpm for 1 minute. The supernatant was then pipetted into a new 1.5 mL microcentrifuge tube. The 16S rRNA gene was amplified using PCR primers 27f (5' AGAGTTTGATCMTGGCTCAG 3') and 1492r (5' TACGGYTACCTTGTTACGACTT 3') (DeLong, 1992). The reaction mixture comprised of 1.25 µL of 10 µM 27f primer, 1.25 µL of 10 µM 1492r primer, 12.5 µL of 2x Econo Taq Master Mix (Lucigen), 8.5 µL of PCR water, and 1.5 µL of template DNA (DeLong, 1992). The PCR program was then run on a BioRad C1000 Touch thermocycler with an initial cycle at 95 °C for 5 minutes, 35 cycles at 95 °C for 1 minute, 50 °C for 30 seconds, and finally 72 °C for 90 seconds. Screening for amplification was done using a 1% agarose/TAE gel and stained with 3x Gel Red (Phenix Research Products) before imaging. After amplification, the PCR products were purified using the Qiagen QIAquick PCR purification kit before Sanger sequencing at Eurofins Genomics. Sequences were run in BLAST (blastn) with environmental isolates excluded in order to identify at the genus level.

3.3 Bacterial Growth Curves

Three isolates were chosen for further study (two from Lake Erie and one from Lake Tai). The isolates will be referred to by their putative genus based upon 16S rRNA sequences. Growth curves were performed with each isolate, in order to determine the bacterial cell densities at various optical densities during the growth phases. In order to calculate the growth curves for the *Exiguobacterium* and *Acidovorax*, a single bacterial

colony was picked from a plate and used to inoculate CT-TY broth (CT with 1 g/L tryptone and 1 g/L yeast extract). The culture was incubated for 48 hours at 26 °C. The optical density of the culture was measured. 100 mL of CT-TY was inoculated to a final optical density of 0.05 with the bacterial culture. The freshly inoculated culture was diluted and plated onto CT-TY agar plates in duplicate to determine cell counts. The fresh culture was incubated at 26 °C in a shaking incubator at 200 rpm. Samples were taken every hour for *Exiguobacterium* and every two hours for *Acidovorax*. The samples taken were diluted and plated. Optical density measurements were taken at the same time. This process was repeated until the culture reached the stationary phase. All of the CT-TY plates were incubated for 48 hours at 26 °C before colonies were counted. Cell densities were calculated to generate the growth curves. To generate a growth curve for *Stenotrophomonas*, a single bacterial colony was used to inoculate CT-TY broth and incubated for 48 hours at 26 °C in a shaking incubator at 200 rpm. The culture was diluted at 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} and optical density was measured. The diluted samples were plated and incubated for 48 hours at 26 °C before colonies were counted. Cell counts were calculated to generate the growth curve.

3.4 Co-Culture Conditions

The following experiments were performed in triplicate. Pure cultures of *Exiguobacterium, Acidovorax*, and *Stenotrophomonas* were grown for 48 hours at 26 °C in CT-TY broth. The flasks were inoculated with 10^5 cells/mL of *Microcystis* and 10^7 cells/mL of bacterial culture (1:100) (Gao *et al*, 2020 and Hoke 2019). The starting volume of *Microcystis* was determined via cell counts of axenic culture using a

hemocytometer. The axenicity of the *Microcystis* was determined before inoculation using fluorescence microscopy. Prior to imaging, 200 mL of *Microcystis* was centrifuged to form a pellet. The supernatant was discarded and the pellet was resuspended in CT media. 100 mL of 300 nM DAPI stain was added in the dark. The sample was incubated in the dark for 60 minutes. 10 µL of the sample was placed on a slide and covered with a cover slip before imaging with a Leica DM6B microscope. The volume of bacterial culture was determined by taking an optical density measurement and calculating the adequate volume based upon the bacterial growth curve data. The control flasks contained only *Microcystis* with an inoculum of 10^5 cells/mL. CT media was added to the flask for a final volume of 300 mL. Each treatment was inoculated in triplicate. All of the flasks were incubated at 26 °C with a 12:12 light:dark cycle for the entirety of the experiment.

3.5 Tracking Growth

Fluorescence readings were taken every 48 hours using a Phyto-PAM-II Compact fluorometer (Walz). The flasks were dark adapted for 20 minutes and an initial fluorescence reading was collected (F_n) by adding 2 mL of culture to a crystal cuvette. Measurements were taken at a gain setting of 14. The gain setting was previously determined by measuring the auto gain of a turbid culture of *Microcystis*. After the F_n reading was recorded, 6 µL of 5 µM DCMU was added to the cuvette to artificially close the photosystem II reaction centers. A second reading was then taken (F_m) (Parkhill *et al*, 2001). The initial (F_n) and final (F_m) readings were used to calculate the F_v/F_m value: Fv/Fm = (Fm - Fn) which was used as a measure of photosynthetic efficiency. 1 mL of culture was also preserved at this time with 35 µL of Lugol's iodine. 10 µL of these

preserved samples were later used for cell counts of *Microcystis* via a hemocytometer under a brightfield microscope.

3.6 RNA Sampling

Mid-exponential and early stationary timepoints were included during the growth curve in order to extract RNA. The mid-exponential timepoint occurred once the total cell concentration was over 1 million cells per mL. The early stationary timepoint occurred when the cell counts began to plateau. To sample, the entire volume of the flask was filtered through a 0.2 µm Sterivex filter and immediately preserved in liquid nitrogen before being stored at -80 °C until extraction. The *Stenotrophomonas* samples were vacuum filtered through 0.2 µm filters before being flash frozen in liquid nitrogen and stored at -80 °C. The extraction, sequencing, and analysis of these samples will not be included here.

4. Results and Discussion

4.1 Response to Co-culture

Before each experiment was started, *Microcystis* was determined to be axenic via DAPI staining and fluorescence microscopy. Notice that no other cells besides *Microcystis* were visible. The axenic *Microcystis* was grown in co-culture with each of the three bacterial isolates (*Exiguobacterium, Acidovorax,* and *Stenotrophomonas*) to determine whether or not there was an effect on the growth and physiology of *Microcystis.* The mid-exponential timepoint occurred after the cell counts reached 1 million *Microcystis* cells/mL. The early stationary timepoint, and the end of the experiment occurred when the cell counts began to plateau. The mid-exponential phase

occurred on day 10 during the *Exiguobacterium* co-culture and on day 14 during the *Acidovorax* and *Stenotrophomonas* co-culture experiments. The early stationary timepoint occurred on day 34 for the *Exiguobacterium* co-culture, and day 32 for the *Acidovorax* and *Stenotrophomonas* co-culture experiments.

The *Exiguobacterium* had a growth promoting effect on *Microcystis* (**Figure 1a and Figure 1b**). The cell densities reached a final of 5,900,000 cells/mL in the *Exiguobacterium* co-culture and 3,453,333.3 cells/mL in the axenic culture. The difference in growth was statistically significant (*P*-value < .05) for both the fluorescence (P=0.0007) and for the cell counts (P=.008) in a t-test. No discernable effect was observed from the *Acidovorax* growth curves (**Figure 2a and Figure 2b**). The *Stenotrophomonas* also had a growth promoting effect on *Microcystis* with the direct cell counts reaching 1,700,000 cells/mL in the control and 1,090,000 cells/mL in the co-culture (**Figure 3a and Figure 3b).** Cell densities reached a maximum of 4,680,000 cells/mL in the *Stenotrophomonas* co-culture relative to 3,233,333.3 cells/mL in the axenic control. The difference in growth was statistically significant (*P*-value < .05) for both the fluorescence (P=.008) and the cell counts (P=.013) in a t-test.

F_v/F_m is a commonly used metric for plant and algae "stress" that specifically measures photosynthetic efficiency. Any values that fall below 0.3 indicate an unhealthy, or "stressed" zone for *Microcystis*. Conversely, any values between 0.4 and 0.6 indicate a healthy zone. In the days following inoculation, both the treatment and the control experienced a dip into the unhealthy zone before a short rise, then plateau in the *Exiguobacterium* and *Stenotrophomonas* co-cultures (**Figure 1c and Figure 3c**). All of the *Acidovorax* co-culture treatments and controls began in the unhealthy zone, but

moved into the healthy zone after day 2 where it remained throughout the duration of the experiment with the exception of the *Acidovorax* treatment on the last day (**Figure 2c**). The *Acidovorax* treatment also had a higher F_vF_m than the *Microcystis* control. The difference in stress was statistically significant (*P*-value < .05) for the photosynthetic efficiency (P=.013) in a t-test.

The genus *Exiguobacterium* occupy a geographically and thermally diverse environment; of which certain species have demonstrated the ability to provide protection via extracellular substances (Armstrong *et al,* 2001). In co-culture experiments with *M. aeruginosa* and *Exiguobacterium*, the numbers of cells of both the algae and bacteria have been shown to increase (Wang *et al,* 2016). Additionally, these studies have shown that *Exiguobacterium* is enhancing the growth by inducing colony formation, though the exact mechanisms remain unclear (Wang *et al*, 2016). Other evidence show that certain species of *Exiguobacterium* are antagonistic towards *Microcystis* (Tian *et al,* 2012). *Exiguobacterium* was chosen for this experiment because of its potential protective effect in high salt environments (Hoke 2019).

Acidovorax (previously *Pseudomonas*) are gram-negative rods that are commonly found in aquatic environments. This specific isolate has genes for the reduction of nitrate and nitrite to ammonium including genes for nitric oxide reductase and nitrous oxide reductase (Hoke 2019) This isolate was chosen because it had previously demonstrated growth promoting effects on *Microcystis* in co-culture (Hoke 2019). It was hypothesized that the ammonium produced by this isolate would satisfy the nitrogen requirements of the *Microcystis* and encourage its growth.

Acidovorax has demonstrated significant impacts on growth in previous co-culture studies (Hoke 2019). These data did not show those same results and it is hypothesized that is due to the inoculum. The technique used to determine bacterial cell density was based off of an optical density. This does not account for the ratio of living:dead cells. The difference in the number of live cells added to the co-culture could change the rate of nutrient depletion, therefore impacting the effect the bacteria had.

Species of *Stenotrophomonas* have been isolated from colonies of *M. aeruginosa* and have shown the ability to promote growth via the release of amino acids and solubilized phosphates (Shi *et al,* 2009). This isolate was chosen for the growth experiment because it had been shown to have a strong impact on the growth of *Microcystis* when grown in co-culture (Gardner 2021).

While the bacteria used for this experiment were isolates collected in 2017 and 2018 from Lake Erie and Lake Tai respectively, they are also found in established *Microcystis* cultures from other locations. Four different *Acidovorax* ASVs were identified in the 16S microbiome analysis from the previous chapter. Additionally, the Xanthomonadaceae family was represented in both the NIES 843 culture (6%) and the CCMP culture (11%) and is the family that *Stenotrophomonas* belongs to. The fact that these two are found both in established cultures as well as in recent field samples from *Microcystis* blooms could have further implications about the importance of relationships within the phycosphere.

It is hypothesized that the nature of all three of these bacterial relationships with *Microcystis* are mutualistic. The growth promoting effect of the *Microcystis* indicates that the algal partner is benefitting from the relationship. The bacteria are likely benefitting

from the accessible forms of carbon that *Microcystis* provides. This could be demonstrated by repeating the experiment and measuring bacterial density throughout.

Growth promoting effects of heterotrophic bacteria have been studied in other freshwater and marine algal species (Gao *et al*, 2020 and Seymour *et al*, 2017). Often, a single heterotroph can substantially affect growth by increasing the nutrient or vitamin concentration (Gao *et al*, 2020). RNA sequencing will provide further insight into the specifics of the bacterial contribution. Further studies using a consortium of bacteria are required in order to get a more accurate replication of what occurs in the field.

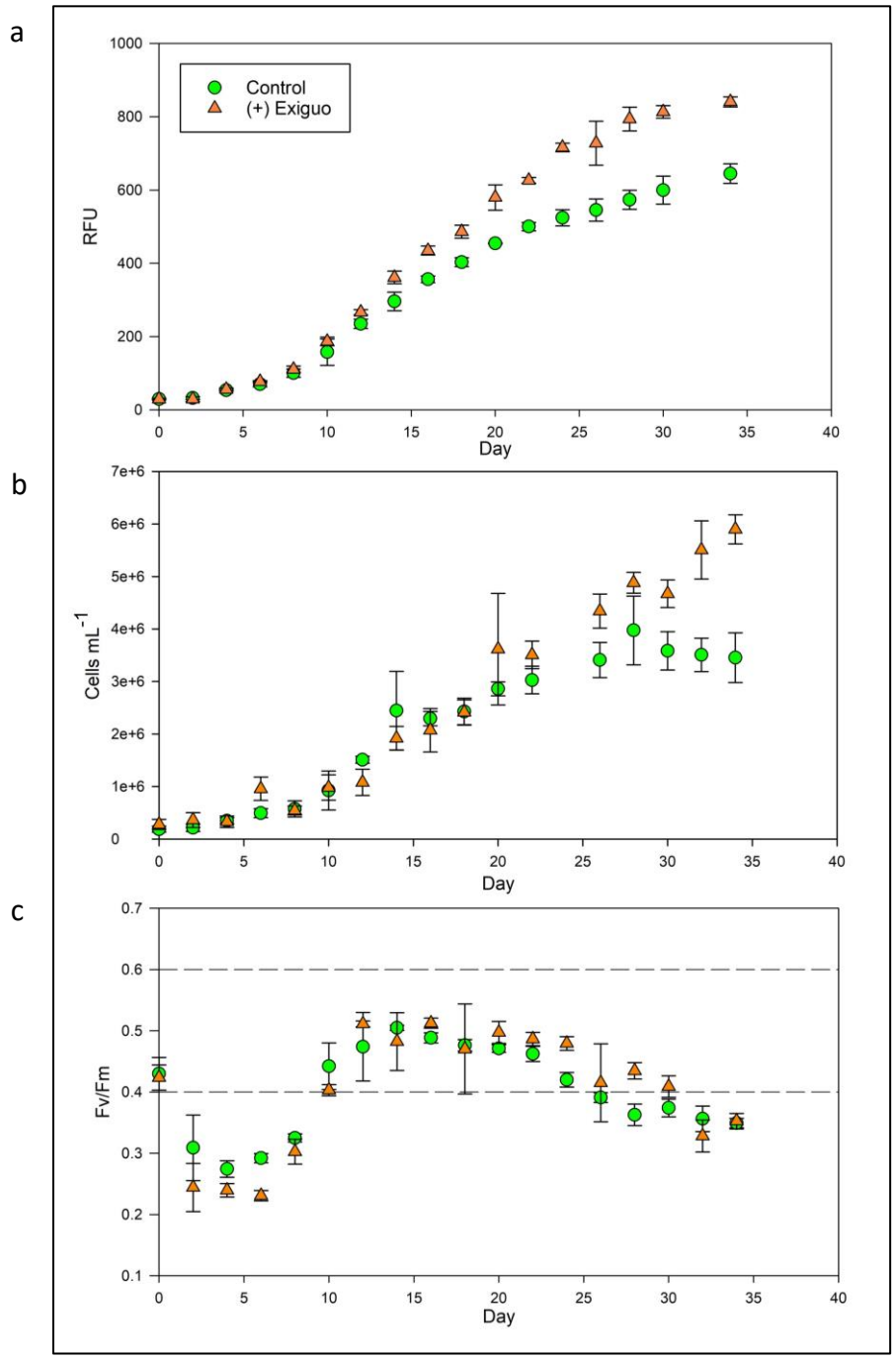

Figure 1. (a) Fluorescence of *Microcystis* cells grown with and without *Exiguobacterium* at 540 nm. Vertical line depicts mid-exponential timepoint (Day 10), (b) direct cell counts of *Microcystis* cells with and without *Exiguobacterium.* Vertical line depicts mid-exponential timepoint (Day 10), (c) F_v/F_m of *Microcystis* cells grown with and without *Exiguobacterium* at 540 nm. Vertical line depicts mid-exponential timepoint (Day 10). The green horizontal lines indicate a healthy zone (between 0.6 and 0.4) while the red line shows a stressed zone (below 0.3).

a

b

c

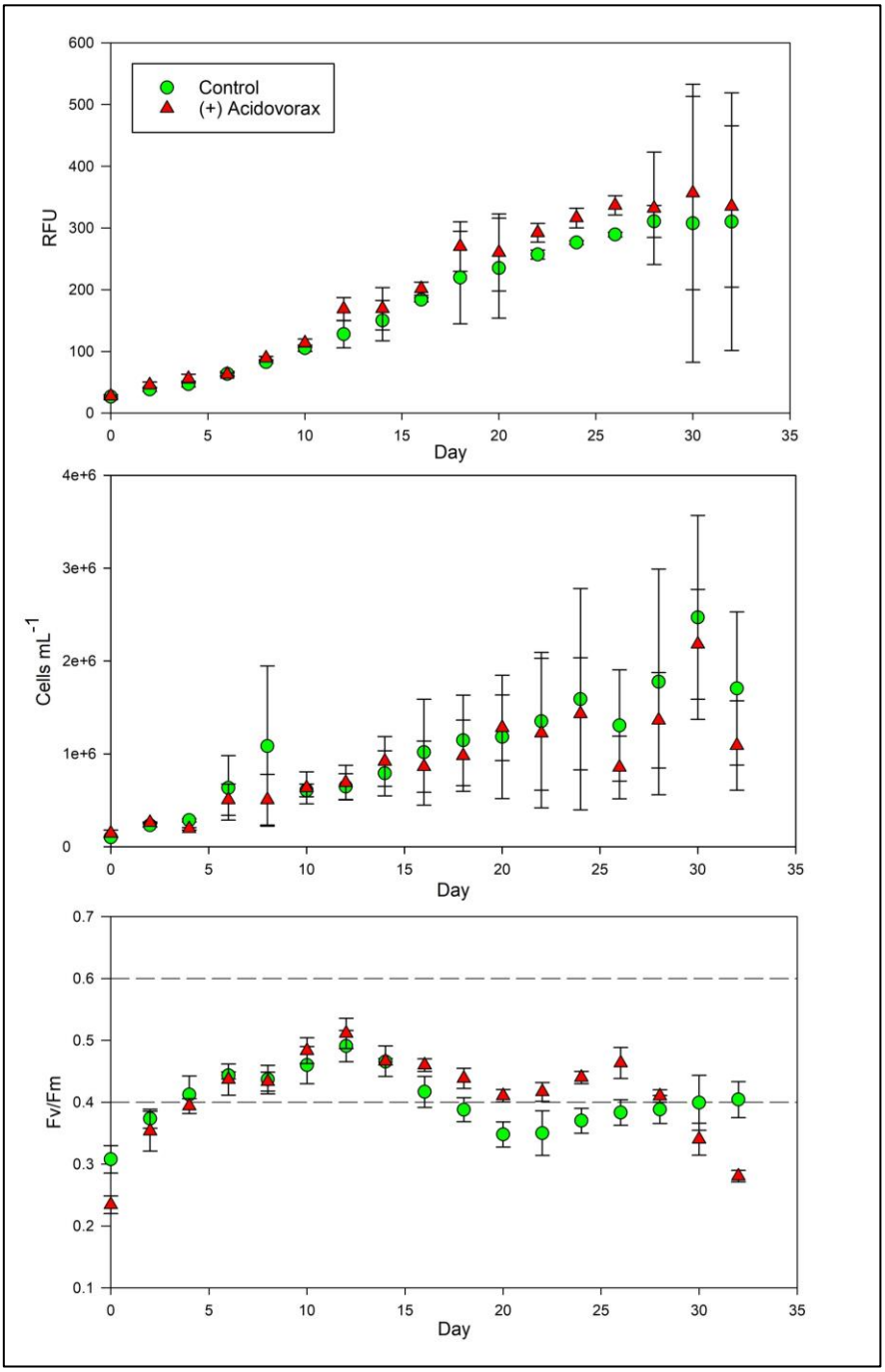

Figure 2. (a) Fluorescence of *Microcystis* cells grown with and without *Acidovorax* at 540 nm. Vertical line depicts mid-exponential timepoint (Day 14), (b) direct cell counts of *Microcystis* cells with and without *Acidovorax.* Vertical line depicts mid-exponential timepoint (Day 14), (c) F_v/F_m of *Microcystis* cells grown with and without *Acidovorax* at 540 nm. Vertical line depicts mid-exponential timepoint (Day 14). The green horizontal lines indicate a healthy zone (between 0.6 and 0.4) while the red line shows a stressed zone (below 0.3).

a

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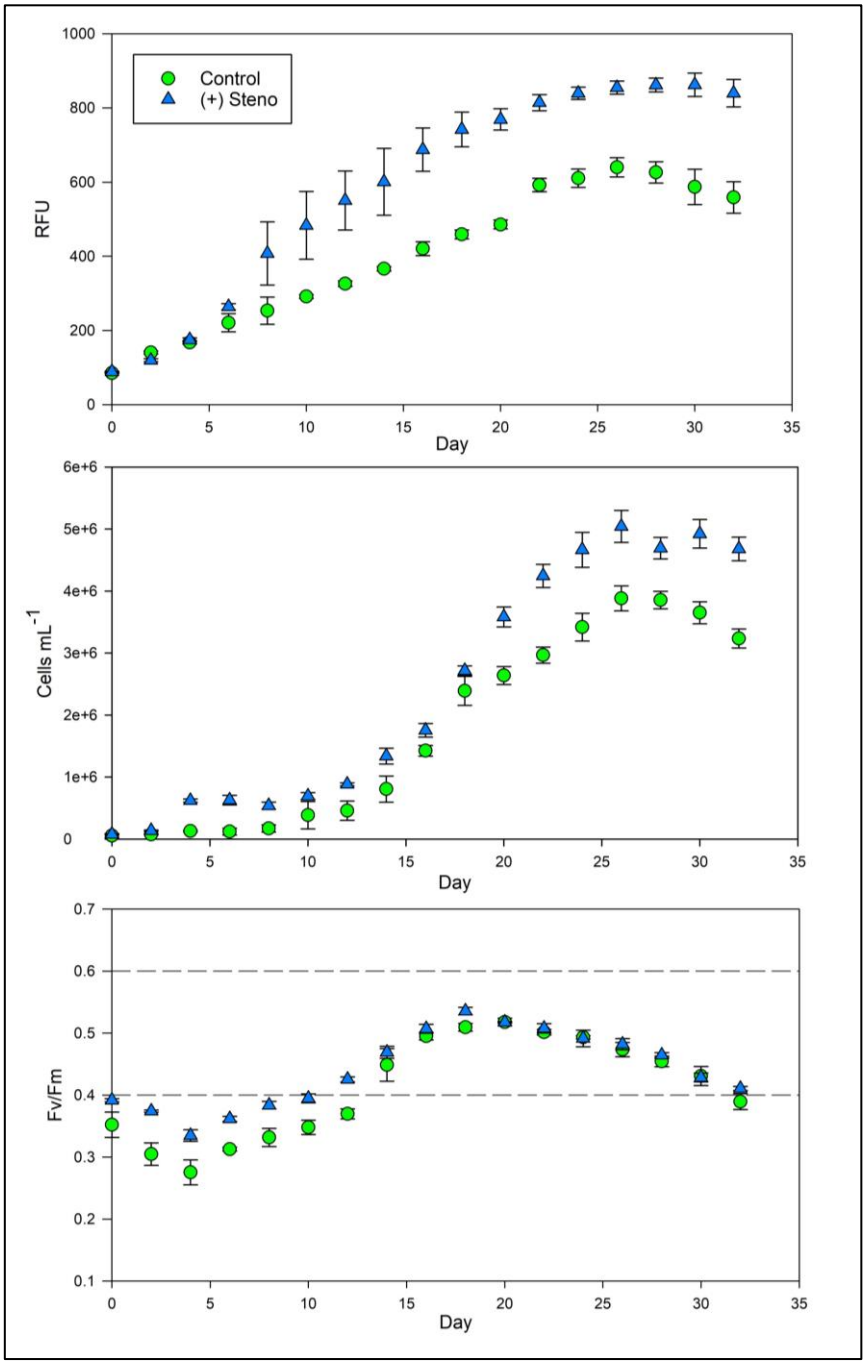

Figure 3. (a) Fluorescence of *Microcystis* cells grown with and without *Stenotrophomonas* at 540 nm. Vertical line depicts mid-exponential timepoint (Day 14), (b) direct cell counts of *Microcystis* cells with and without *Stenotrophomonas.* Vertical line depicts mid-exponential timepoint (Day 14), (c) F_v/F_m of *Microcystis* cells grown with and without *Stenotrophomonas* at 540 nm. Vertical line depicts mid-exponential timepoint (Day 14). The green horizontal lines indicate a healthy zone (between 0.6 and 0.4) while the red line shows a stressed zone (below 0.3).

5. Conclusions

These growth data (fluorescence and cell counts), as well as photosynthetic efficiency (F_vF_m) allow us to establish a positive effect in co-culture for *Exiguobacterium* and *Stenotrophomonas.* The *Acidovorax*, while not directly having a positive impact on growth, does have an effect on photosynthetic efficiency. Including the timepoints for RNA extraction allows for further investigation into this theory via transcriptomics.

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Chapter IV

Conclusions

Blooms of *Microcystis* are a growing concern with the current rates of global climate change combined with eutrophic conditions of freshwater environments. While not only creating an unsightly sludge on the top of the water, there are serious public health and economic costs that are associated. The microcystin toxin, that is produced by some species, is a potent liver toxin whose effects are not entirely known. N and P inputs, largely from fertilizer runoff, have been implicated as limiting factors. In addition to abiotic factors, there exist living components that also play a role. Heterotrophic bacteria that reside within the phycosphere are being studied as biotic factors that contribute to blooms of *Microcystis*. They work together, turning the region around the algal cell into a community that acts as a system rather than a single agent.

Interactions between an algal cell and the heterotrophic bacteria that are embedded in its phycosphere are proving to be equally as important in the establishment of blooms as abiotic factors are. Taking both biotic and abiotic factors into account is critical in protecting the Earth's freshwater resources.

In this thesis, 16S microbiome analysis was performed of four high-biomass bloom-forming algae in order to determine community structure. A clustering pattern between the two strains of *M. aeruginosa* was seen, indicating host control of the microbiome. The overlap of ASVs between *Microcystis* and the other cyanobacterium also revealed some generalist species. The lack of similarity to a marine eukaryote (outgroup) further enforces the theorized specificity of the microbiome. Several of the ASVs identified were also isolated from filtered samples collected in Lake Erie and Lake Tai. Three of these colony-associated heterotrophs (*Exiguobacterium*, *Acidovorax*, and *Stenotrophomonas*) were used in detailed growth experiments. Out of these three,

Exiguobacterium and *Stenotrophomonas* appeared to have a growth-promoting effect; determined by the fluorescence and direct cell counts of *Microcystis*. The *Acidovorax* species, while not having any apparent effect on growth, was not an antagonist and did appear to have some protective aspect; as reflected by the F_vF_m of *Microcystis*.

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