8-2021

A stable-isotope probing approach to modeling bidirectional nutrient exchange in the Microcystis phycosphere

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A stable-isotope probing approach to modeling bidirectional nutrient exchange in the *Microcystis* phycosphere

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A thesis submitted to the Graduate Faculty of

JAMES MADISON UNIVERSITY

In

Partial Fulfillment of the Requirements

for the degree of

Master of Science

Department of Biology

August 2021

FACULTY COMMITTEE:

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Abstract

Cyanobacterial harmful algal blooms (cHABs) plague freshwater systems worldwide and are projected to increase in intensity in the coming decades. cHABs damage aquatic ecosystems by blocking light penetration into the water column, creating hypoxic conditions, and releasing toxins. One of the most prolific cHAB formers is the cosmopolitan genus of cyanobacteria *Microcystis.* Global climate change and anthropogenic loading of nutrients such as nitrogen (N) and phosphorus (P) fuel *Microcystis* bloom formation. Increasing global temperatures favor *Microcystis* because of its high optimal growth temperature. N input is of particular importance for *Microcystis* because it is unable to fix atmospheric N, unlike other co-occurring genera of cyanobacteria such as *Dolichospermum*. Understanding these abiotic factors is essential for managing bloom formation, but the lesser understood biotic factors, including microbial interactions, may also be critical to bloom formation and success. *Microcystis* spp. co-occur with a consortium of microorganisms in the microenvironment surrounding the phytoplankton cells, called the phycosphere. This close association with microbes gives way for interactions between *Microcystis* and the constituents of its phycosphere. The *Microcystis* phycosphere is rich in nutrients that are produced by its members, and other members benefit from the provision of these nutrients. To better understand bidirectional nutrient exchange between *Microcystis* and heterotrophic bacteria in the phycosphere, two stable isotope (SIP) experiments were performed to trace the movement of nutrients within two lab strains of *M. aeruginosa*. DNA-SIP was used with ^{15}N-nitrate and ^{15}N-urea to track the N utilization capabilities of bacteria within the *Microcystis* cultures and identify bacteria that could use these N sources and those that

could not. These results give insight into how N is used in the phycosphere and the potential for N exchange between the phycosphere partners. To trace carbon (C) exchange in the *Microcystis* phycosphere, 36 heterotrophic bacteria were isolated from a 2018 *Microcystis* bloom in Lake Tai (Taihu) to be used in an RNA-SIP experiment. This consortium of 36 bacterial isolates promoted growth of axenic *Microcystis aeruginosa* NIES 843 during a batch culture experiment. *M. aeruginosa* NIES 843 was then labeled with ^{13}C to trace C exchange from *Microcystis* to the Taihu bacterial consortium. Analysis of the transcriptomes from this experiment will identify the bacteria capable of utilizing *Microcystis*-derived C and give insight to the gene actively being used during this exchange of C. These SIP experiments give direct evidence of bidirectional nutrient exchange in the *Microcystis* phycosphere and further provide evidence of the important role heterotrophic bacteria play in cHAB formation.

Chapter I. Biotic interactions within *Microcystis* blooms

1. Introduction

Freshwater and marine ecosystems are threatened by harmful algal blooms (HABs) worldwide. In recent decades, blooms have occurred with increasing frequency, with this trend expected to continue as global temperatures rise and nutrient loading increases (Lurling, 2017; Preece et al., 2017). Cyanobacterial harmful algal blooms (cHABs) can negatively impact the ecology, health, and economy of aquatic environments and surrounding communities due to their potential to release toxins and cause animal, and rarely, human deaths. It is important to focus on bodies of freshwater that are plagued by cHABs because they are frequently sources of potable water and locations of recreation, tourism, and fisheries. *Microcystis* spp. commonly form cHABs in freshwater and estuarine systems all over the world (Harke et al., 2016).

2. Cyanobacterial Harmful Algal Blooms (cHABs)

Cyanobacteria are ubiquitous in aquatic environments, and although they are essential primary producers in aquatic ecosystems, some species can form cHABs when they proliferate. There is a wide array of freshwater bloom-forming cyanobacteria, including members of the genera *Dolichospermum (Anabaena)*, *Aphanizomenon*, *Lyngbya*, *Nodularia*, *Planktothrix*, *Oscillatoria*, and *Microcystis* (Paerl et al., 2001). Cyanotoxin production by these cyanobacteria is one primary reason for the destructive nature of these blooms.

The cyanobacterial genus *Microcystis* is the most commonly reported cause of cHABs in lakes and reservoirs globally (Wu, 2019), forming blooms on every continent except Antarctica (Zurawell et al., 2005). Since the 1970s *Microcystis* has been the dominant plankton in two of the world's largest lakes, Lake Erie and Lake Tai (*Taihu* in

Mandarin) (Lehman et al., 2017; Levy, 2017; Zhu et al., 2016). This is only expected to continue, as *Microcystis spp.* can survive in diverse environmental conditions and thrive in warm, high-nutrient waters. In North America, Lake Erie has been notoriously plagued by *Microcystis* blooms since the 1990s, with disruption in access to drinking water in the city of Toledo, OH, as recently as 2014 (Brittain et al., 2000; Steffen et al., 2017). Taihu, the third-largest lake in China, has annual *Microcystis* blooms, which threaten the drinking water for the tens of millions of people in the watershed (Qin et al., 2010).

cHABs have adverse effects on aquatic ecosystems due to biomass and toxin production, as the sheer biomass of the blooms can prevent light penetration in the water column (Anderson et al. 2002), hindering photosynthesis of aquatic plants beneath the surface scum (Landsberg, 2002). When blooms decline, heterotrophic bacteria consume the dissolved oxygen in the water during respiration, causing oxygen depletion and leading to plant and animal deaths (Anderson, 2009). Cyanotoxins can contaminate freshwater sources, leading to domestic and agricultural animal deaths (Huisman et al., 2005). Furthermore, cyanotoxins are acquired through the food web when contaminated organisms are consumed, leading to death as toxins accumulate in higher trophic levels (Huisman et al. 2005).

In addition to the ecological impacts of cHABs, there are also economic and public health consequences for surrounding communities. cHABs can cause mass fish deaths due to oxygen depletion and toxin accumulation in fish, having devastating impacts on fishing and tourism industries (Wolf et al., 2017). Fisheries have increased processing costs when blooms occur and may even stop production (US EPA, 2015); these costs to fisheries due to blooms can be millions of dollars annually (Sanseverino et

al., 2016). Consumer demand for seafood can decrease due to media reports of toxin contamination even for unaffected seafood (Wessells et al., 1995), and tourism industries are hindered by blooms because of aversion to the discolored water, unpleasant odor, and presence of large fish kills (Sanseverino et al., 2016). The tourism and fishing industries of Lake Erie provide millions of dollars in revenue and are threatened by the increasingly frequent *Microcystis* blooms (Michalak et al., 2013). Toxins from cyanobacterial blooms are also frequently linked with mammal deaths. In July of 2020, microcystin-contaminated water was the cause of the death of 330 elephants in Botswana, South Africa (Swails and Rahim, 2020; TWC India, 2020). Toxic algae blooms are the cause of dog deaths every year in the United States. By July of 2020, there were six dog deaths linked to toxic algae reported across the United States, from New York to Utah (Persellin, 2020).

Furthermore, cHABs can become harmful to humans when there is direct contact with cyanobacteria, ingestion of toxin-contaminated water, and consumption of contaminated fish (Anderson et al., 2008; Poste et al., 2011). Direct contact with toxin-contaminated water can cause skin rashes and ingestion can have gastrointestinal or neurological effects (Backer et al., 2015). In May of 2021, the cyanotoxin cylindrospermopsin was detected in the water supply of West Palm Beach, forcing the city to issue an advisory against tap water consumption (Rose and Miller, 2021). Cyanotoxins have also caused human fatalities when water used for dialysis was contaminated with the cyanobacterial toxins microcystin and cylindrospermopsin at a dialysis center in Caruaru, Brazil in 1996 (Azevedo et al., 2002; Carmichael et al., 2001).

3. *Microcystis* spp.

Microcystis spp. are unique among most cyanobacteria, as they grow both as single cells and colonially (**Figure 1**). Individual *Microcystis* cells are spherical and range in size from approximately 1.7 to 7 µm, while colonies of cells can reach 200 µm (Hu and Wei, 2006; Reynolds, 2006). *Microcystis* is the dominant genus of plankton in freshwater ecosystems globally and this success is partially attributed to its colonial morphology (Xiao et al., 2017). Colony formation may be influenced by the presence of heterotrophic bacteria, as well as zooplankton grazing and competition with other cyanobacteria, but the exact cause and ecological function of colony formation remains unresolved (Shen et al., 2011; Burkert et al., 2001; Mello et al., 2012). Polysaccharide secretions by the cyanobacterium also induce colony formation (Shen at al., 2011; Wang et al., 2015). These secretions are extracellular polymeric substances (EPS) that are rich in carbohydrates and form a mucilage around the *Microcystis* colonies (**Figure 1B)** (Pereira et al., 2009). *Microcystis* spp. also have several other unique morphological characteristics, including the ability to regulate buoyancy through formation of gas vesicles (Walsby et al., 1997) (**Figure 1C**).

Some *Microcystis* species produce cyanotoxins and are best known for producing microcystins, potent hepatotoxins originally called Fast-Death Factor (Bishop at al., 1959). Microcystins are hepatotoxins and the most common type of cyanotoxin, with over 100 variants (Vesterkvist at al., 2012). Microcystins act by inhibiting serine protein phosphatases, which can lead to excess phosphorylated proteins in the liver, resulting in cell necrosis, hemorrhaging and death (MacKintosh et al, 1990; Bhattacharya et al., 1997; Merel at al., 2013). Additionally, microcystins are suspected carcinogens (Massey et al.,

2018). Chronic exposure to microcystin has been shown in mice to be a potential tumor promoter in the development of prostate cancer (Pan et al., 2021). A study by Li et al. (2011) suggests that chronic exposure to microcystin may be associated with liver damage in children who regularly drink microcystin-contaminated water. Long-term exposure to microcystin has also been shown to cause lung impairment in mice (Li et al., 2016). The ability to produce potent toxins like microcystin emphasizes the importance of understanding the drivers of *Microcystis* blooms.

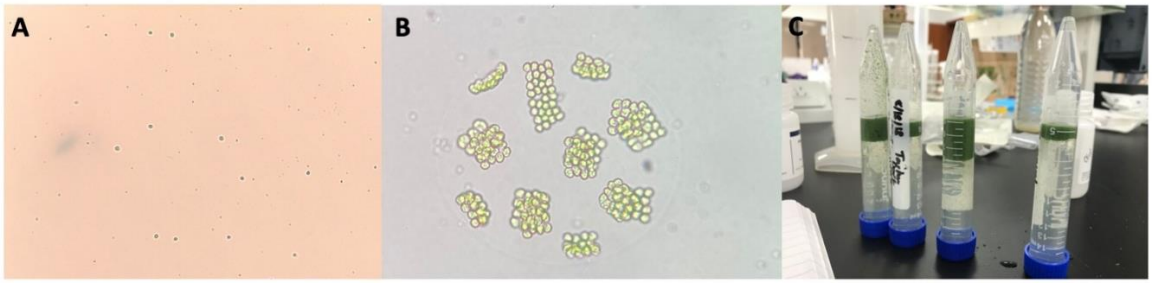

Figure 1. 1. Morphological variations of Microcystis growth in A) single celled, B) colonial, and C) buoyant *Microcystis*.

4. Drivers of cHABs

cHAB proliferation is heavily influenced by the availability of nutrients and by climate change, including the increase in storm events. Specifically, increased anthropogenic loading of nitrogen (N) and phosphorus (P) is correlated with the prevalence of *Microcystis* blooms (Bláha, 2009; Paerl et al., 2014). N and P are essential and limiting nutrients for *Microcystis* (Conley et al., 2009; Paerl et al., 2014a). *Microcystis* cannot fix atmospheric N_2, so cells must acquire N exogenously from environmental sources (Harke et al., 2016), such as agricultural runoff and wastewater, which also are a source of P (Conley et al., 2009). The availability of N is important to *Microcystis* as well as the form of N available. *M. aeruginosa* prefers reduced forms of N like ammonium over oxidized forms (nitrate and nitrite) (Vallino et al., 1996; Chaffin & Bridgeman, 2014), due to the energetic cost for assimilation of the oxidized forms (Syrett, 1981; Gardner et al., 2004; Flores and Herrero, 2005).

Dominance of cHAB-formers like *Microcystis* is not only influenced by this increased availability of nutrients but is also influenced by global climate change. Climate change is predicted to favor cyanobacteria over other phytoplankton in freshwater systems (Carey, 2011), because increasing global temperatures favor cyanobacteria due to their high optimum temperature compared to other phytoplankton (Jöhnk et al., 2008). *Microcystis* in particular is favored due to its unique ability to regulate buoyancy in turbid and light limited eutrophic waters, moving vertically through the water column to depths with optimum light and/or nutrient levels. Storm events are expected to increase in frequency which will lead to increased precipitation and nutrient loading due to increased runoff (Michalak et al., 2013). In elevated or depleted CO_2

concentrations, *Microcystis* spp. are easily able to regulate gene expression for carbon acquisition, giving it an advantage over other phytoplankton in blooms with rising CO_2 levels (Steffen et al., 2015; Sandrini et al., 2015). Despite decades of research into the impact of these abiotic drivers of *Microcystis* bloom formation, we still have not definitively identified the specific suite of factors that make these organisms successful. One reason for this may be the failure to consider potential biotic factors that may also contribute to *Microcystis* cHAB proliferation.

5. The *Microcystis* Phycosphere

Phytoplankton cells are surrounded by a zone rich in extracellular products, a microenvironment termed the **phycosphere** (Bell & Mitchell, 1972) which can be considered analogous to the rhizosphere in plants. The phycosphere allows for close proximity and interactions between the microbes that co-occur with phytoplankton. cHABs are not only composed of the harmful alga, but also include all of the microorganisms that make up the wider bloom community, including those microbes which live in the phycosphere. The phycosphere is a favorable environment for co-occurring heterotrophic bacteria through provision of nutrients, including organic carbon (Paerl, 1984; Jiang et al., 2007; Briand et al., 2016), and protection from predation (Casamatta and Wickstrom, 2000; Dziallas and Grossart, 2012). Many mutualistic interactions observed between phytoplankton and bacteria have been well-studied in marine eukaryotic systems. Eukaryotic phytoplankton such as some strains of *Gymnodinium aureolum* and *Aureococcus anophagefferens* must acquire vitamins exogenously because they are unable to synthesize them and require these vitamins for growth (Tang et al., 2010). These phytoplankton have obligate relationships with bacteria

that synthesize vitamins (Grant et al., 2014; Kazamia et al., 2012; Xie et al., 2013). The marine bacterium *Ruegeria pomeroyi* has a mutualistic relationship with the diatom *Thalassiosira pseudonana* in which the bacterium provides the diatom with vitamin B_{12} and the diatom provides sugar derivatives and organic N compounds in return (Durham et al., 2017). The cyanobacteria *Richelia intracellularis* and *Calothrix rhizosoleniae,* are thought to provide fixed nitrogen to several genera of diatoms, including *Hemiaulus, Rhizosolenia,* and *Chaetoceros* (Foster et al., 2011). While nutrient exchange like that seen in eukaryotic phytoplankton has not been closely studied in *Microcystis* spp., similar interactions most likely occur between *Microcystis* and associated heterotrophic bacteria.

We know that one key reason heterotrophic bacteria are closely associated with *Microcystis* spp. is because their extracellular polymeric substance (EPS) provides a unique environment for bacteria to grow, and in fact it is difficult to remove these bacteria when attempting to isolate pure cultures of *Microcystis* (Shirai et al., 1989; Xu et al., 2013). The attached bacterial community composition is unique to *Microcystis* when compared to the free-living bacteria from the same environment (Dzialles and Grossart, 2012). Bacteroidetes and Alphaproteobacteria have been found in both free-living and attached communities, Gammaproteobacteria are usually attached to *Microcystis,* while Betaproteobacteria are mainly free-living (Dzialles and Grossart, 2012). The relationship between a subset of these heterotrophic bacteria and *Microcystis* has previously been shown to be growth-promoting for both partners (Hoke, 2019; Shen et al., 2011; Kim et al., 2019).

The purpose of this thesis is to probe the potential for nutrient exchange between members of the *Microcystis* phycosphere using stable isotope probing (SIP) paired with sequencing for one of the first direct measurements of nutrient utilization and exchange between members of the *Microcystis* phycosphere.

6. Stable Isotope Probing Approach

SIP is a technique that allows the identification of microorganisms that use specific molecules during metabolic processes, without the need for laboratory culture (Dumont et al. 2005). It can be applied as a direct measure of microbial metabolic function, allowing us to move beyond inference. A microbe's ability to metabolize certain sources of N or carbon (C) can be determined by enrichment and isolation on defined media with known compositions, but this is limited to organisms that can be cultured in a lab. Organisms that can utilize isotopically labeled compounds, such as ^{13}C and ^{15}N, incorporate the labeled molecules into their nucleic acids and other macromolecules. The isotopically labeled nucleic acids can then be separated from unlabeled nucleic acids using a cesium chloride density gradient and ultracentrifugation (Meselson and Stahl, 1985; Neufeld et al., 2007). This will form two fractions: the heavier nucleic acid containing the isotope and the lighter nucleic acid lacking the isotope **(Figure 2**). The nucleic acid from the organisms that utilized the isotopically labeled compound will be in the "heavy" fraction, while the nucleic acid of all the other organisms will be in the "light" fraction. The nucleic acid fractions can be extracted from the gradient and purified for downstream analysis, including sequencing applications for community composition and/or function. This technique has been applied broadly in aquatic ecosystems to measure both C (Zhang et al., 2019; Sapp et al., 2008; Cunliffe et

al., 2017) and N (Wawrik et al., 2009; Terrado et al., 2017) dynamics in microbial consortia.

Unraveling the complex interactions in the *Microcystis* phycosphere is essential to understanding the success of *Microcystis* blooms. Pairing stable isotope probing (SIP) with metatranscriptomics or metagenomics is a powerful way to target the specific group of organisms able to uptake and incorporate a specific labeled substrate. DNA-SIP can directly link the identity of multiple organisms to their function. For bacteria, utilization of specific nutrients can be traced with SIP by supplementing the bacteria with an isotopic nutrient and sequencing the labeled DNA for the community profile that utilized the nutrient. DNA-SIP can directly identify bacteria involved in important nutrient cycling. While DNA-SIP is useful for identification of nutrient utilization, combining metatranscriptome analysis with SIP is a more robust way to unravel the complex interactions of microorganisms. Transcriptome analysis, RNA-SIP, allows for targeting of functional and taxonomic insight into microbial systems at the molecular level. RNA-SIP gives a more specific grasp of the active microbial populations because of the sequencing of genes being actively transcribed (Manefield et al., 2002; Lueders et al., 2016). DNA-SIP and RNA-SIP are both powerful tools to study the exchange of nutrients between *Microcystis* and the active heterotrophic bacteria by tracing nutrient exchange from one partner to the other.

While we know that positive interactions occur within the *Microcystis* phycosphere, nothing is known about the mechanism of nutrient exchange between heterotrophic bacteria and freshwater cyanobacteria, despite a robust effort to characterize these relationships between marine eukaryotic algae and bacteria. This thesis

research seeks to establish a molecular model for nutrient exchange in the *Microcystis* phycosphere and potentially answer the question, “Do microbial interactions in the phycosphere drive *Microcystis* bloom formation and success?”

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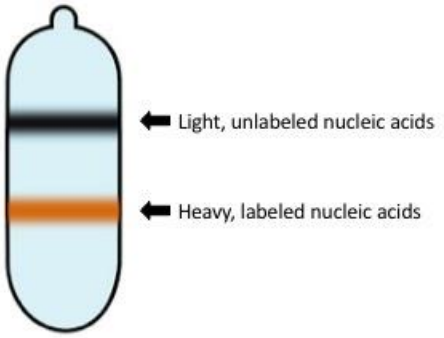

Figure 1. 2. Representative tube of nucleic acids separated using a CsCl gradient and ultracentrifugation. The black, top line represents the nucleic acids of the organisms that did not incorporate the stable isotope into its macromolecules. The bottom, orange line represents the nucleic acids of the organisms that were able to incorporate the stable isotope into its macromolecules. Adapted from Ghori et al. (2019).

Chapter II. The presence of *Acinetobacter* and *Methylobacterium* in *Microcystis* cultures indicates active assimilation of nitrate and urea

Abstract

Anthropogenic nutrient loading of nitrogen (N) and phosphorus (P) leads to widespread cyanobacterial harmful algal blooms (cHABs). N is an important limiting nutrient for the *Microcystis* spp. as they are unable to fix atmospheric N and must acquire N from other exogenous sources. *Microcystis* spp. live in close proximity with heterotrophic bacteria in the microenvironment surrounding the cyanobacterium, called the phycosphere. It is important to understand the N utilization capabilities of the *Microcystis* phycosphere because N is an important driver of blooms. The N utilization capabilities of these bacterial partners will provide insight to how N loading influences the *Microcystis* phycosphere. To unravel the N dynamics of the *Microcystis* phycosphere, stable isotope probing (SIP) was used to trace the utilization of urea and nitrate in two non-axenic lab cultures of *Microcystis aeruginosa.* Sequencing of the populations that could and could not use urea and nitrate as N sources revealed that the diversity of the microbial communities were foremost driven by the strain of *M. aeruginosa* then secondly by the fraction. The N utilization capabilities of the bacteria aligned with known N metabolisms, indicating successful separation of labeled and unlabeled DNA. However, incomplete labeling of *Microcystis* suggests a longer incubation time with the isotopes or a period of N starvation is needed. When looking more closely at the bacteria that were able to use the added N sources, nitrate utilization was indicated by *Acinetobacter* spp. and urea utilization was indicated by *Methylobacterium* spp. Not all bacteria present in these cultures were able to use urea and nitrate, and some were capable of fixing atmospheric N. The diverse N utilization capabilities suggests N

exchange within the *Microcystis* phycosphere and highlights the importance of N input into freshwater systems.

1. Introduction

Anthropogenic loading of the growth-limiting nutrients such as phosphorus (P) and nitrogen (N) leads to hypereutrophic lakes (Paerl et al., 2014; Elser et al., 2007; Lewis et al., 2011). Once these limiting nutrients become available in excess, cyanobacterial harmful algal blooms (cHABs) often dominate freshwater ecosystems (Conley et al., 2009; Paerl et al., 2001; Dodds et al., 1989). Since the 1970s there has been a notable increase in N input in freshwater systems due to increased agricultural, urban, and industrial wastes (Chislock et al., 2013; Howarth et al., 2011). This increase in N loading leads to increased frequency in bloom formation, especially of non-N_2 fixing cyanobacteria like *Microcystis* spp. cHABs caused by *Microcystis* spp. occur on all continents except Antarctica (Cook et al., 2020; Zurawell et al., 2005) and diminish water quality. The potent hepatotoxin, microcystin, is associated with *Microcystis* spp. and can negatively impact human health (Mankiewicz-Boczek et al., 2011; Azevedo et al., 2002).

Phytoplankton, including *Microcystis*, are known to be closely associated with heterotrophic bacteria. In numerous -omics style studies, blooms of *Microcystis* have been shown to be incredibly diverse, often dominated by members of the Proteobacteria and other non-cyanobacterial phyla (Steffen et al., 2012; Cook et al., 2020; Li et al., 2018). *Microcystis* spp. may enrich for bacterial constituents through production of an extracellular polymeric substance (EPS), which surrounds its large colonies (up to 200 µm) and in which bacteria are often found embedded (Kim et al., 2019). The EPS and its surrounding microenvironment make up the *Microcystis* phycosphere, a metabolite-rich area surrounding phytoplankton cells (Bell and Mitchell, 1972) where nutrient exchange has been shown to take place between bacteria and marine phytoplankton. Bidirectional

nutrient exchange has been observed between the diatom *Pseudo-nitzschia multiseries* and the bacterium *Sulfitobacter* sp. SA11, in which the diatom provides organosulfur molecules and the bacterium provides ammonium in return (Amin et al., 2015). A similar exchange of chemical currency may take place between members of the *Microcystis* phycosphere, however, little is known about the interactions which may occur in this microenvironment.

Stable isotope probing (SIP) and metagenomics can be used in combination to directly trace nutrient utilization and exchange. SIP uses stable isotopes, like ^{15}N, to isotopically label organisms that can use those specific nutrient sources. These isotopes will then be incorporated into the nucleic acids and other macromolecules of the organism, which can be extracted and separated out via isopycnic ultracentrifugation. By sequencing isotopically labeled DNA, the organisms capable of targeted metabolic functions can be directly identified. SIP has previously been used to identify bacteria actively utilizing carbon in the *Microcystis* phycosphere (Zhang et al., 2019).

Little is known about the exchange of N between *Microcystis* and heterotrophic bacteria in its phycosphere. To gain insight into nitrogen assimilation of constituents of the *Microcystis* phycosphere, we have applied SIP with metagenomics to directly trace utilization of urea and nitrate in two lab cultures of *M. aeruginosa*. Because N is a growth limiting nutrient for blooms, it is important to know how different sources of N are being used within the bloom communities. This approach allows us to identify heterotrophic bacteria that are capable of using urea and nitrate as forms of N and assess their potential role in *Microcystis* proliferation.

2. Methods

2.1 Nitrogen Utilization Incubation

To determine which members of *Microcystis* lab culture consortia are able to use exogenous sources of urea and/or nitrate, we incubated non-axenic cultures of two *Microcystis aeruginosa* strains, NIES 843 and CCMP 3462, with equimolar concentrations of the N stable isotopes ^{15}N-urea (25 µM) and ^{15}N-NO_3 (50 µM). Uptake control flasks had no added ^{15}N source. Incubations were performed in triplicate 500 mL sterile acid-washed flasks with a total volume of 300 mL of 10^6 cells/mL *Microcystis* culture per flask. Flasks were incubated for 24 hours (Connelly et al., 2014) at 26 ℃ in a 12:12 light:dark cycle at 114.7 ft-cd. After 24 hours, biomass was filtered onto 47 mm 0.2 µm polycarbonate Isopore ™ filters (MILLIPORE, Cork, Ireland) and stored at -80 ℃ until extraction. DNA was extracted from the filters using the DNeasy® PowerWater® DNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and eluted into water. Quantification of the DNA was done using a NanoVue™ Plus Spectrophotometer 4282 V2.0.3 (GE Healthcare).

2.2 DNA Isopycnic CsCl Ultracentrifugation Separation

The extracted DNA was separated into labeled and unlabeled fractions using a cesium chloride density gradient ultracentrifugation protocol adapted from Neufeld et al. (2007). This was developed for 15 mL, Quick-Seal® Bell-Top Polypropylene tubes (Beckman Coulter, Brea, CA, U.S.A.) in a fixed angle rotor (Beckman Coulter Type Ti 50.2).

The cesium chloride gradient was prepared by mixing the DNA with gradient buffer (GB) (0.1 M Tris, 0.1 M KCl and1 mM EDTA). The volume of DNA and GB was

determined using the following equation: GB/DNA volume = (CsCl stock solution density − desired final density) × volume of CsCl stock added × 1.52 (Rickwood, 1983). The CsCl stock solution (7.163 M CsCl) was then added to the GB/DNA mixture. The mixture was then added to the ultracentrifuge tube using a sterile syringe and needle, filling the tube approximately halfway. The tube was then topped with sterile mineral oil, leaving a small air bubble on the top. The tubes were balanced within 10 mg and sealed with a Cordless Tube Topper kit PN 358312 (Beckman Coulter). Ultracentrifuge settings were 32,000 rpm at 20 ℃ for 217:24 (hr:min) with vacuum, maximum acceleration and no brakes.

After isopycnic separation, the tubes were then fractionated using a syringe pump within 10 minutes of ultracentrifuge cessation. The tube was pierced with a needle on the top and bottom of the tube, then mineral oil was pumped with a NE-1000 “Just Infusion” ™ pump (0.700 mL/minute) through the top of the tube and twenty ~300 µL fractions were collected from the bottom of the tube. The weight of the fractions was used to determine the density of each fraction (**Figures 1 and 2**). Labeled fractions were combined into one fraction and unlabeled fractions were combined into one fraction. The labeled and unlabeled fractions were then purified according to Neufeld et al. (2007).

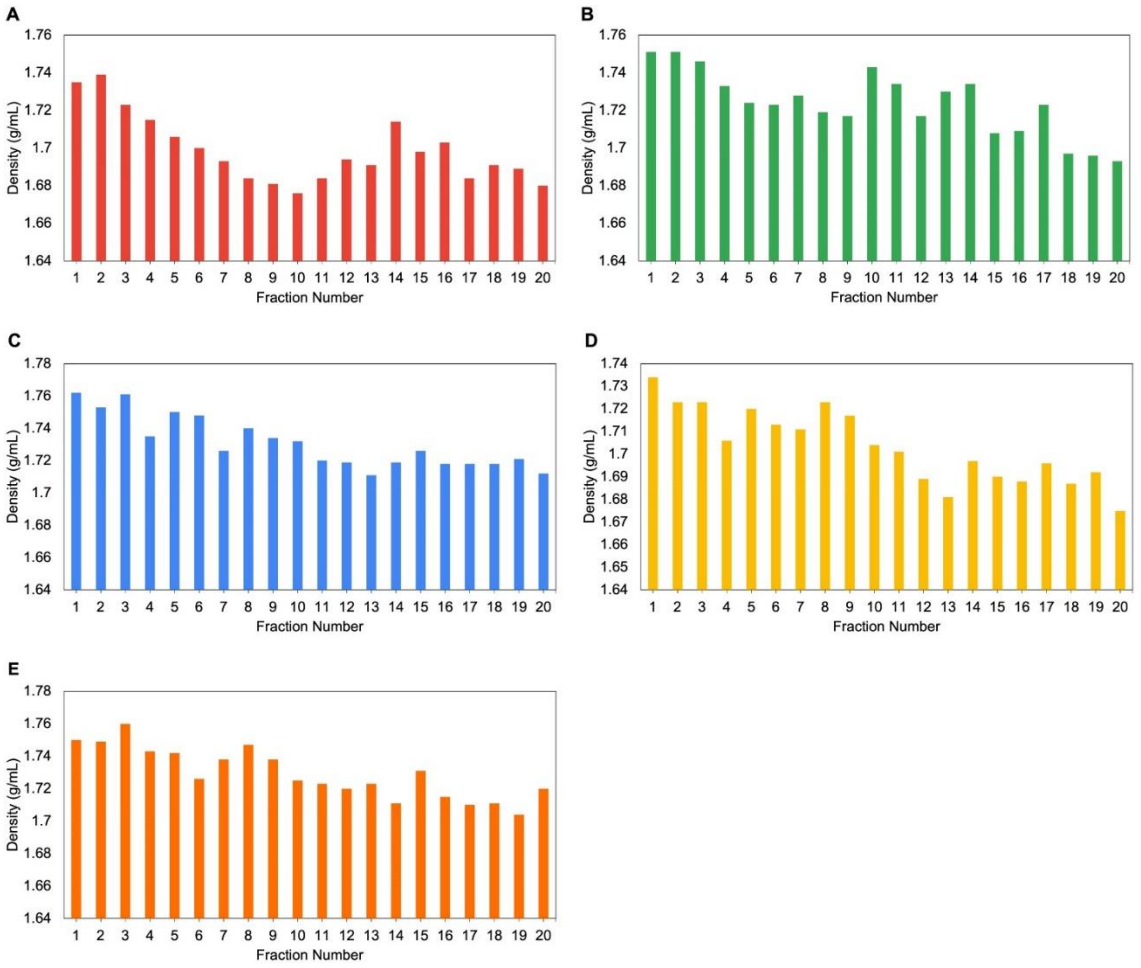

Figure 2. 1. Densities of the fractions from the CsCl isopycnic separation of the *M. aeruginosa* NIES 843 DNA for urea (A, B) and nitrate (C, D, E) treatments.

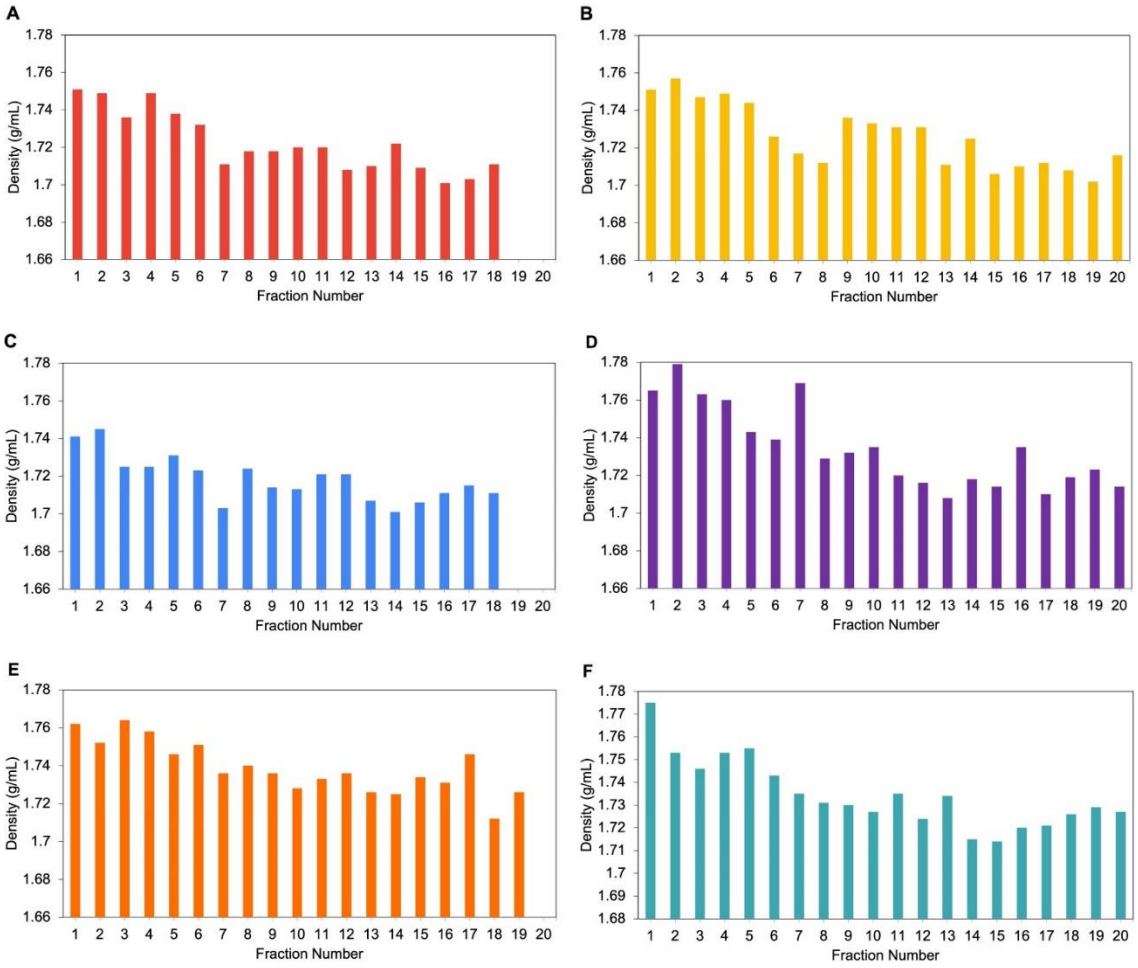

Figure 2. 2. Densities of the fractions from the CsCl isopycnic separation of the *M. aeruginosa* CCMP 3462 DNA for urea (A, B, C) and nitrate (D, E, F) treatments.

2.3 16S Library Preparation and Sequencing

Purified total genomic DNA was sent to GENEWIZ, Inc. (South Plainfield, NJ, U.S.A) for 16S-EZ rDNA next generation sequencing library preparation and Illumina MiSeq sequencing. The sequencing library was prepared using a MetaVx™ 16s rDNA Library Preparation kit (GENEWIZ, Inc., South Plainfield, NJ, USA). The GENEWIZ 16S-EZ workflow included multiplexed PCR amplification of the V3 and V4 hypervariable regions of the bacterial 16S rDNA. Sample-specific barcodes were added to each sample during a limited cycle second round PCR. Libraries were pooled together and sequenced with an Illumina MiSeq (Illumina, San Diego, CA, USA) with a 2 x 250 bp paired-end (PE) configuration.

2.4 Sequence Analysis and Data Visualization

The 16S rRNA data analysis was completed by GENEWIZ, Inc. using the QIIME (1.9.1) data analysis package. Forward and reverse reads were joined and assigned to samples based on barcode, and the barcode and primer sequence were removed. The joined sequences were filtered for quality, and the sequences that did not fulfill the following criteria were discarded: sequence length < 200 bp, no ambiguous bases, mean quality score greater than or equal to 20. Chimera sequences were removed by comparison with the reference database (RDP Gold database) using UCHIME algorithm to detect chimeric sequences. The resulting sequences were then used for further analysis. Sequences were grouped into operational taxonomic units (OTUs) using the clustering program VSEARCH (1.9.6) against the Greengenes database pre-clustered at 97% sequence identity. The Ribosomal Database Program (RDP) classifier was used to assign a taxonomic category to all OTUs at a confidence threshold of 0.8. Sequences were

rarefied prior to calculation of alpha and beta diversity statistics. Alpha diversity indexes were calculated in QIIME from rarefied samples using for diversity the Shannon index, for richness the Chao1 index.

Beta diversity was calculated using non-parametric multidimensional scaling (NMDS). The NMDS analysis was performed using Bray-Curtis dissimilarity on the OTU data with the R package vegan version 2.5-7 (Oksanen et al., 2020). Analysis of similarity (ANOSIM) was completed using the communities' OTU abundance and Bray-Curtis as a measure of dissimilarity with the R package vegan version 2.5-7 (Oksanen et al., 2020). The ANOSIM R statistical value indicates the separation between groups with numbers closer to 0 showing similarity and numbers closer to 1 showing dissimilarity. Statistical analyses were done using Sigma Plot v. 14, base R version 3.6.3 (R Studio Team, 2019), or specific R packages described below. Kruskal-Wallis rank sum tests and t-tests were used to compare community composition at the phylum and genus levels among strains, N source, and fraction. Indicator species were determined using the indicspecies package in R (De Cáceres et al., 2011). Most data were visualized with the package ggplot2 version 3.3.3 (Wickham 2016), R base graphics, Microsoft Excel, Primer-e v. XX or Sigma Plot v. 14.

3. Results and Discussion

The 16S rRNA sequencing results included 22 samples from the separated DNA from both strains and ^{15}N treatments. For all treatments, 12,766,412 reads were generated and after quality optimization 5,798,370 reads with an average quality score of 35.47 were used for analysis.

3.1 Community Composition

The ^{15}N-labeled fractions were primarily composed of the phylum Cyanobacteria, followed by Proteobacteria and Bacteriodetes (**Figure 3**). The unlabeled fractions were largely comprised of the same three phyla, but at different proportions (**Figure 3**). This composition is consistent with previous studies of *Microcystis* associated microbial communities in field populations (Steffen et al., 2012; 2017; Parveen et al., 2013). In particular, the dominance of Proteobacteria and Bacteroidetes is not surprising, as members of these two phyla are often associated with *Microcystis* spp. (Shi et al. 2009, 2012; Parveen et al. 2013; Cai et al. 2014; Wu et al., 2019). Proteobacteria and Bacteriodetes were significantly more abundant in the unlabeled fractions than labeled fractions of the *M. aeruginosa* CCMP 3462 strain. As expected, sequences from members of the Cyanobacteria were significantly more abundant in the labeled fractions than the unlabeled for both strains, as *Microcystis* spp. can use both urea and nitrate as their sole source of N (**Table 1**).

The following genera were present in all treatments: *Microcystis*, *Hydrogenophaga, Agrobacterium, Rhodobacter, Devosia, Blastomonas, Bradyrhizobium, Mesorhizobium,* and *Sphingopyxis* (**Figure 4**). There were 21 genera that appeared in both the labeled and unlabeled fractions of the nitrate treatment and 32 genera that were present in both fractions of the urea treated samples, including *Microcystis*. *Microcystis* spp. are known to be able to utilize both urea and nitrate, so this suggests that a subset of genera that could utilize the N sources did not have enough time to assimilate them in all cells of the population within the 24 h incubation window. This may be in part due to N replete culture conditions immediately prior to the start of the ^{15}N incubation. We argue

that the incubation time for similar SIP experiments with *Microcystis* needs to increase to a longer incubation period of 48 hours for the organisms to be able to fully incorporate these N isotopes or begin with N starved conditions.

Significant differences in abundance at the genus level were found between treatments for 18 of the genera present (**Table 2**). In the *M. aeruginosa* CCMP 3462 cultures, there were significantly more sequences belonging to the genera *Agrobacterium, Devosia, Blastomonas, Stenotrophomonas, Emticicia, Methyloversatilis, Bifidobacterium* than in the *M. aeruginosa* NIES 843 cultures. In the *M. aeruginosa* NIES 843 cultures, *Rhodobacter Mesorhizobium, Sphingopyxis,* and *Limnobacter* were significantly more abundant. The bacterial composition of the two strains varied significantly at the genus level which demonstrates that the host strain of *M. aeruginosa* strongly influences its bacterial microbiome.

Genus abundance also varied between the labeled and unlabeled fractions (**Table 2**). *Microcystis* and *Bacillus* abundance was significantly higher in the labeled fractions of both strains for both N sources. The labeled fractions (both urea and nitrate) for *M. aeruginosa* NIES 843 had an increased abundance of *Oscillospira,* a genus which has been proposed as capable of degrading high concentrations of DMS and is often found in rumen microbiomes as butyrate producers (Gophna et al., 2017). These three genera were significantly increased in the labeled fractions and were therefore able to incorporate both forms of N provided.

The genera *Rhodobacter, Devosia, Emticicia,* and *Erythromicrobium* all were significantly increased in the unlabeled fraction of both strains. Some *Rhodobacter* spp. and *Devosia* spp. are capable of fixing atmospheric N (Wang et al., 2016; Masepohl et

al., 2005; Rivas et al., 2003). *Emticicia* spp. vary in their ability to reduce nitrate or hydrolyze urea (Tan et al., 2019). *Erythromicrobium* belongs to the family Erythromicrobium and produces carotenoids that have protective antioxidant capabilities, but they are not known diazotrophs (Yurkov, 2015). In the *M. aeruginosa* CCMP 3462 culture, *Hydrogenophaga* and *Methyloversatilis* were significantly increased in the unlabeled fraction. *Hydrogenophaga* have been found to fix atmospheric N (Willems et al., 1991) and *Methyloversatilis* spp. vary in their ability to utilize urea and nitrate or fix atmospheric N (Smalley et al., 2015; Doronina et al., 2014; Kalyuzhnaya et al., 2006). In the unlabeled fractions of the *M. aeruginosa* NIES 843 cultures, the genera *Agrobacterium*, *Mesorhizobium*, *Blastomonas*, and *Sphingopyxis* were significantly increased. *Mesorhizobium* spp. are commonly found in the N-fixing nodules of legume trees and shrubs (Ferraz Helene et al., 2019). *Blastomonas* spp. usually cannot reduce nitrate (Sly and Hugenholtz, 2015). Members of several of the genera which were significantly enriched in the unlabeled fractions could be diazotrophic, obtaining N via fixation. Furthermore, members of the phycosphere which can use the provided N sources may be supplementing these bacteria with reduced forms of N when urea and nitrate cannot be used.

When comparing the N source as well as the fraction of each sample, there were few significant differences at the genus level. Unsurprisingly, in the nitrate treatments *Microcystis* was significantly increased in the labeled fractions, given that the cultures are maintained with nitrate as the sole N source. *Emticicia* was increased in the nitrate treated labeled fractions over the urea treated labeled fractions; this may be due to the varying capabilities of *Emticicia* spp. to use nitrate and urea (Tan et al., 2019). *Erythromicrobium*

was significantly increased in the nitrate treated unlabeled fraction over the nitrate labeled and urea labeled fractions. However, *Erythromicrobium* was not present in any urea treated labeled fractions for the *M.aeruginosa* NIES 843 strain. The varying N utilization capabilities of the microbial communities of the two *M. aeruginosa* strains used in this study was highlighted by the use of SIP. The genus abundance profiles varied with strain, N source, and fraction, which is also reflected in the community diversity of the samples.

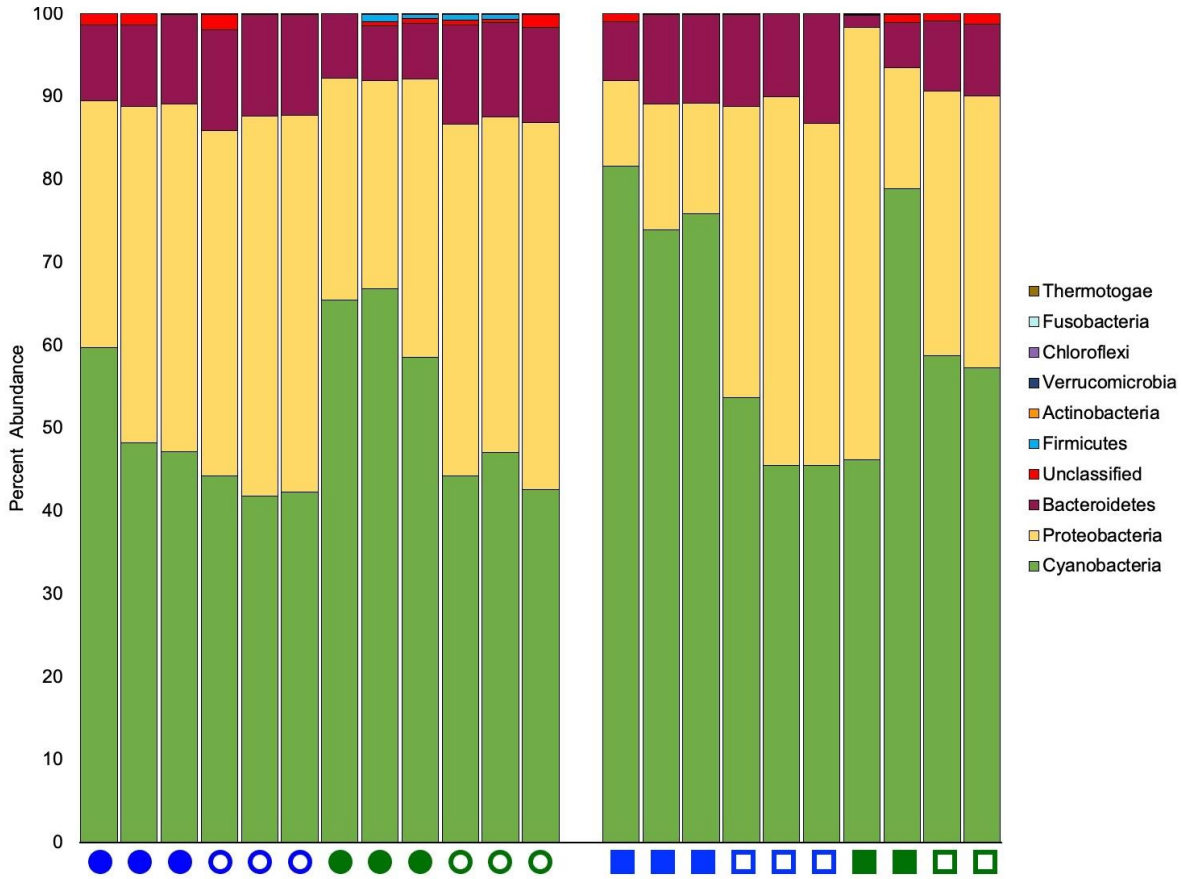

Figure 2. 3. Relative abundances of the bacterial community taxa at the phylum level of the different strains (CCMP 3462 and NIES 843), different N sources (control, nitrate, urea), and fraction(heavy, light). The circles represent the *M. aeruginosa* CCMP 3462 and the squares represent *M. aeruginosa* NIES 843. The nitrate treated samples are blue and the urea treated samples are green. The closed symbols represent the labeled fractions and the open symbols represent the unlabeled fractions.

Table 2. 1. Significant differences at the phylum level between the variables: strain, nitrogen, fraction, and nitrogen combined with fraction.

Phylum	Comparison	p-value
Cyanobacteria		
	Fraction	
	Labeled > Unlabeled	0.002
	CCMP Labeled > CCMP Unlabeled	0.008593
	NIES Labeled > NIES Unlabeled	0.03735
	Nitrogen & Fraction*	0.009788
	Nitrate Labeled vs. Nitrate Unlabeled	0.037
	CCMP Nitrogen & Fraction*#	0.02408
Proteobacteria		
	Fraction	
	Unlabeled > Labeled	0.01134
	CCMP Unlabeled > CCMP Labeled	0.014
Bacteroidetes		
	Nitrogen	
	Nitrate > Urea	0.02791
	Fraction	
	Unlabeled > Labeled	0.003948
	CCMP Unlabeled > CCMP Labeled	0.003747
	Nitrogen & Fraction*	0.002126
	Labeled Nitrate vs. Labeled Urea	0.0027
	CCMP Nitrogen & Fraction*	0.01556
	CCMP Labeled Nitrate vs. CCMP Labeled Urea	0.025
Firmicutes		
	Strain	
	CCMP > NIES	0.03879
	Nitrogen	
	Urea > Nitrate	0.03879

		CCMP Urea > CCMP Nitrate	0.03686
Actinobacteria			
	Nitrogen		
		Urea > Nitrate	0.0202
		CCMP Urea > CCMP Nitrate	0.02998
Verrucomicrobia			
	Nitrogen		
		NIES Urea > NIES Nitrate	0.02503

* Kruskal-Wallis test; # Posthoc showed no significant difference between individual treatments

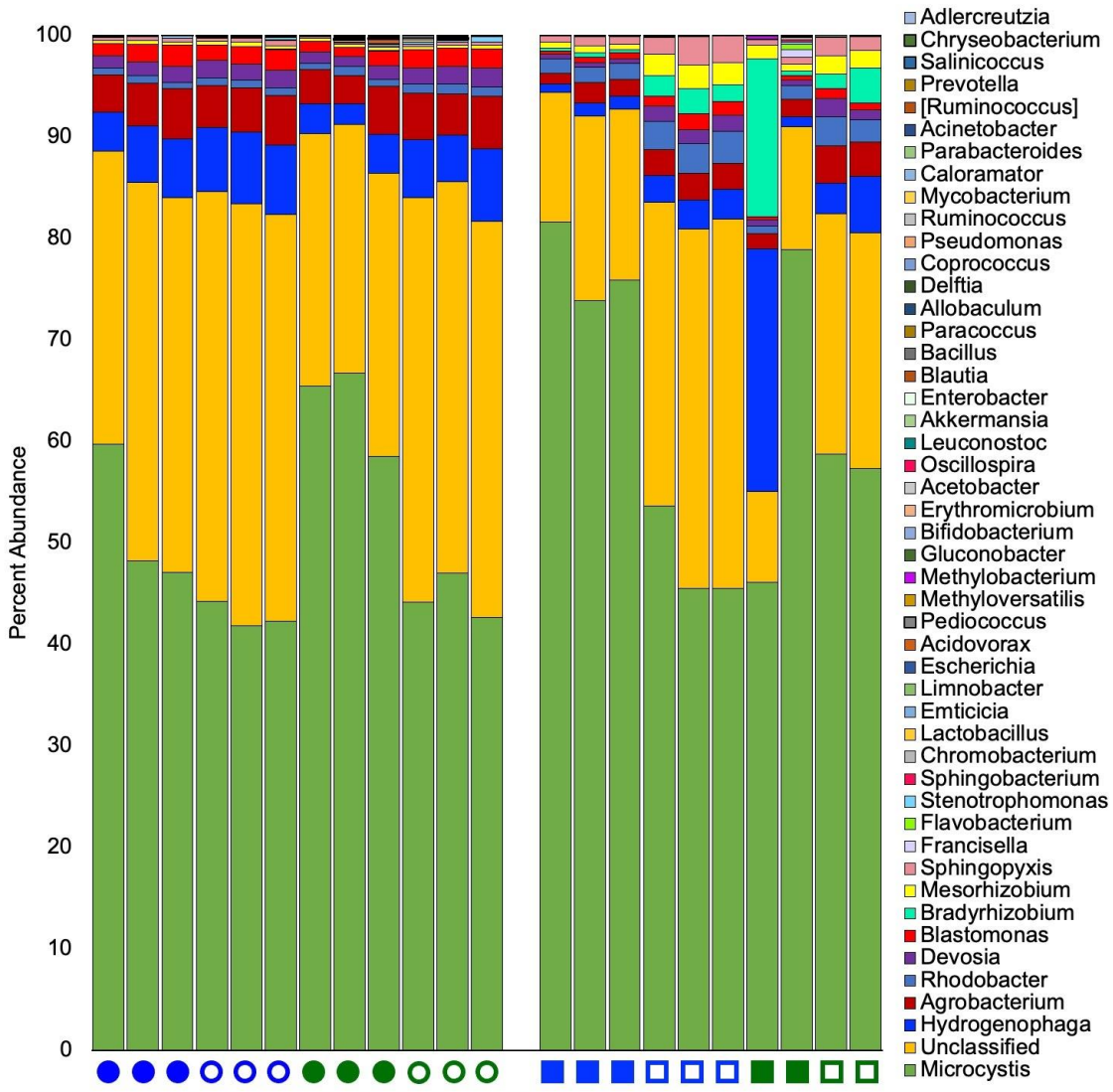

Figure 2. 4. Relative abundances of the bacterial community taxa at the genus level of the different strains (CCMP 3246 and *M. aeruginosa* NIES 843), different N sources (control, nitrate (NO3), urea), and fraction(heavy, light). The circles represent the *M. aeruginosa* CCMP 3462 and the squares represent *M. aeruginosa* NIES 843. The nitrate treated samples are blue and the urea treated samples are green. The closed symbols represent the labeled fractions and the open symbols represent the unlabeled fractions.

Table 2. 2. Significant differences at the genus level between the variables: strain, nitrogen, fraction, and nitrogen combined with fraction. The differences between strain, nitrogen, and fraction were compared using t-tests.

Genus	Comparison	p-value
Microcystis		
	Fraction	
	Labeled > Unlabeled	0.002003
	CCMP Labeled > CCMP Unlabeled	0.008481
	NIES Labeled > NIES Unlabeled	0.03738
	Nitrogen x Fraction*	0.1019
	Nitrate Labeled v. Nitrate Unlabeled	0.039
	CCMP Nitrogen x Fraction*#	0.02607
Hydrogenophaga		
	Fraction	
	CCMP Unlabeled > CCMP Labeled	0.01158
Agrobacterium		
	Strain	
	CCMP > NIES	1.57E-05
	Fraction	
	NIES Unlabeled > NIES Labeled	0.002233
Rhodobacter		
	Strain	
	NIES > CCMP	7.92E-04
	Fraction	
	Unlabeled > Labeled	0.04729
	CCMP Unlabeled > CCMP Labeled	0.01869
	NIES Unlabeled > NIES Labeled	0.0002008
Devosia		
	Strain	
	CCMP > NIES	0.01668
	Fraction	
	Unlabeled > Labeled	0.0004262
	CCMP Unlabeled > CCMP Labeled	0.00127
	NIES Unlabeled > NIES Labeled	0.001982

	Nitrogen x Fraction*#	0.00738
	CCMP Nitrogen x Fraction*#	0.02862
Blastomonas		
	Strain	
	CCMP > NIES	1.82E-04
	Fraction	
	Unlabeled > Labeled	0.02973
	NIES Unlabeled > NIES Labeled	0.009036
Mesorhizobium		
	Strain	
	NIES > CCMP	1.22E-03
	Nitrogen	
	CCMP Nitrate > CCMP Urea	0.04847
	Fraction	
	NIES Unlabeled > NIES Labeled	0.0001415
	NIES Nitrogen x Fraction*#	0.04601
Sphingopyxis		
	Strain	
	NIES > CCMP	0.003063
	Nitrogen	
	CCMP Nitrate > CCMP Urea	0.004289
	Fraction	
	Unlabeled > Labeled	0.04746
	NIES Unlabeled > NIES Labeled	0.007556
	CCMP Nitrogen x Fraction*#	0.03245
Stenotrophomonas		
	Strain	
	CCMP > NIES	0.002586
Lactobacillus		
	Nitrogen	
	Urea > Nitrate	0.03909
	NIES Urea > NIES Nitrate	0.04117
Emticicia		

	Strain	
	CCMP > NIES	0.04717
	Fraction	
	Unlabeled > Labeled	0.0006919
	CCMP Unlabeled > CCMP Labeled	0.006642
	NIES Unlabeled > NIES Labeled	0.004728
	Nitrogen x Fraction*	0.004889
	Labeled Urea v. Labeled Nitrate	0.034
	CCMP Nitrogen x Fraction*#	0.04745
Limnobacter		
	Strain	
	NIES > CCMP	6.92E-05
Methyloversatilis		
	Strain	
	CCMP > NIES	6.31E-06
	Fraction	
	CCMP Unlabeled > CCMP Labeled	9.08E-04
	CCMP Nitrogen x Fraction*#	0.04413
Bifidobacterium		
	Strain	
	CCMP > NIES	3.24E-02
	Nitrogen	
	Urea > Nitrate	0.03551
	CCMP Urea > CCMP Nitrate	0.02859
Erythromicrobium		
	Fraction	
	Unlabeled > Labeled	3.90E-04
	CCMP Unlabeled > CCMP Labeled	0.001149
	NIES Unlabeled > NIES Labeled	0.03798
	Nitrogen x Fraction*	0.002053
	Nitrate Labeled v. Nitrate Unlabeled	0.017
	Urea Labeled v. Nitrate Unlabeled	0.049
	CCMP Nitrogen x Fraction*#	2.20E-02

Oscillospira		
	Nitrogen	
	CCMP Urea > CCMP Nitrate	0.04287
	Fraction	
	NIES Labeled > NIES Unlabeled	0.01613
Akkermansia		
	Nitrogen	
	NIES Nitrate > NIES Urea	0.02503
Bacillus		
	Fraction	
	Labeled > Unlabeled	0.01998
	Nitrogen x Fraction*#	3.68E-02

* Kruskal-Wallis test; # Posthoc showed no significant difference between individual treatments

3.2 Community Diversity

There were significant differences in the bacterial community diversity across the 22 samples and the 1,369 unique OTUs within those samples. At the level of α-diversity, there were few significant differences between samples. Community diversity was measured using Chao 1 to estimate the species richness and Shannon diversity for species diversity. Significant differences in species richness were observed between the strains and between the N sources in the *M. aeruginosa* NIES 843 cultures (**Figure 5**). *M. aeruginosa* CCMP 3462 had a higher species richness than *M. aeruginosa* NIES 843, indicating that the *M. aeruginosa* CCMP 3462 culture supports significantly more unique bacterial species. The *M. aeruginosa* NIES 843 samples treated with urea had a higher species richness than the nitrate treated of the same strain. Perhaps more organisms in the *M. aeruginosa* NIES 843 culture were able to survive on urea than nitrate. The Shannon diversity index differed significantly between the labeled and unlabeled fractions, with the unlabeled fractions having a higher diversity than the labeled (**Figure 6**). When comparing the fractions for the *M. aeruginosa* NIES 843 and *M. aeruginosa* CCMP 3462 strains individually, the unlabeled fractions also had a significant increase in diversity (**Figure 6**). Shannon diversity only differed significantly when the samples were compared by fraction (labeled vs. unlabeled). The decreased diversity of the labeled fraction indicates an uneven distribution of species, most likely because the labeled fractions were dominated by *Microcystis*.

When comparing the OTU composition between the samples (β-diversity), there were significant differences between samples based on the strain of *Microcystis*, N source, and fraction. Based on Bray-Curtis distances, the NMDS ordination analysis

showed distinct groupings between the strains and between the fractions (**Figure 7**). For all samples, there were significant differences in OTU composition based on culture strain, N source, and fraction (**Table 3**). When comparing the communities of the two strains separately, there were no significant differences between the N sources for either strain, but both strains had significant differences in OTU composition in the labeled vs. unlabeled fractions (**Table 3**). To examine the impact of individual community members on community diversity, we compared abundance patterns of the 18 OTUs that comprised > 1% of the sequence libraries across all samples (**Figure 8**). The most abundant OTU was a *Microcystis* sp., followed by an OTU belonging to the family Chitinophagaceae, which has been seen associated with cyanobacterial blooms (Guedes et al., 2018). The clustering of these OTUs showed distinct grouping by strain of *M. aeruginosa*, and secondarily by fraction within the two larger strain clusters (**Figure 8**).

Microcystis strain had the largest influence on the microbial community, more so than nitrogen or fraction (**Figures 7 and 8, Table 3**). Bacterial community composition is often associated with the primary producer, including cyanobacteria, in freshwater systems (Guedes et al., 2018). Individual *Microcystis* colonies have been found to have distinct bacteria in their phycosphere, suggesting that physiological differences even between the strains plays a role in shaping bacterial community composition (Smith et al., 2021). The microbiome of rhizospheres have also been found to be unique to the species of plant (Turner et al., 2013). The differences seen in the labeled vs. unlabeled fractions for the alpha and beta diversity are likely due to the different N utilization capabilities of the constituents of each sample. The distinct differences between the labeled and unlabeled fractions confirm we were successfully able to isotopically label

bacteria in the *M. aeruginosa* cultures that were able to assimilate urea or nitrate as N sources in a 24 h incubation window, although full labeling of all cells in the community would likely require an extended incubation period.

3.3 Individual Constituents of Culture Consortia

To look more closely at the OTUs driving the diversity of the samples, Indicator Species analysis was done (De Cáceres et al., 2010). Indicator species are found more frequently in one treatment group compared to another and are therefore considered diagnostic of a specific condition, i.e., urea as an N source. The labeled fractions contained three OTUs as indicator species belonging to the genera *Microcystis, Bacillus,* and *Acidovorax* (**Figure 9**). The genus *Bacillus* was only found in labeled fractions, but not the unlabeled, indicating all cells of this genus in the culture consortia were able to fully incorporate both nitrate and urea within the 24 hour incubation window (**Tables 4 and 5**). *Acidovorax* was only present in the labeled fractions for all treatments, except it was present in the urea treated *M. aeruginosa* CCMP 3462 unlabeled fraction (**Table 4**). *Microcystis, Bacillus,* and *Acidovorax* can reduce nitrate (Nakano et al., 1998) and bacteria belonging to these genera can also hydrolyze urea (Willems and Gillis, 2015; Mols and Abee, 2008; Kim et al., 2005; Rasko et al., 2004). The presence of members of the genera *Bacillus* and *Acidovorax* may be indicative of nitrate and/or urea utilization in *Microcystis*-associated samples. Furthermore, a Lake Erie strain of *Acidovorax*, JMULE5, is known to have growth promoting capabilities when grown in co-culture with *Microcystis* (Hoke, 2019).

An *Acinetobacter* sp. was diagnostic of nitrate utilization in the NIES 843 culture, while a *Methylobacterium* sp. was indicative of urea utilization in that same culture

(**Figure 9**). Several species of *Acinetobacter* are able to reduce nitrate (Thornley, 1967) and *Methylobacterium* spp. can utilize urea (Minami et al., 2016). *Acinetobacter* and *Methylobacterium* have both been identified as members of *Microcystis* blooms previously (Li et al., 2016; Cai et al., 2014). A species of *Ruminococcus* was also diagnostic for urea utilization for both cultures of *Microcystis* (**Figure 8**). *Ruminococcus* spp. have been found to be able to use urea as a N source (Kim et al., 2014). *Acinetobacter* and *Methylobacterium* have been seen in *Microcystis* blooms before which points to some beneficial relationship between these organisms. *Microcystis* may benefit from the association with these indicator species because they are able to use these abundant forms of N and provide *Microcystis* with a more bioavailable reduced form of N which has been seen in between a diatom and a bacterium before (Amin et al., 2015).

The unlabeled fractions contained a much larger number of indicator species: 140 OTUs with six belonging to the genus *Devosia,* five *Blastamonas* spp., two of the genus *Hydrogenophaga,* and two *Rhodobacter*. The *Devosia* individuals could be fixing atmosphere N instead of using the exogenous forms added, as members of this genus are known to be diazotrophic and have mutualistic relationships with phototrophs: *D. neptuniae* is capable of fixing N_2 and is symbiotic with an aquatic legume (Rivas et al., 2003; Wolińska et al., 2017) *Blastomonas* spp. are unable to fix nitrate and are negative for urease, the enzyme necessary for hydrolysis of urea (Glaeser and Kämpfer, 2014; Xiao et al., 2015), so they are likely incapable of using either N source. *Hydrogenophaga* was also found to be significantly increased in the unlabeled fraction of *M. aeruginosa* CCMP 3462. *Hydrogenophaga* and *Rhodobacter* are also capable of fixing atmospheric N (Willems et al., 1991; Wang et al., 2016; Masepohl et al., 2005).

The bacteria that were only present or were significantly more abundant in the unlabeled fractions may have not been able to utilize urea and nitrate as N sources, did not need to, or were unable to incorporate urea and/or nitrate in the 24 h incubation period. Out of the 140 OTUs identified as indicator species for the unlabeled fractions, 68 belonged to the order Rhizobiales, members of which can fix atmospheric N(Beeckmans and Xie, 2015). Atmospheric N fixation is likely a contributing factor for why the bacteria did not need the exogenous N sources provided and appeared in the unlabeled fractions. Since *Microcystis* spp. cannot fix atmospheric N, it would be advantageous for *Microcystis* spp. to maintain a bacterial community in culture that can fix atmospheric N and act as a source of N when dissolved N sources are depleted. The large number of bacteria in the unlabeled fractions includes those that are unable to use either nitrate or urea as N sources. These bacteria most likely acquire N from exchange within the *Microcystis* culture. Other bacteria can fix atmospheric N or use nitrate and urea can provide other bacteria with more bioavailable forms of N. Diazotrophic bacteria could be supplementing *Microcystis* with its preferred source of N, ammonium (Chaffin & Bridgeman, 2014), through N fixation. These bacteria could also be reducing organic (urea) and inorganic N (nitrate) into the more bioavailable form of ammonium. The ability of cyanobacterial-associated bacteria to assimilate these nitrogen sources has been suggested through metagenomic analysis. Genes for nitrogen fixation, nitrate reduction, and urea metabolism have been found in the metagenomes of bacteria associated with cyanobacterial blooms (Steffen et al., 2012).

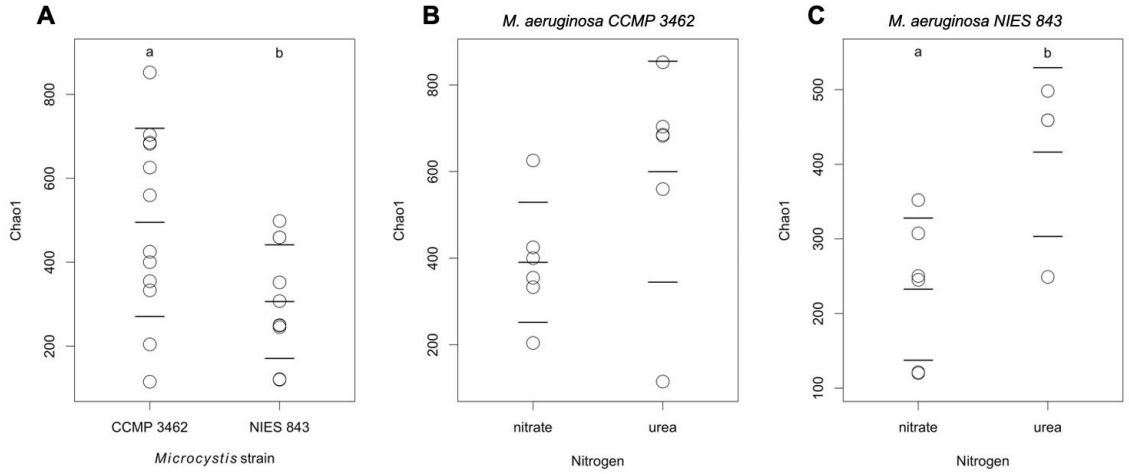

Figure 2. 5. Chao 1 indices plotted for (A) *M. aeruginosa* strain, (B) *M. aeruginosa* CCMP 3462, and (C) *M. aeruginosa* NIES 843. Lowercase letters indicate significant differences where the p-value < 0.05.

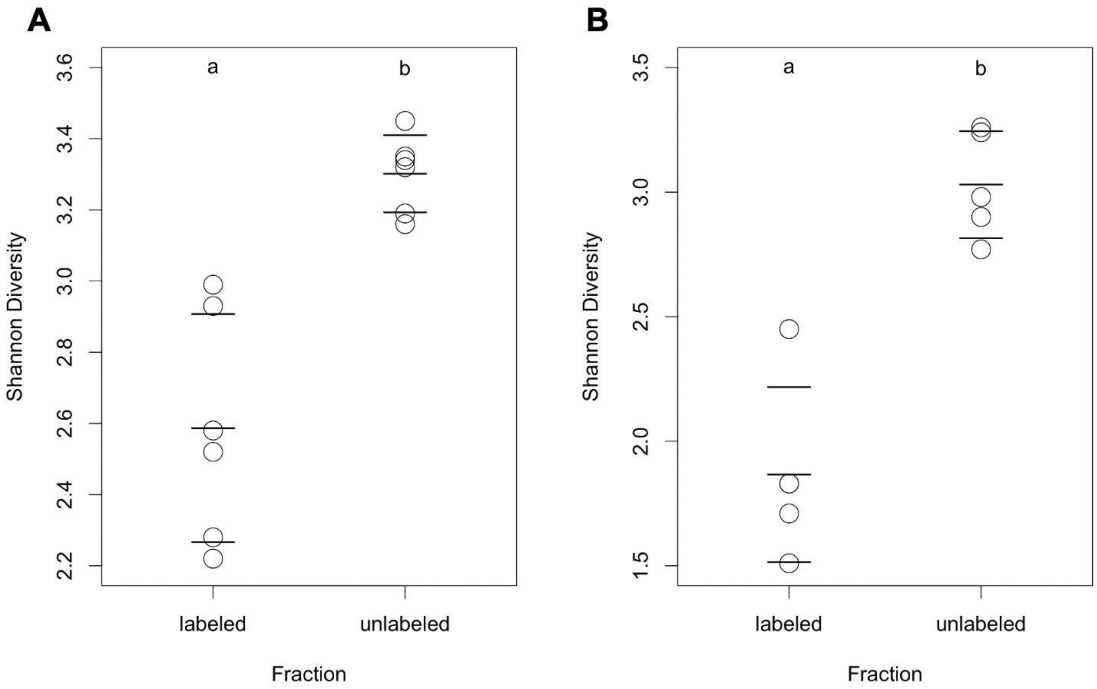

Figure 2. 6. Shannon diversity indices plotted for (A) *M. aeruginosa* CCMP 3462 and (B) *M. aeruginosa* NIES 843. Lowercase letters indicate significant differences where the p-value < 0.01.

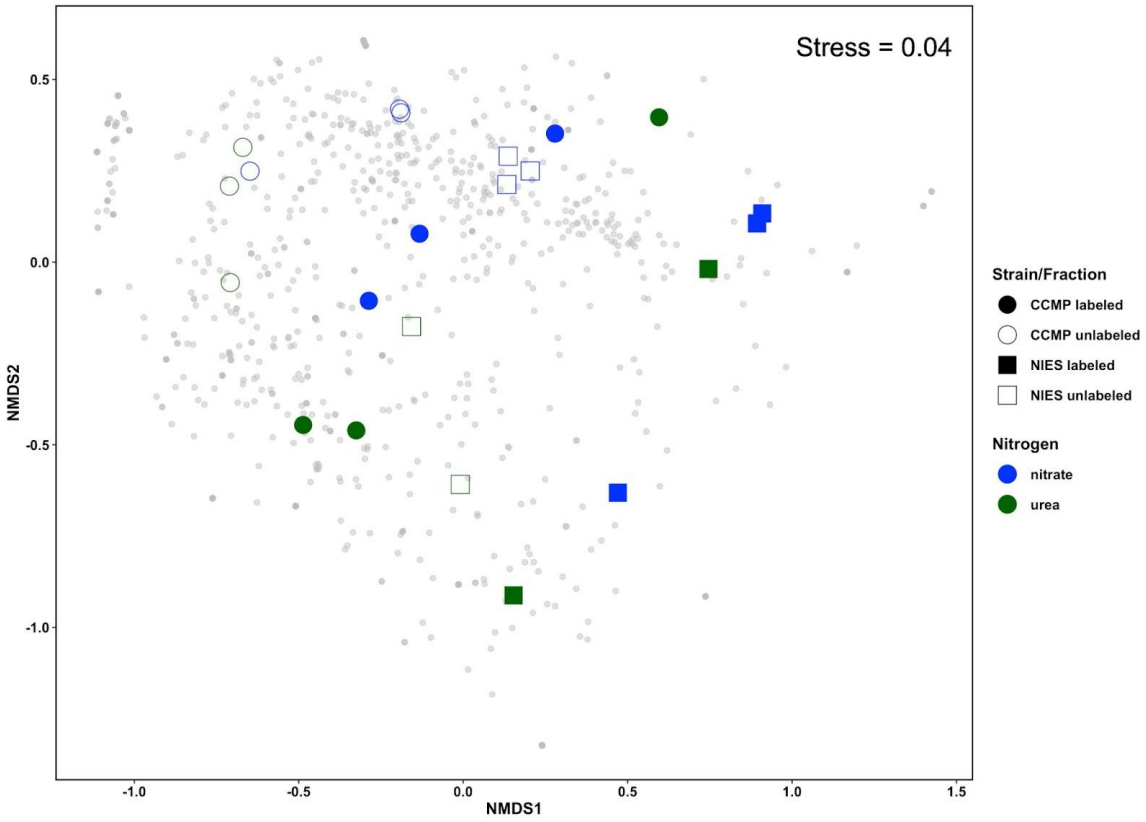

Figure 2. 7. Non-parametric multidimensional scaling (NMDS) analysis of OTU data for the nitrogen utilization experiment using the Bray-Curtis algorithm to calculate distance between samples. The gray dots represent the individual OTUs.

Table 2. 3. Analysis of similarity (ANOSIM) of the bacterial community structure of the samples among strain, nitrogen source, and fraction based on OTU abundance.

Comparison	**Sample Statistic R**
Strain	
CCMP vs. NIES	0.6411***
Nitrogen	
Urea vs. Nitrate	0.2887**
CCMP Urea vs. CCMP Nitrate	0.04815
NIES Urea vs. NIES Nitrate	0.05556
Fraction	
Labeled vs. unlabeled	0.2181**
CCMP Labeled vs. CCMP Unlabeled	0.5944**
NIES Labeled vs. NIES Unlabeled	0.592**

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

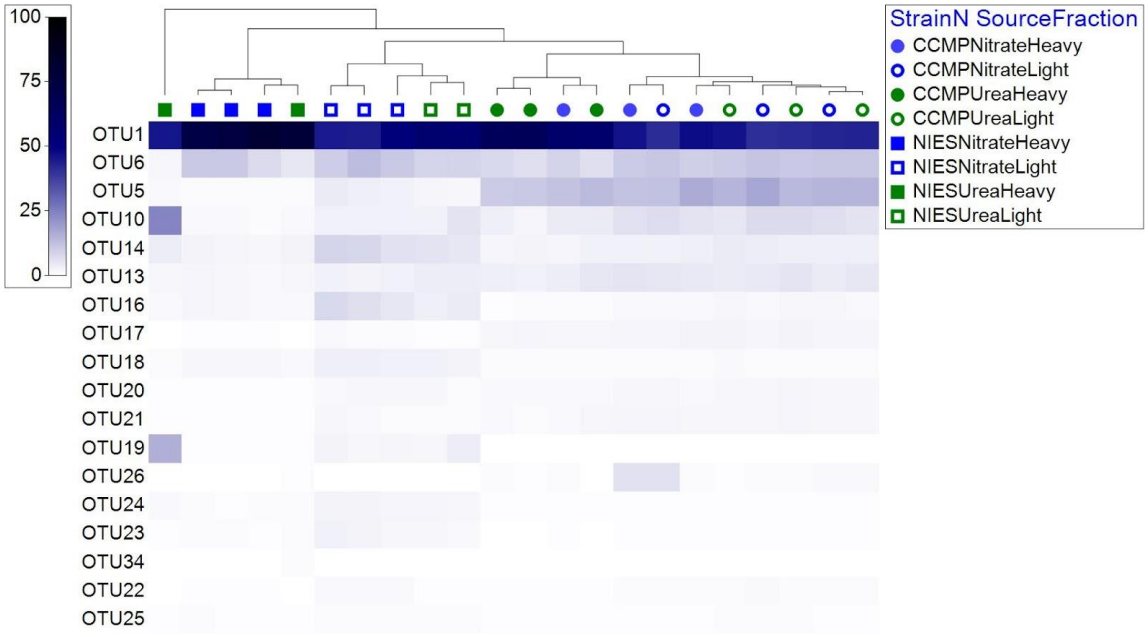

Figure 2. 8. Heatmap showing the percent relative abundance of the 18 most abundant OTUs in the treatments with added ^{15}N. The samples are clustered at the top using Bray-Curtis dissimilarities.

Table 2. 4. List of genera that was only present in the labeled or unlabeled fraction of the urea treatments.

CCMP 3462		NIES 843	
Labeled	Unlabeled	Labeled	Unlabeled
Blautia	*Flavobacterium*	*Francisella*	*Erythromicrobium*
Bacillus	*Lactococcus*	*Flavobacterium*	*Pseudomonas*
Pseudomonas		*Stenotrophomonas*	
Caloramator		*Sphingobacterium*	
Clostridium		*Chromobacterium*	
Prevotella		*Escherichia*	
		Acidovorax	
		Gluconobacter	
		Oscillospira	
		Bacillus	
		Ruminococcus	
		Mycobacterium	
		Salinicoccus	
		Adlercreutzia	

Table 2. 5. List of genera that was only present in the labeled or unlabeled fraction of the nitrate treatments.

CCMP 3462		NIES 843	
Labeled	Unlabeled	Labeled	Unlabeled
Escherichia	*Francisella*	*Stenotrophomonas*	*Bifidobacterium*
Acidovorax	*Flavobacterium*	*Acidovorax*	*Acetobacter*
Gluconobacter	*Sphingobacterium*	*Pediococcus*	*Pseudomonas*
Bifidobacterium	*Erythromicrobium*	*Gluconobacter*	
Bacillus	*Acetobacter*	*Bacillus*	
Coprococcus	*Akkermansia*	*Delftia*	
Ruminococcus	*[Ruminococcus]*	*Chryseobacterium*	
Mycobacterium	*Clostridium*	*Moraxella*	
Prevotella	*Fusobacterium*	*Fervidobacterium*	
Moraxella			
Propionibacterium			
Streptococcus			

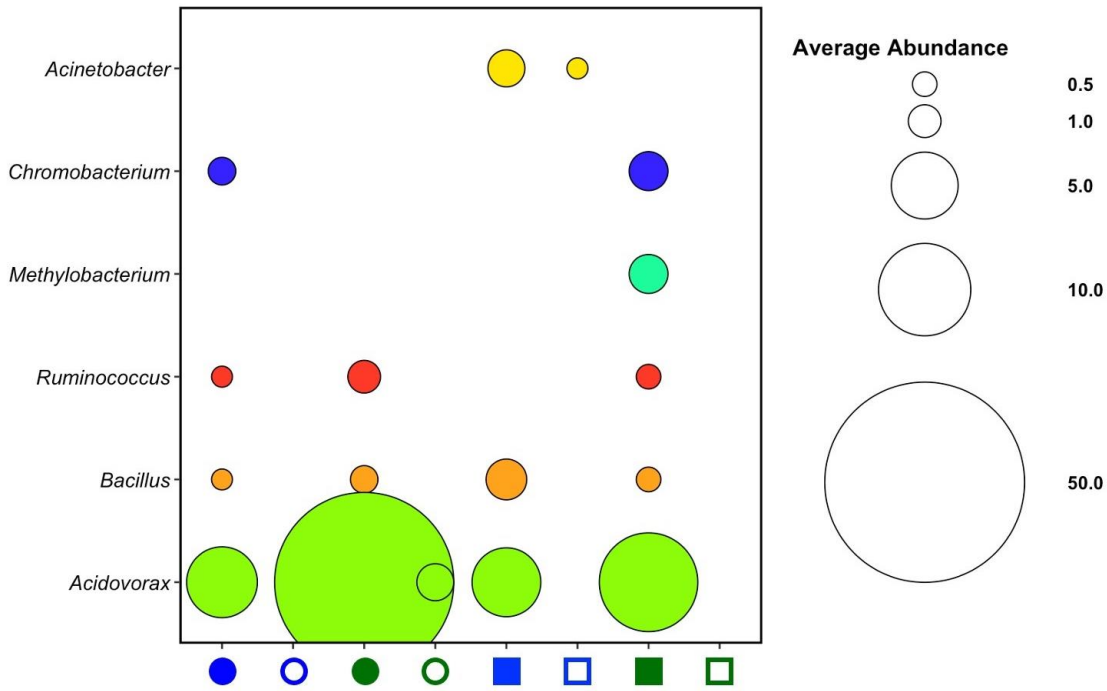

Figure 2. 9. Indicator species average abundance across the samples. The circles represent the *M. aeruginosa* CCMP 3462 and the squares represent *M. aeruginosa* NIES 843. The nitrate treated samples are blue and the urea treated samples are green. The closed symbols along the x-axis represent the labeled fractions and the open symbols represent the unlabeled fractions.

4. Conclusions

The bacterial communities of two non-axenic *M. aeruginosa* strains (CCMP 3462 and NIES 843) were tested for nitrate and urea utilization using DNA-SIP metagenomics. *Microcystis* was present in both the labeled and unlabeled fractions indicating that the incorporation of the N sources was incomplete and future SIP studies with *Microcystis* should have the cultures in N starved conditions prior to incubation or extend the incubation time to 48 hours. The differences in alpha and beta diversity indicate that host *Microcystis* strain is the primary driving factor in microbial community composition in *Microcystis* laboratory cultures. The nitrogen capabilities of specific genera in the labeled and unlabeled fractions from each N source aligned with previous knowledge of those genera's N metabolisms, indicating adequate separation of the labeled and unlabeled DNA. Two genera stand out as potential indicators for specific N utilization capabilities in *Microcystis*-associated bacteria communities: *Acinetobacter* spp. appear to be indicative of nitrate utilization and *Methylobacterium* spp. could be diagnostic for urea utilization. The diverse N metabolisms found in the *Microcystis* cultures suggest the potential for N exchange within the bacterial community associated with the *Microcystis* phycosphere. Heterotrophic bacteria in the *Microcystis* phycosphere could be supplementing *Microcystis* and other phycosphere partners with N during N limitation. Phycosphere partners could be integral in formation and sustaining blooms.

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Chapter III. The development of a synthetic consortium of heterotrophic bacteria isolated from a Taihu *Microcystis* bloom for RNA-SIP nutrient exchange

Abstract

Microorganisms are ubiquitous on earth and inhabit many different environments. Every unique environment has its own unique set of microbes that comprise its microbiome. Phytoplankton, including the cyanobacterium *Microcystis,* have mutualistic relationships with their microbiome in the phycosphere, a microenvironment surrounding phytoplankton cells where nutrient exchange and other interactions can take place. To unravel the interactions in the *Microcystis* phycosphere between the cyanobacterium and its associated heterotrophic bacteria, bacterial strains were isolated from a 2018 *Microcystis* bloom in Lake Tai (Taihu) in China. This consortium of 36 isolates was used to develop a synthetic bloom community with ^{13}C-labeled *Microcystis* to determine the ecophysiological impact of interaction on this important bloom-forming organism and track bacterial uptake of *Microcystis*-derived carbon using RNA-SIP metatranscriptomics. *Microcystis* growth was improved when grown with the Taihu consortium in comparison to axenic *Microcystis*. Tracking incorporation of the ^{13}C isotope from *Microcystis* to the Taihu consortium will provide a direct measure of *Microcystis*-derived C uptake by bloom-associated bacteria. Transcriptome analysis of the RNA-SIP experiment will give insight into the mechanisms of nutrient exchange in the *Microcystis* phycosphere.

1. Introduction

Microorganisms are ubiquitous, colonizing environments from deep-sea hydrothermal vents to the rumen of cattle. Every unique environment has its own associated microbiome consisting of a range of microbes including archaea, bacteria, and fungi. The relationships between host environments and their individual microbiomes were long thought to be commensal, but we now know that these relationships are more dynamic, including mutualistic relationships. One of the most widely studied mutualistic relationships occurs between plants and microorganisms in the narrow zone surrounding plant roots called the rhizosphere. Nutrients such as nitrogen are provided by the microbiome to the plant and fixed carbon is provided to the microbes (Mendes et al., 2013). Similarly, we know that mutualistic exchange also occurs between algae and closely associated bacteria in a microenvironment termed the phycosphere (Bell and Mitchell, 1972).

Within the phycosphere, the photobiont (alga) is often a source of organic carbon while the heterobionts (heterotrophic bacteria) provide essential vitamins, as well as other growth promoting metabolites (Croft et al., 2005; Amin et al., 2015; Durham et al., 2017). The phycosphere attracts bacteria because it is rich in dissolved carbon and other nutrients from phytoplankton secretion and cell lysis (Bell and Mitchell, 1972; Smriga et al., 2016). These complex interactions have been studied in marine eukaryotic phytoplankton, but less is known about the mutualistic relationships between cyanobacteria and their associated bacteria, particularly in freshwater systems.

Freshwater ecosystems are threatened by cyanobacterial harmful algal blooms (cHABs) worldwide. cHABs are devastating to local ecology by causing hypoxic waters

and producing toxins (Anderson, 2009; Huisman et al., 2018). In the past few decades, blooms have increased in frequency, and this trend is expected to continue with global temperatures rising and increases in nutrient loading (Lurling et al., 2017; Pearl et al., 2014). *Microcystis* is a genus of cyanobacteria that is a cosmopolitan cHAB former, including in Lake Erie in North America and Lake Tai (Taihu) in China. *Microcystis* spp. secrete a carbon-rich extracellular polymeric substance (EPS) that surrounds the cells (Pereira et al., 2009) in which heterotrophic bacteria are found embedded (Kim et al., 2019). A subset of these heterotrophic bacteria in the *Microcystis* phycosphere likely have an important role in *Microcystis* growth and proliferation (Kim et al., 2019; Smith et al., 2021; Hoke, 2019).

Microcystis bloom community dynamics are complex with many microbes present in the blooms across all domains of life (Steffen et al., 2017; McKindles et al., 2020). Recent evidence suggests members of this community have growth-promoting potential for *Microcystis*, but little is known about the complex mechanism of exchange that underlie the relationship between the growth-promoting bacteria and their host. In an effort to disentangle the potential nutrient exchange in the *Microcystis* phycosphere, we developed a synthetic consortium of bloom-associated bacteria to pair direct tracing exchange of *Microcystis*-derived C uptake by the bacterial community with consortium gene expression profiles using a combined SIP-metatranscriptomics approach. Thirty-six heterotrophic bacteria were isolated from a 2018 Taihu *Microcystis* bloom and incubated with ^{13}C-labeled axenic *M. aeruginosa*. To directly measure carbon exchange from *Microcystis* to the synthetic consortium of heterotrophic bacteria from Taihu, labeled and unlabeled RNA were separated via isopycnic ultracentrifugation and used to generate

metatranscriptomes. Paired with growth data and photosynthetic efficiency, the molecular expression profiles will provide new insight into the mechanism of bidirectional exchange between bloom-associated heterotrophic bacteria and *Microcystis* in the phycosphere.

2. Methods

2.1 Isolation and Identification of Bacterial Isolates

Water samples were collected from Taihu in Wuxi, China, on August 18, 2018. Samples were taken from the dock at the Taihu Laboratory for Lake Ecosystem Research (TLLER) (N 31° 25.12', E 120° 12.51'). A 20μm mesh plankton net was used to collect samples from the site and stored at room temperature in ambient light until processed for bacterial isolations. Water samples were then plated on a variety of media: R2-a, CT with tryptone and yeast extract (CT-TY) (Hoke, 2019), CT without nitrogen, CT with urea (Steffen et al., 2014), Luria broth (LB), and tryptic soy agar (TSA). Plates were then incubated at 26°C until colonies grew, up to 14 days. Individual colonies were re-streaked onto new plates of the same media and this was repeated until the isolates were determined pure by Gram stain. Once pure, the isolates were all grown on CT-TY.

Identity of the individual isolates was done via 16S Sanger sequencing. For each isolate, one pure colony was used to inoculate 5 mL of CT-TY broth and incubated in a shaking incubator for 24 h at 26 °C. Then 1 mL of the turbid broth was transferred to a 1.5 mL microcentrifuge tube for centrifugation at 13,000 rpm for 1 minute. The supernatant was removed, and the pellet was resuspended in 0.5 mL of sterile water and heated on a heating block for 15 minutes at 95°C. The microcentrifuge tube was then centrifuged again for one minute. The supernatant, containing the bacterial DNA, was

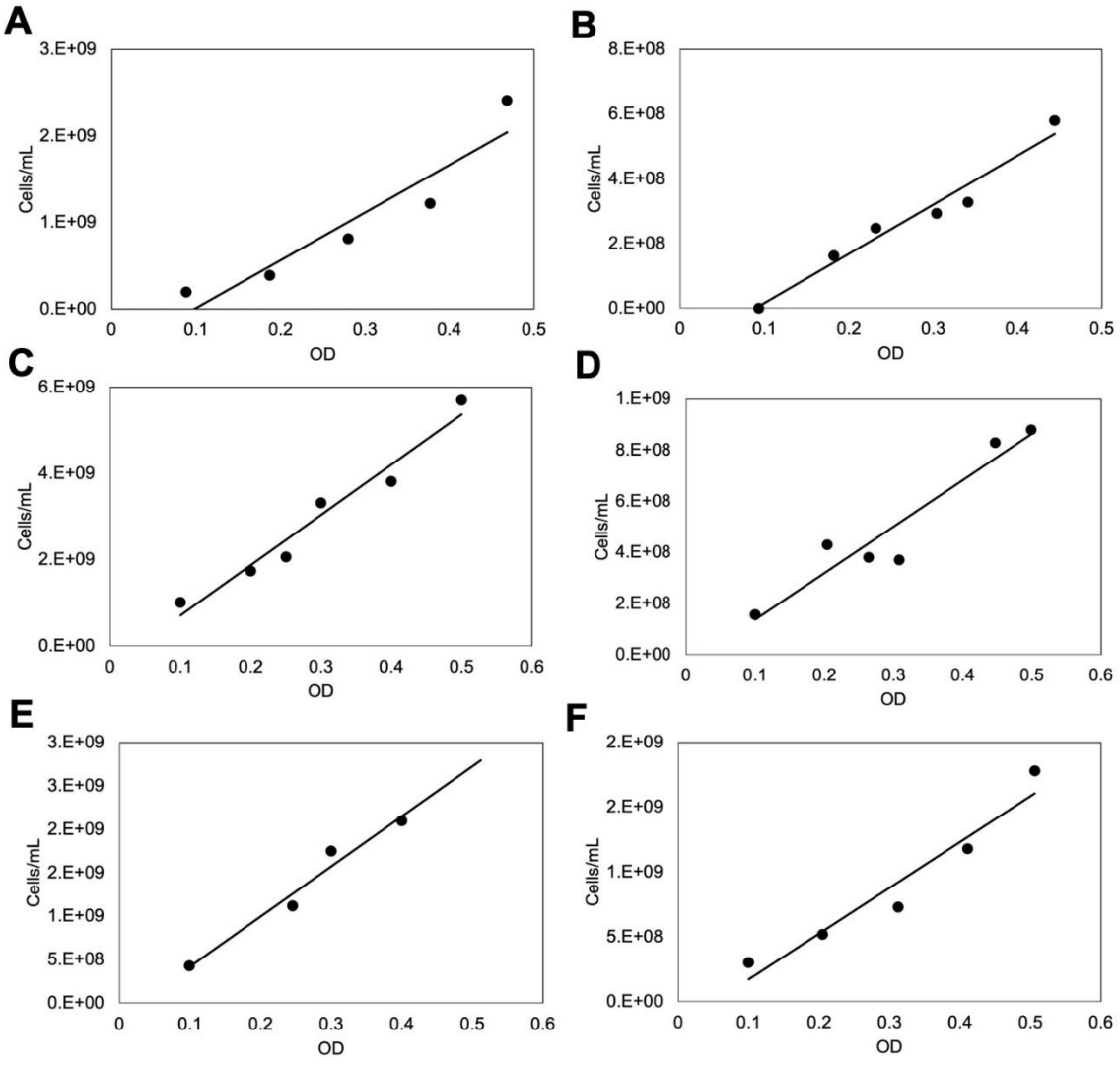
then transferred to a new microcentrifuge tube. The 16S rRNA gene was then amplified using PCR primers 515f (5' GTGYCAGCMGCCGCGGTAA 3') and 806r (5' GGACTACNVGGGTWTCTAAT 3') (Caporaso et al., 2018). The reaction mixture included 0.04 μM of the 515f and 806r primers, 12.5 μL of 2x Econo Taq Master Mix (Lucigen), 10.5 μL of PCR water, and 1.0 μL of template DNA (DeLong, 1992). The PCR was performed with an initial 94 °C for 3 minutes, and 34 cycles of 94 °C for 45 seconds, 50 °C for 60 seconds, and 72 °C for 90 seconds using a BioRad C-1000 Touch thermocycler. Amplification was confirmed with a 1% agarose/TAE gel and stained with 3x Gel Red (Phenix Research Products). The PCR products were purified using the Qiagen QIAquick PCR purification kit for Sanger sequencing by Eurofins Genomics. The resulting sequences were identified at the genus level using the Basic Local Alignment Search Tool (BLASTn) for nucleotides using the nr database using the default settings and excluding environmental isolates.

The growth rate of each isolate was determined by generating growth curves. For each isolate, 100 mL of CT-TY was inoculated with a single colony and incubated at 26 °C for 24-48 hours using a shaking incubator. After incubation, the optical density (OD) of the turbid culture was measured at 600 nm. The turbid culture was then diluted to the ODs of 0.1, 0.2, 0.25, 0.3, 0.4, and 0.5. For each of the five ODs, a serial dilution was done and dilutions were plated for cell counts. Growth curves were generated by graphing OD vs. cell counts for each isolate (**Figure 1**).

2.2 Consortium Growth Dynamics

To determine the effect of the presence of heterotrophic bacteria on *Microcystis,* axenic *Microcystis* and *Microcystis* grown with the Taihu consortium were monitored for

growth until the cultures reached stationary phase. Cultures of the 36 bacterial isolates were grown in CT-TY broth at 26 °C for 48 hours. The bacteria were added together at equal cell densities for a final cell density of 10^8 cells/mL and then the bacterial consortium was washed with CT to remove residual carbon and other nutrients prior to inoculation with *Microcystis*. The synthetic consortium was grown in three acid-washed sterile 500 mL flasks with *Microcystis aeruginosa* NIES 843 at a density of 10^6 cells/mL for a 1:100 (*Microcystis*:bacteria) ratio in a total volume of 300 mL CT. Control flasks were grown in three 500 mL flasks with axenic *Microcystis* at a density of 10^6 cells/mL in a total volume of 300 mL CT. The cultures were incubated for 56 days at 26 °C in a 12:12 light:dark cycle. Axenic conditions of *Microcystis* cultures were confirmed with acridine orange staining. Fluorescence readings were taken at 48 hour intervals as a proxy for growth with a Phyto-PAM-II Compact fluorometer (Walz) with a gain setting of 14 at 625 nm. Prior to fluorescence readings, flasks were dark adapted for 30 minutes and an initial reading was collected (Fo). Then 6 μL of 5 μM DCMU was added to the sample to close the Photosystem II reaction centers for a second reading (Fm) (Campbell et al., 1998). Photosynthetic efficiency was measured using the Fo and Fm readings to calculate Fv/Fm, or variable fluorescence (Fv/Fm = (Fm-Fo)/Fm). During the fluorescence readings, one additional mL of culture was collected and preserved with Lugol's iodine for *Microcystis* cell counts. Growth rate was calculated using cell counts for the 24th and 28th days of the growth experiment. Fluorescence, cell counts, and growth rates between the axenic *Microcystis* and the *Microcystis* grown with the Taihu consortium were compared using t-tests. Graphs were made in Microsoft Excel.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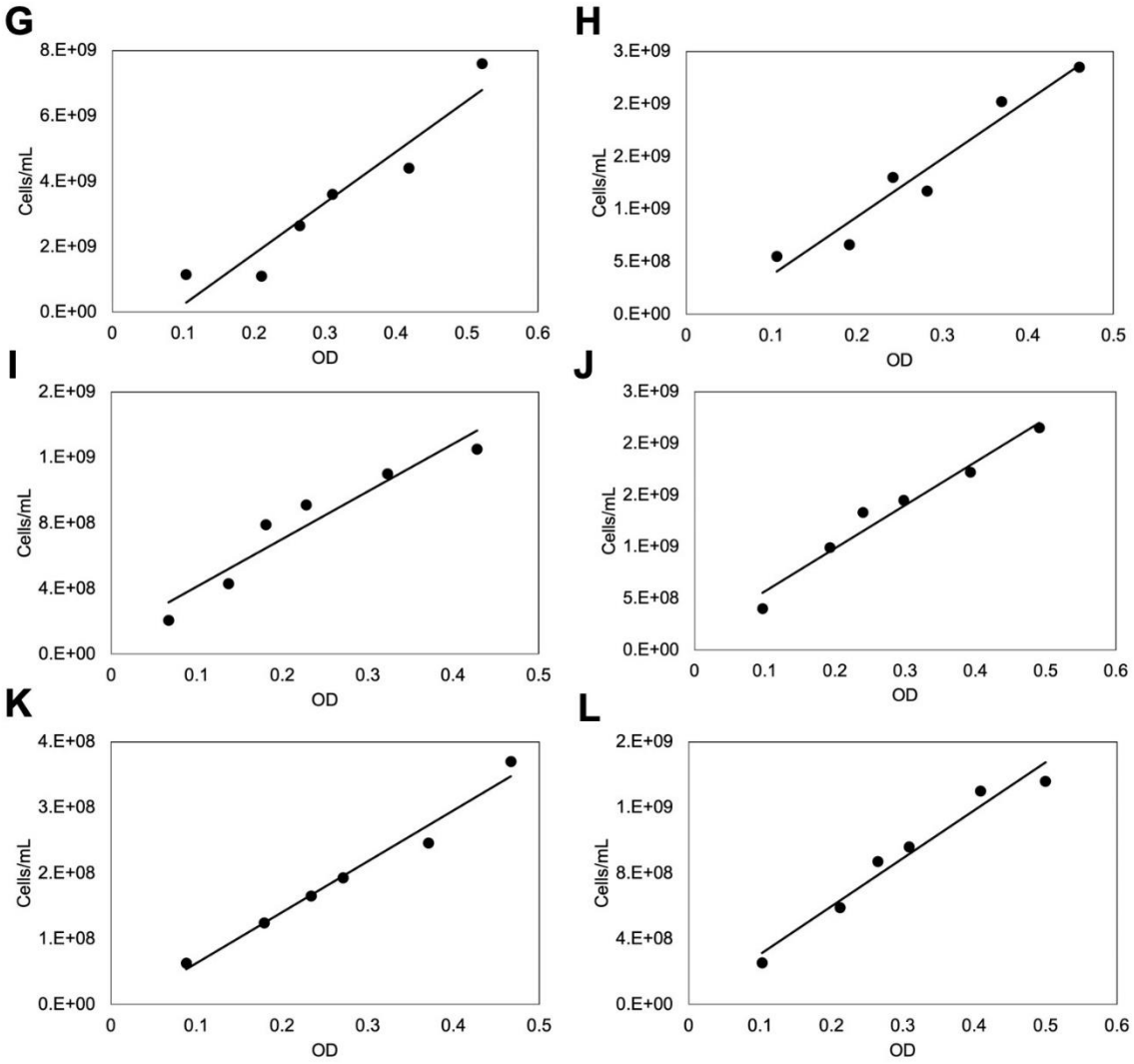
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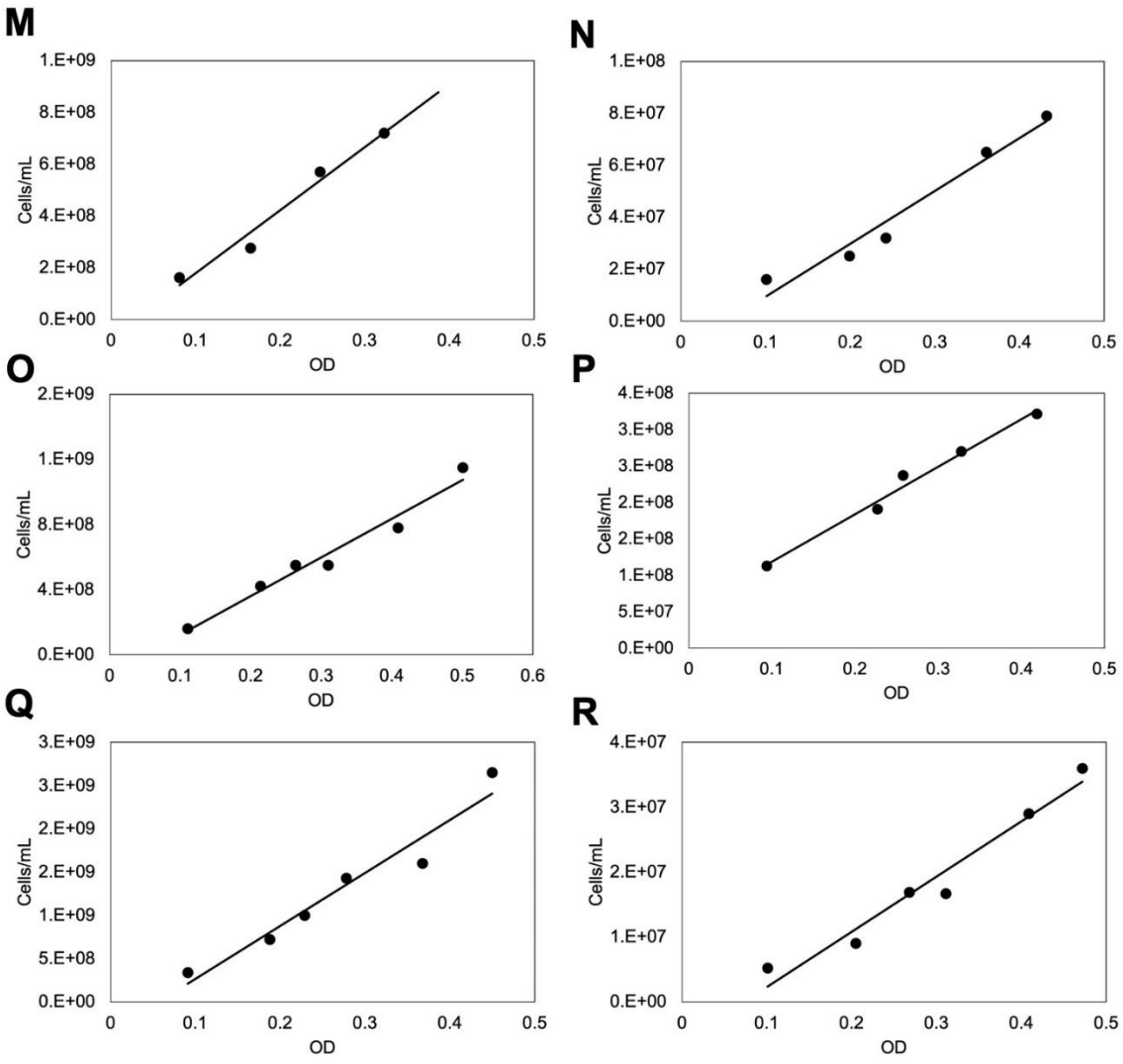
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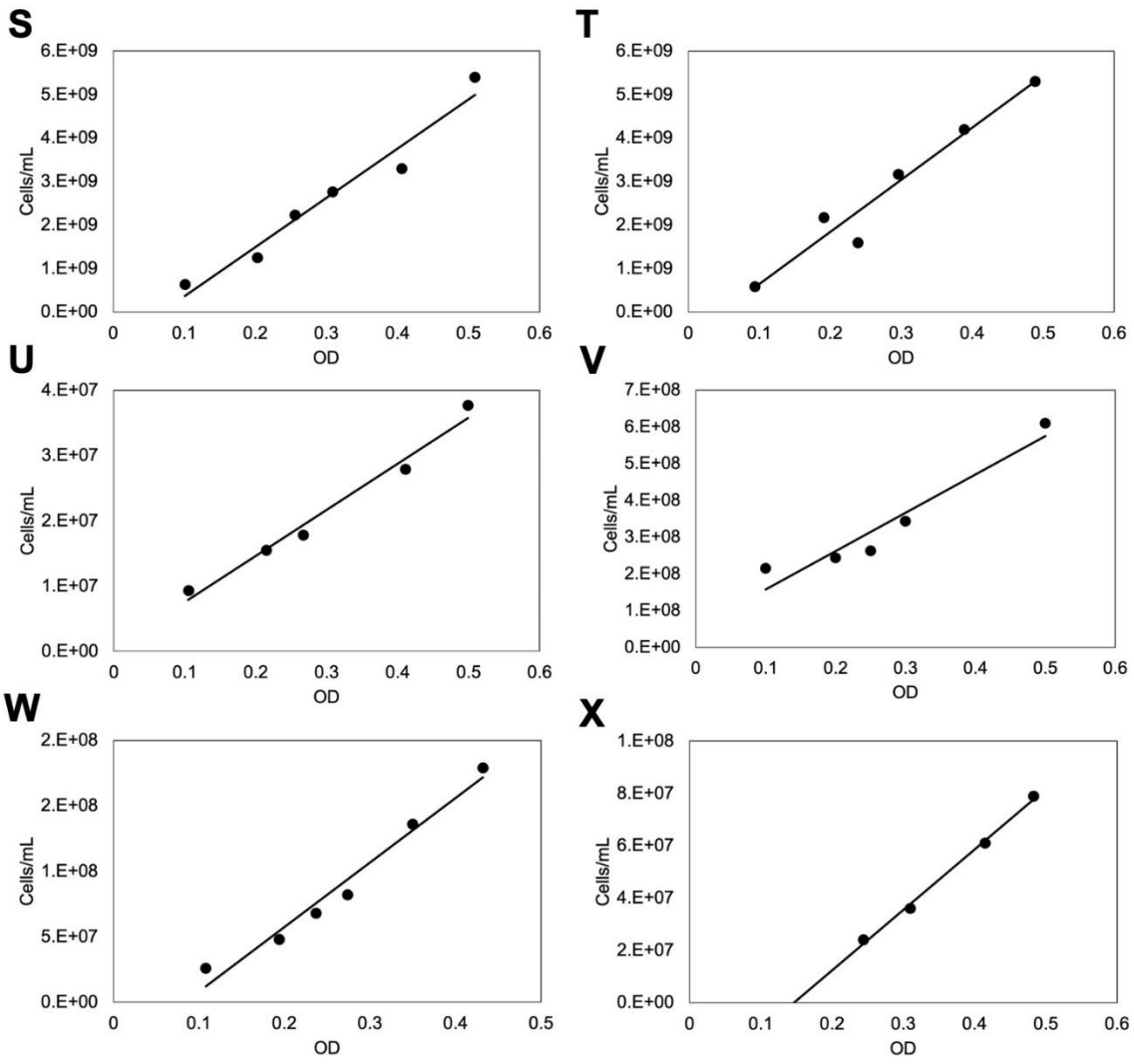
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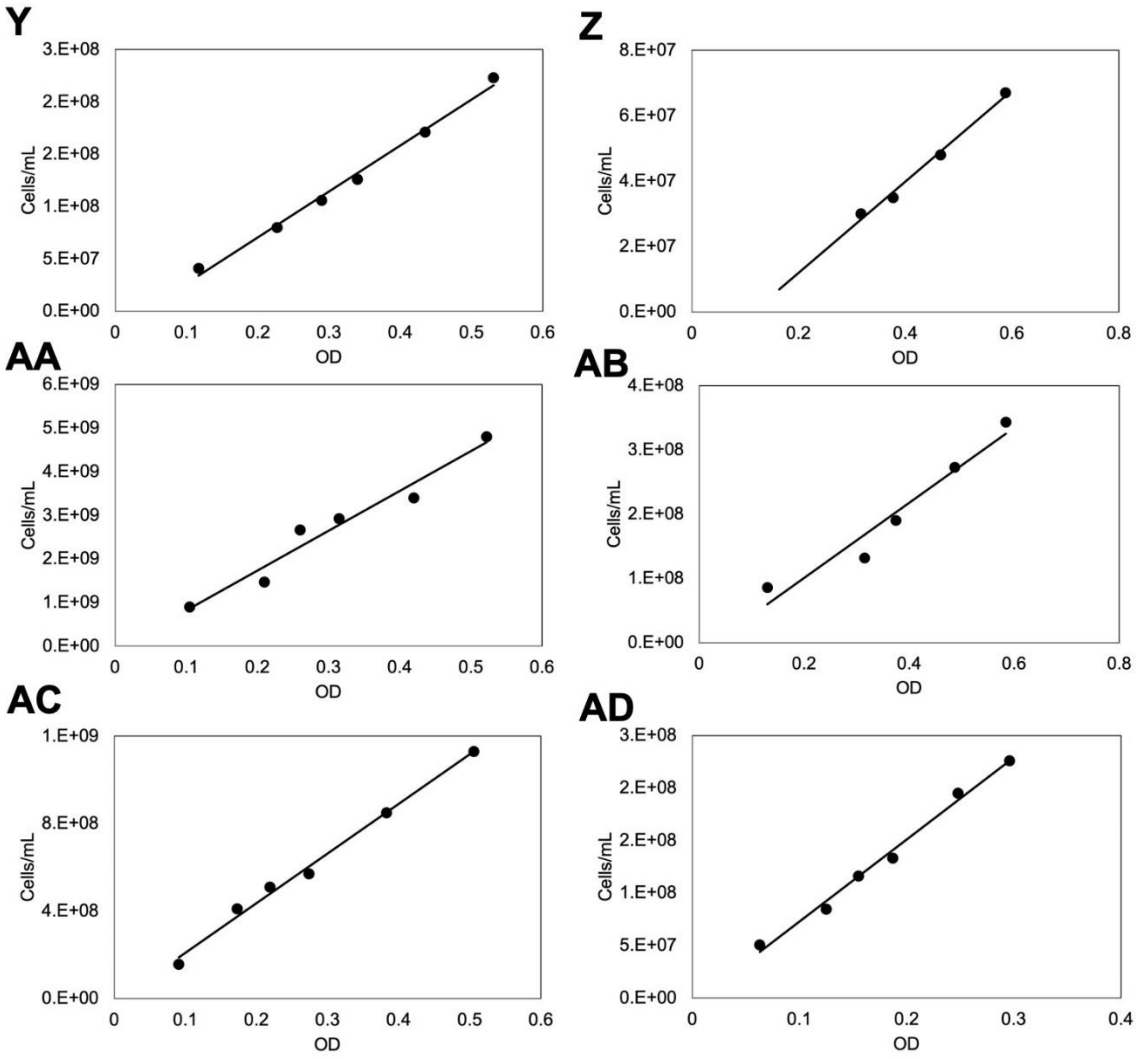
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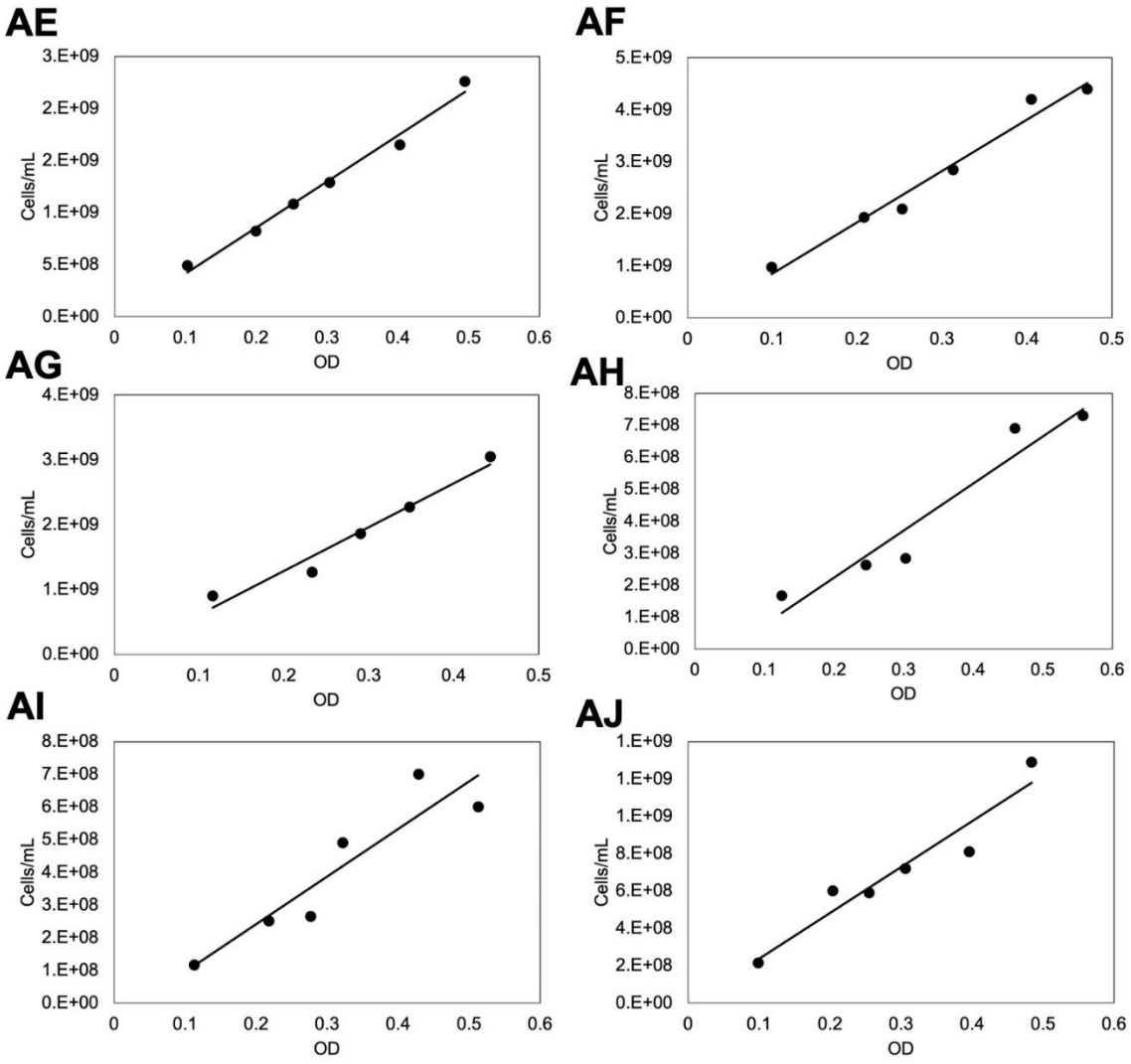

Figure 3. 1. Growth curves for the 36 bacterial isolates from a 2018 Taihu *Microcystis* bloom. A) *Stenotrophomonas sp.*, B) *Stenotrophomonas sp.*C) *Brevundimonas sp.* D) *Stenotrophomonas sp.*, E) Unidentified, F) *Stenotrophomonas sp.*, G)*Pseudoxanthomonas sp.* , H) *Stenotrophomonas sp.*, I) *Exiguobacterium sp.* , J) *Stenotrophomonas sp.*, K) *Stenotrophomonas sp.*, L) *Pseudomonas sp.*, M) *Bacillus sp.*, N) *Exiguobacterium sp.* , O) *Bacillus sp.*, P) *Algicola sp.*, Q) *Stenotrophomonas sp.*, R) *Bacillus sp.*, S) *Stenotrophomonas sp.*, T) *Stenotrophomonas sp.*, U) *Bacillus sp.*, V) *Enterbacter sp.*, W) *Exiguobacterium sp.*, X)*Bacillus sp.*, Y) *Bacillus sp.*, Z) *Pseudoxanthomonas sp.*, AA) *Stenotrophomonas sp.*, AB) *Exiguobacterium sp.* , AC) *Aeromonas sp.* , AD) *Paenibacillus sp.* , AE) *Rhizobium sp.* , AF) *Cloacibacterium sp.*, *AG) Cloacibacterium sp.* , AH) Unidentified, AI) Unidentified, and AJ) *Pseudomonas sp.*The minimum cutoff for R^2 was 0.8 and the average $R^2 = 0.927$.

2.3 RNA-SIP

To trace carbon exchange in the *Microcystis* phycosphere, RNA-SIP was used with a stable carbon isotope and total RNA extracted. Initially, cultures of axenic *Microcystis aeruginosa* NIES 843 were isotopically labeled via incubation with 6 mM ^{13}C - sodium bicarbonate at 26 °C in a 12:12 light:dark cycle for 4 days (Fortunato and Huber, 2016). The 36 bacterial isolates from Taihu were grown in CT-TY broth with ammonium (NH_4) instead of nitrate at 26 °C for 48 hours. The labeled *Microcystis* cells were washed with standard CT to remove residual ^{13}C not incorporated into cells and resuspended in standard CT media. The consortium was inoculated as described above for the growth experiment with a few differences. The control flask with *Microcystis* had CT for media and the control flask with just the bacterial consortium had CT-TY (NH_4). All flasks were incubated at 26 °C in a 12:12 light:dark cycle at 114.7 ft-cd for 48 hours. After 48 hours, cultures were harvested by using Sterivex 0.22 µm polyethersulfone membrane filters (MILLIPORE, Burlington, MA, U.S.A) and sterile 50 mL syringes, then flash frozen in liquid nitrogen and stored at -80 °C until extraction. Samples of the consortium were also visualized via fluorescent microscopy using acridine orange.

RNA was extracted from the filters using the RNeasy PowerWater Kit (Qiagen) according to the manufacturer's instructions, modified for Sterivex filters according to Cruaud et al. (2017). Labeled and unlabeled RNA from the *Microcystis* was separated out and purified using the adapted protocol from Neufeld et al. (2007). This was adapted for 15 mL, Quick-Seal® Bell-Top Polypropylene tubes (Beckman Coulter, Brea, CA, U.S.A.) in a fixed angle rotor (Beckman Coulter Type Ti 50.2).

The cesium chloride gradient was prepared by mixing the RNA with gradient buffer (GB) (0.1 M Tris, 0.1 M KCl and1 mM EDTA). The volume of RNA and GB was determined using the following equation: GB/DNA volume = (CsCl stock solution density − desired final density) × volume of CsCl stock added × 1.52 (Rickwood, 1983). The CsCl stock solution (7.163 M CsCl) was then added to the GB/DNA mixture. The mixture was then added to the ultracentrifuge tube using a sterile syringe and needle, filling the tube approximately halfway. The tube was then topped with sterile mineral oil, leaving a small air bubble on the top. The tubes were balanced within 10 mg and sealed with a Cordless Tube Topper kit PN 358312 (Beckman Coulter). Ultracentrifuge settings were 32,000 rpm at 20 ℃ for 217:24 (hr:min) with vacuum, maximum acceleration and no brakes.

After isopycnic separation, the tubes were then fractionated using a syringe pump within 10 minutes of ultracentrifuge cessation. The tube was pierced with a needle on the top and bottom of the tube, then mineral oil was pumped with a NE-1000 “Just Infusion” ™ pump (0.700 mL/minute) through the top of the tube and twenty ~300 µL fractions were collected from the bottom of the tube. The weight of the fractions was used to determine the density of each fraction. Labeled and unlabeled fractions of RNA were combined then purified according to Neufeld et al. (2007). The NanoVue™ Plus Spectrophotometer 4282 V2.0.3 (GE Healthcare) was used to determine quality and concentration of the RNA samples. The total RNA samples were sent to GENEWIZ, Inc. (South Plainfield, NJ, U.S.A) for standard RNA sequencing.

3. Results and Discussion

3.1 Taihu Bacterial Consortium

To model bacterial interaction in *Microcystis* blooms, a synthetic consortium was made by isolating and characterizing heterotrophic bacteria present in a 2018 Taihu *Microcystis* bloom. There were 36 bacterial isolates that were isolated from the Taihu bloom samples and the 16S rRNA gene was used to identify the isolates at the genus level (**Table 1**). The majority of the bacteria belonged to the phylum Proteobacteria (55.6%), followed by Firmicutes (30.6%) and Bacteroidetes (0.06%). Of the bacteria belonging to Proteobacteria, two isolates belonged to the class Alphaproteobacteria and 18 to the class Gammaproteobacteria. Eleven of the Gammaproteobacteria isolates were identified as *Stenotrophomonas* spp. and six of the Firmicutes isolates were identified as *Bacillus* spp.. The two Bacteroidetes isolates were both identified as the genus *Cloacibacterium*. *Stenotrophomonas* and *Bacillus* have been previously isolated from blooms in Taihu and have the ability to degrade microcystins (Hu et al., 2012; Chen et al., 2010). Metagenomic analysis of lab cultures of *Microcystis* have had *Stenotrophomonas* and *Bacillus* present as well (**Chapter II**). These two genera may be common members of the *Microcystis* phycosphere and have mutualistic relationships with *Microcystis*. Species of *Stenotrophomonas* and *Bacillus* are known growth promoting rhizobacterium (Hashem et al., 2019; Alexander et al., 2019) and other species belonging to these genera could have similar interactions with *Microcystis*. These bacteria could be benefiting from association with *Microcystis* by using it as a source of carbon. The carbon-rich phycosphere of *Microcystis* may provide these bacteria with essential carbon, which will be proven with the RNA-SIP transcriptomes.

Synthetic microbial consortia are useful for studying the metabolic interactions in a model community that is not possible in uncontrolled natural microbial communities (Grosskopf and Soyer, 2014). Because natural microbial communities can contain up to thousands of species (Curtis et al. 2002), it is not feasible to experimentally pair individual species with specific functions. Using a synthetic microbial community from a *Microcystis* bloom allows for control of the particular species, their numbers, labeling of specific members, and all abiotic factors that otherwise cannot be controlled in field studies. A bacterial synthetic consortium has previously been isolated from the cyanobacterium, *Prochlorococcus,* and the consortium promoted *Prochlorococcus* growth (Sher et al., 2011). SIP has also been used to trace metabolite exchange in a small bacterial synthetic consortium (Ranava et al., 2021). Synthetic consortia create an opportunity for understanding the complex interactions between microorganisms in a controlled way.

Table 3. 1. Identification of the 36 isolates that were cultured and identified based on 16S sequencing.

Phylum	Class	Genus	Number of Isolates
Proteobacteria	Alphaproteobacteria	*Brevundimonas*	1
Proteobacteria	Alphaproteobacteria	*Rhizobium*	1
Proteobacteria	Gammaproteobacteria	*Aeromonas*	1
Proteobacteria	Gammaproteobacteria	*Algicola*	1
Proteobacteria	Gammaproteobacteria	*Enterobacter*	1
Proteobacteria	Gammaproteobacteria	*Pseudomonas*	2
Proteobacteria	Gammaproteobacteria	*Pseudoxanthomonas*	2
Proteobacteria	Gammaproteobacteria	*Stenotrophomonas*	11
Firmicutes	Bacilli	*Bacillus*	6
Firmicutes	Bacilli	*Exiguobacterium*	4
Firmicutes	Bacilli	*Paenibacillus*	1
Bacteroidetes	Flavobacteria	*Cloacibacterium*	2
Undetermined			3

3.2 Consortium Growth Experiment

To test the impact of the synthetic consortium on *Microcystis* growth and physiology, axenic *Microcystis* was grown with the synthetic consortium of 36 Taihu bacterial isolates. The *Microcystis* was able to grow unhindered by the consortium as shown by the cell counts, fluorescence, and photosynthetic stress (**Figures 2-4**). When grown with the Taihu consortium, there was no significant difference in daily cell abundance between the consortium and axenic *Microcystis,* however, the cell counts were consistently higher for the consortium (**Figure 2**). The *Microcystis* did show a significant increase in fluorescence at 625 nm 17 out of the 56 days over the axenic *Microcystis* control (**Figure 3**). Both the axenic *Microcystis* and the *Microcystis* grown with the Taihu consortium did not exhibit signs of photosynthetic stress as measured via Fv/Fm (**Figure 4**). The maximum growth rate for the axenic *Microcystis* was 0.1666706 for cell counts and 0.05275795 for fluorescence while the maximum growth rate for *Microcystis* growth with the consortium was 0.02304709 for cell counts and 0.1943986 for fluorescence. There was no significant difference between the average maximum growth rates of the axenic *Microcystis* and the *Microcystis* growth with the consortium. The consortium of bacteria isolated from a *Microcystis* bloom appeared growth-promoting for *Microcystis.* This is consistent with previous studies that have found heterotrophic bacteria isolated from *Microcystis* blooms have growth-promoting capabilities, although the mechanism for growth promotion has yet to be resolved (Hoke, 2019; Gao et al., 2020).

3.3 RNA-SIP

^{13}C-SIP allows for direct measurement of carbon exchange *Microcystis* and heterotrophic bacteria from a *Microcystis* bloom. Combining the ^{13}C-SIP with

transcriptome analysis will give insight into the genes actively being used during the exchange of carbon. The density of the RNA fractions from isopycnic ultracentrifugation show the two fractions of RNA: the heavy labeled RNA and the lighter unlabeled RNA (**Figure 5)**.

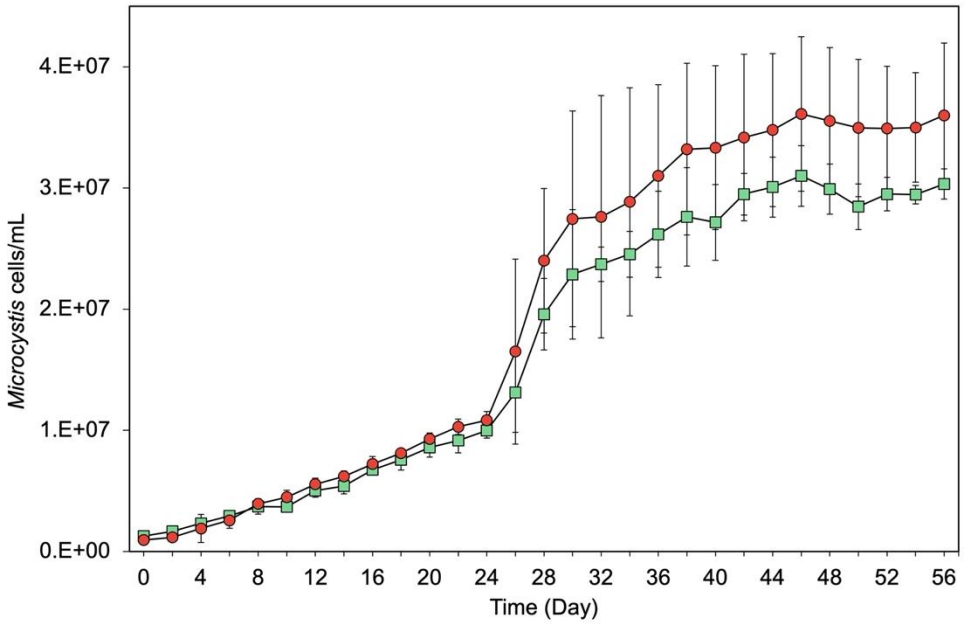

Figure 3. 2. Cell counts of *Microcystis* cells grown with and without the Taihu Consortium. The green squares represent the cultures with axenic *Microcystis* and the red circles represent *Microcystis* grown with the Taihu consortium. Error bars indicate +/- standard deviation.

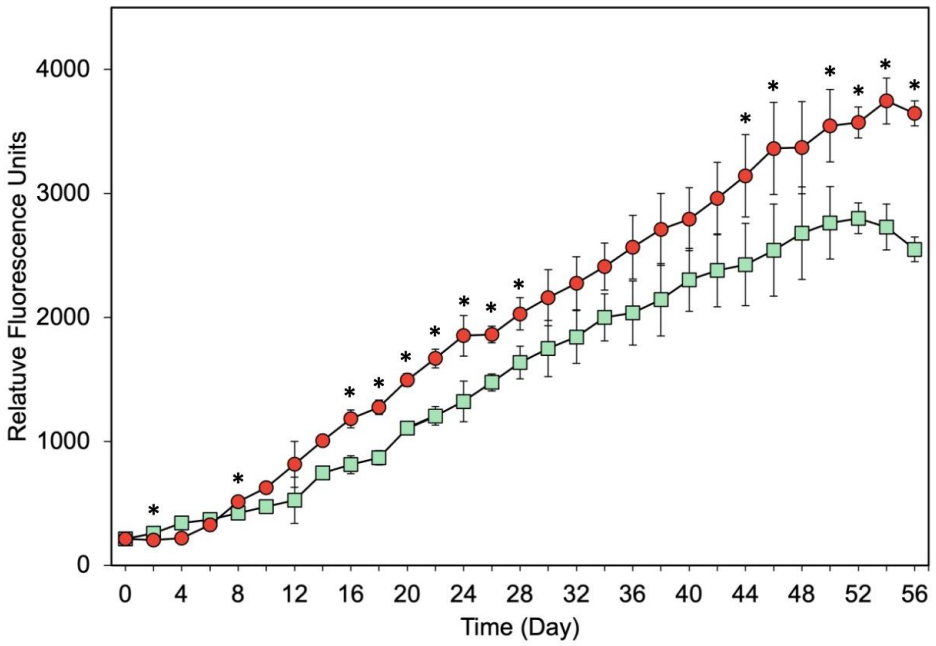

Figure 3. 3. Fluorescence of *Microcystis* cells when grown with and without the Taihu consortium. The green squares represent the cultures with axenic *Microcystis* and the red circles represent *Microcystis* grown with the Taihu consortium. Error bars indicate +/- standard deviation. * indicate significant differences between the two groups with a $p < 0.05$.

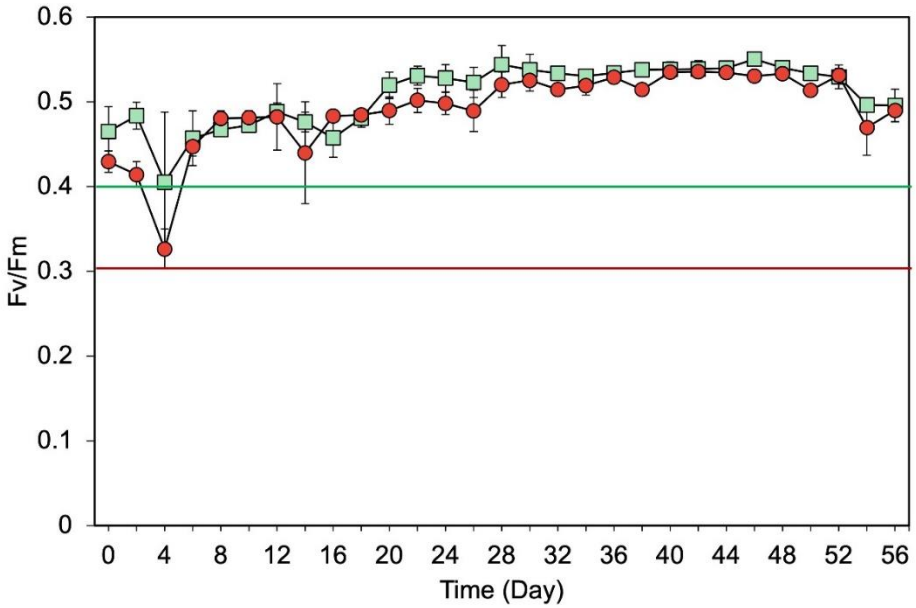

Figure 3. 4. Fv/Fm of *Microcystis* when grown with the Taihu consortium and without. The green horizontal line and above indicates a healthy zone while the red horizontal line shows a stressed zone (below 0.3). The green squares represent the cultures with axenic *Microcystis* and the red circles represent *Microcystis* grown with the Taihu consortium. Error bars indicate +/- standard deviation.

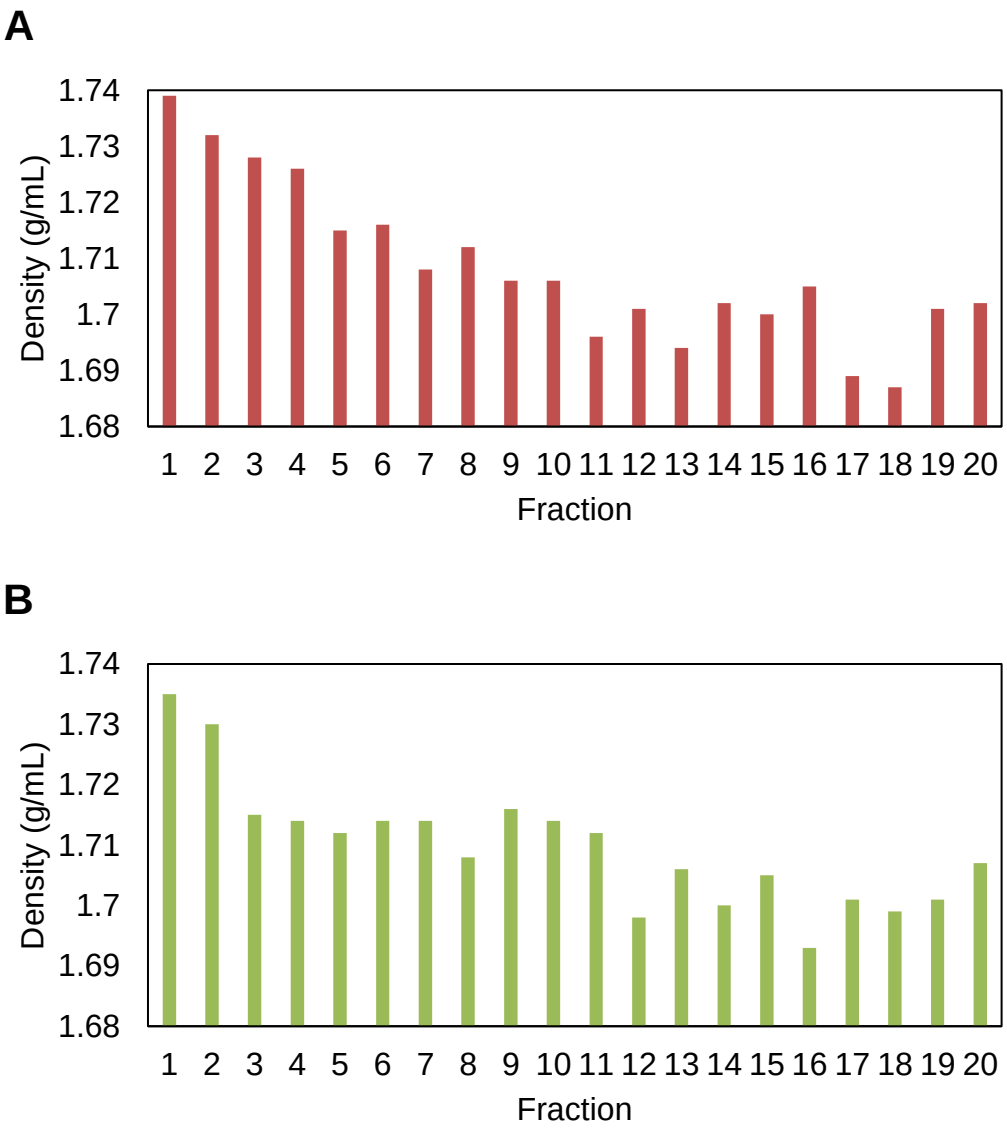

Figure 3. 5. Densities of the fractions from the CsCl isopycnic separation of RNA from the C exchange experiment between *Microcystis* and the bacterial consortium.

4. Conclusions

A synthetic consortium of heterotrophic bacteria was assembled from bacteria isolated from a 2018 Taihu *Microcystis* bloom. This consortium allowed for control of the members of the consortium as well as the abiotic factors which is not possible in field experiments. This consortium was then grown with axenic *Microcystis* and showed growth promotion for the cyanobacteria. The RNA-SIP experiment results will not only identify the bacteria capable of utilizing *Microcystis*-derived carbon, but the genes being actively used in this exchange of nutrients. This study will provide evidence of direct carbon exchange between *Microcystis* and the heterotrophic bacteria in a bloom.

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Chapter IV. Conclusions

Nutrient utilization capabilities of the *Microcystis* phycosphere were investigated using stable isotope probing (SIP) combined with metagenomics and transcriptomics. DNA-SIP was used to trace nitrogen (N) utilization in lab strains of *Microcystis aeruginosa.* Bacteria that could and did not use the added ^{15}N-urea and ^{15}N-nitrate were identified in these cultures. *Microcystis*, *Bacillus*, and *Oscillospira* were able to use both urea and nitrate as forms of N. Bacteria that did not use the N sources included the genera *Devosia*, *Blastamonas*, *Hydrogenophaga* , and *Rhodobacter*. Some bacteria may not have needed to use the added N sources because they can fix atmospheric N like the genera *Devosia*, *Hydrogenophaga* ,and *Rhodobacter.* Other bacteria like those belonging to the genus *Blastamonas* are not known to be able to use urea and nitrate as N sources. The diverse range of N utilization capabilities suggest that not all of these bacteria can use these exogenous forms of N and may be dependent upon other bacteria in the consortium for N. *Microcystis* could also benefit from the association with bacteria in these cultures that can use many forms of N and potentially provide *Microcystis* with more bioavailable reduced forms of N. Future experiments with ^{15}N-SIP should be done to trace N exchange between the bacterial partners of the *Microcystis* phycosphere to prove this hypothesis. Indicator species analysis showed two OTUs diagnostic of specific N utilization. An *Acinetobacter* sp. was found to be indicative of nitrate utilization and a *Methylobacterium* sp. was diagnostic of urea utilization. These genera could be used as indicators of active N utilization in future and previous *Microcystis* bloom community analysis. The microbial communities did not differ between the N sources added, but there was significant differences between the microbial communities of the two strains of *Microcystis* used in this experiment.

To trace *Microcystis*-derived carbon utilization by phycosphere partners, 36 heterotrophic bacteria were isolated from a 2018 *Microcystis* bloom in Taihu. The genera *Stenotrophomonas* and *Bacillus* have been found in Taihu *Microcystis* blooms previously, which may indicate a mutualistic relationship between these heterotrophic bacteria and *Microcystis.* The consortium of bacteria from Taihu were first tested for its effect on *Microcystis* growth and were found to have positive effects. The *Microcystis* grown with the bacterial consortium showed a significant increase in growth when measured by fluorescence. RNA-SIP was used to trace ^{13}C from *Microcystis* to the Taihu consortium. The labeled and unlabeled RNA were successfully separated from each other using isopycnic separation. The resulting transcriptomes from this experiment will identify the bacteria that are using *Microcystis*-derived carbon as well as the genes being actively used during this exchange. We will be able to compare the gene expression of the *Microcystis* grown in consortium to the individual partners. These results will provide direct evidence of *Microcystis* providing C to the heterotrophic bacteria in its phycosphere and give insight into the mechanisms of C exchange.

These SIP experiments give insight into the relationship between the cHAB forming *Microcystis* and the complex interactions it has with heterotrophic bacteria in its phycosphere. This thesis clearly demonstrates that biotic factors leading to bloom formation are critical to understanding bloom dynamics when considering abiotic factors that lead to bloom formation because other organisms in these blooms interact with the abiotic factors as well as the bloom formers.

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