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The Role of Nitrogen and Phosphorus in the Growth, Toxicity, and Distribution of the Toxic Cyanobacteria, Microcystis aeruginosa

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This Master's Project

The Role of Nitrogen and Phosphorus in the Growth, Toxicity, and Distribution of the Toxic Cyanobacteria, *Microcystis aeruginosa*, in Aquatic Ecosystems

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Chapter 1 - Introduction

Nutrients are crucial to sustaining the life of aquatic ecosystems. Nitrogen (N) and phosphorus (P), and their respective chemical forms, are two of the most influential nutrients in stimulating primary production. Phytoplankton feed on N and P forms that are either introduced to or recycled through the water system, and in turn are fed on by zooplankton such as krill or shrimp. The energy gained by phytoplankton consumption is transferred through the food web from small fish and mollusks, to larger fish, to sea mammals, and even to humans. However, these nutrients crucial for supporting life in the water can be just as much toxic as they are healthful.

At the base of the food web, where N and P nutrients feed primary producing phytoplankton to support the rest of the food web, toxic species of phytoplankton are also feasting – and thriving – off of these nutrients. When exposed to nutrient-rich waters, these toxic phytoplankton can rapidly grow, forming dense, toxin-releasing colonies known as harmful algal blooms (HABs). In addition to the anoxic conditions created by these HABs, their toxins can make their way through the food web, accumulating into the tissues of shellfish and other fish, and consequently threatening the health of marine ecosystems while poisoning marine mammals and humans alike.

The growth, distribution, and toxicity of HABs are influenced by a complex interaction of ecology, nutrients, and local environmental forces. It is important to understand these components to evaluate current management efforts, expand the knowledge base, and formulate new strategies to control them.

1.1 Ecology of *Microcystis aeruginosa*

Microcystis aeruginosa is one of the most common harmful algal-blooming species in the world (Moisander et al., 2009; Straub et al., 2011), yet its growth dynamics are poorly understood. Unlike other phytoplankton, *M. aeruginosa* is not true algae, but rather photosynthetic bacteria known as *cyanobacteria,* named after the color of their blue-green algal blooms. Cyanobacteria are the most ancient of phytoplankton species, and they thus have a wide distribution and diverse dynamic that makes understanding their growth mechanics complicated (O'Neil et al., 2011). While *M. aeruginosa* commonly inhabits freshwater lakes during eutrophic seasons, it can also be found in estuarine systems and along marine coasts, and it is continuing to increase in frequency and intensity around the world (Davis et al., 2009; Lehman et al., 2013). It has only recently started to infest water bodies in Northern

California, blooming for the first time in the brackish waters of the San Francisco Bay estuary in 1999, and in the fresh waters of the Copco and Iron Gate reservoirs of the Klamath River in 2005, making consistent returns in health-concerning abundance levels every year (Lehman et al., 2013; Moisander et al., 2009). Of special concern is the introduction of *M. aeruginosa* in the San Francisco Bay estuary, as this is indicative of its global spread not only geographically, but from its common freshwater habitat and into harsher, more saline conditions. Toxic strains of *M. aeruginosa* release highly noxious metabolites from their blooms, which are absorbed by zooplankton, clams, and juvenile fish, and are subsequently spread throughout all trophic levels, causing detrimental and sometimes lethal effects to aquatic wildlife and their ecosystems.

As part of the *Microcystis* genus, *M. aeruginosa* is one in a spectrum of *Microcystis* species, although growth and response patterns may be similar between them. Some studies reviewed in this document examine *M. aeruginosa* among its *Microcystis* counterparts. For the sake of clarity, the term *Microcystis* as it is discussed in study results will refer to a range of *Microcystis* species that includes *M. aeruginosa*.

1.2 Nutrient Influences on *Microcystis aeruginosa*

As a primary producer, *M. aeruginosa* depends on N and P forms for food. Nutrient-rich wastewater effluents discharged from wastewater treatment plants and into aquatic ecosystems, like the San Francisco Bay Estuary, are loading them with N and P that can spur harmful algal blooms (HABs) dominated by cyanobacteria. P has traditionally been accepted as having a dominant role in primary production, and as such, it has been commonly considered the limiting nutrient in freshwater ecosystems (O'Neil et al., 2011). Consequently, current management efforts are stringently regulating P loads in wastewater effluents, dramatically decreasing eutrophication potential and increasing the N-to-P (N:P) concentration ratio (Paerl et al., 2012). An example of P regulation increasing the N:P ratio can be observed in the San Francisco Bay Estuary, where the N:P ratio has almost doubled in the past 35 years, from a dissolved inorganic N-to-total P (DIN:TP) ratio of 2 in 1975 to almost 4 in 2010 by weight (SFBRWQCB, unpublished). This approach can be concerning, however, as recent research suggests that N may be just as influential, if not more influential, as P, in promoting cyanobacterial blooms like *M. aeruginosa* (O'Neil et al., 2011).

Although more stringent N regulation in wastewater is being considered, a growing amount of research suggests that toxic *M. aeruginosa* strains actually dominate under low N:P ratios (Beversdorf et al., 2013; Chaffin et al., 2011; Fuhimoto et al., 1997; X. Liu et al., 2011; Y. Liu et al., 2011; Marinho et al.,

2007; Orihel et al., 2012; Otten et al., 2012; Smith, 1983; Xu et al., 2010). Thus, expensive regulation of wastewater effluents may actually exacerbate the growing problem of *M. aeruginosa* by manipulating nutrient concentrations into lower N:P ratios that can benefit the cyanobacterium.

1.3 Local Environmental Influences on *Microcystis aeruginosa*

In addition to the influence of N and Ps loadings, environmental forces driven by the growing threat of climate change may provide for a more ideal habitat for *M. aeruginosa*, explaining its worldwide increase in abundance. Factors such as water temperature, sunlight, turbidity, salinity, and water velocity—among others—may complement or surpass the role of nutrients in influencing the growth, toxicity, and distribution of *M. aeruginosa.*

With a comprehensive examination of the role of nutrients respective of other environmental factors on *M. aeruginosa*, a clearer understanding of the growth, toxicity, and distribution dynamics of this cyanobacterium may offer valuable insight into effective strategies for controlling these potentially devastating blooms.

1.4 Management Efforts to Control *Microcystis aeruginosa*

Management of toxic strains of *M. aeruginosa* is a complicated task because its mechanisms of growth and toxicity, in addition to what influences those mechanisms, are not yet fully understood. Moreover, conflicting theories as to these influences clutter the realm of scientific literature, and little research has been conducted for *M. aeruginosa* in marine waters due to its abundant freshwater invasions. Although P is heavily regulated in both point and non-point sources of pollution, the concurrent and almost synchronized global expansion of *M. aeruginosa* suggests a different, perhaps more comprehensive approach to managing its growth and distribution may be more effective.

With the recent spread of *M. aeruginosa* to Northern California waters, considerations for the cause of this onset revolve around possible flawed or insufficient management strategies surrounding nutrient regulations and the influential factors associated with climate change. The potential of innovative and efficient management options in managing *M. aeruginosa* can increase with a clarified understanding of the drivers behind *M. aeruginosa* dominance in aquatic systems. Whether to treat the *M. aeruginosa* problem directly through biotic or abiotic biomass removal or indirectly through nutrient manipulations and policy adjustments must be analyzed by well-informed decision-makers to guide these management strategies.

1.5 Research Focus

Despite being one of the most common harmful algal-blooming species within the most ancient genus of phytoplankton, the mechanisms behind the growth, distribution, and toxicity of *Microcystis aeruginosa* cyanobacteria are poorly understood. The toxins released by *M. aeruginosa* blooms can devastate aquatic ecosystems and be lethal to the organisms that inhabit them and benefit from their services. Phosphorus has traditionally been considered the limiting nutrient of freshwater ecosystems, thus stringent control of both point and non-point sources of discharge have significantly reduced its input into these ecosystems. However, the threat of *M. aeruginosa* is continuing to grow, making consistent returns and expansions on a global scale, afflicting not only freshwater systems in which it is commonly found, but also more saline aquatic ecosystems in estuaries and along marine coastlines. This global expansion suggests that other factors may be more influential than phosphorus in the dominance of toxic *M. aeruginosa*, with studies supporting the significant influences of N and local environmental factors intensified by climate change in *M. aeruginosa* regulation. Nonetheless, conflicting theories and study results create a tangled and unreliable web of information in the realm of scientific literature. A comprehensive examination into the influences of N, P, and the environmental factors associated with climate change can identify trends and clarify the drivers behind *M. aeruginosa* expansion for effective management strategies in controlling their growth, distribution, and toxicity in aquatic ecosystems.

This research will explore the ecology of *M. aeruginosa* (**Chapter 2 - Ecology and Toxicity of *Microcystis aeruginosa***) in order to effectively explore role of N and P in its growth, distribution, and toxicity (**Chapter 3 - Nutrient Influences**). Additionally, relative to N and P, local environmental influences on *M. aeruginosa* will be explored in the context of climate change (**Chapter 4 - Environmental Influences**). Currently, the dynamics between *M. aeruginosa*, nutrients, and the surrounding environment on its growth, toxicity, and distribution are poorly understood. *M. aeruginosa* is a relevant environmental concern that threatens the health of both aquatic ecosystems and humans, and its current, global expansion in the face of climate change renders it a contemporary threat necessary of a comprehensive investigation. Through a clear understanding of the influencing factors behind the growth of *M. aeruginosa*, more appropriate and effective management strategies can be developed to improve control of toxic, and potentially devastating, blooms (**Chapter 5 - Research Conclusions and Management Recommendations**).

Chapter 2 - Ecology and Toxicity of *Microcystis aeruginosa*

Microcystis aeruginosa is comprised of a dynamic physiology, resulting in a complex relationship with its environment that can be difficult to simplify. Thus, the mechanisms behind its growth, toxicity, and distribution are poorly understood, despite being one of the most common and ancient genera of harmful algal blooms (HABs) in the world (O'Neil et al., 2011; Moisander et al., 2009; Montagnolli et al., 2003; Straub et al., 2011). As cyanobacteria, it is a prokaryotic species that uses chlorophyll to convert light into energy, resembling its eukaryotic photosynthetic counterparts, and hence its misnomer identification as "algae". Among those algal counterparts are diatoms and dinoflagellates, which, along with cyanobacteria, comprise the most abundant and harmful phytoplankton species in the world, afflicting aquatic ecosystems with toxic blooms (Gilbert et al., 2005; Vasconcelos, 2006).

Like other primary producers, *M. aeruginosa* feeds off of nutrients that are either introduced to or cycled throughout the water, and, with the help of sunlight, rapidly blooms in dense colonies under eutrophic conditions. Not all strains of *M. aeruginosa* are toxic, nor are all strains colony-forming (Ma et al., 2013). However, even non-toxic strains of *M. aeruginosa* can be fatal, as sufficient concentrations of nutrients can result in rapid, dense bloom formations that block sunlight from penetrating aquatic ecosystems and create anoxic conditions during nighttime respiration, resulting in fish kills and an overall decrease in biodiversity (O'Neil et al., 2011). Moreover, *M. aeruginosa* has a unique relationship with nitrogen (N), phosphorus (P), and its environment in comparison to diatoms, dinoflagellates, and other cyanobacteria (explored further in **Chapter 3 - Nutrient Influences**). Unlike most cyanobacteria, it is a non-diazotrophic species, meaning it does not fix N from the atmosphere, yet little evidence suggests this is a constraint or a disadvantage (Beversdorf et al., 2013; Smith, 1983.). This inability to fix N may be indicative of a unique relationship *M. aeruginosa* shares with N and N-fixing phytoplankton for survival in N-limited aquatic ecosystems, or in aquatic ecosystems where availability of N is insufficient to satisfy phytoplankton nutritional needs. The magnitude of the *M. aeruginosa* problem is increasing with its expanding presence in the environment, influenced by its toxicity, population dynamics, and physiology within the phytoplankton realm.

2.1 Presence in the Environment

Although *M. aeruginosa* is most common in freshwater ecosystems, it can also be found in the saltier waters of estuarine systems and marine coasts (Belyk et al.,2012; Beversdorf et al., 2013; Lehman et al., 2013; Montagnolli et al., 2003; Robson and Hamilton, 2003). It is believed that the majority of *M.*

aeruginosa enters estuarine and marine ecosystems through flushing of *Microcystis*-contaminated streamflows and runoff from freshwater systems, where it is then able to survive and establish itself in the harsher conditions of more brackish waters (explored further in **Chapter 4 - Environmental Influences**) (Miller et. al., 2010). Such cases of *Microcystis*-polluted freshwater infiltrating brackish ecosystems have been documented in the Swan River estuary of Western Australia, the St. Lucie River estuary in Florida (Ross et al., 2006), and the San Francisco Bay estuary and Monterey Bay of California, during the wet season (Lehman et al., 2013; Lehman et al., 2005; Miller et al., 2010 Mosiander et al., 2009; Robson and Hamilton, 2003). However, some cases have detected *in situ* growth in brackish waters, as observed in Puget Sound in the State of Washington (Lehman et al., 2005).

M. aeruginosa has typically been observed to bloom annually during the warmer months, beginning in May and ending after November (Lehman et al., 2013; Lehman et al., 2005; Xiu et al., 2011; Yoshida et al., 2007). However, dense blooms *M. aeruginosa* have also been observed in the winter months between December and February (Tas et al., 2006). Though these blooms are often ephemeral, they are also extensive, and their ability to degrade aquatic ecosystems has relevant consequences (Miller et al., 2010). During these blooms, toxic taste and odor compounds released degrade the quality of water, which can cause severe ecological, economical, and public health impedances; aquatic wildlife is harmed, recreational and fishery markets are impacted, and drinking water is contaminated (Lehman et al., 2013; Ma et al., 2013). Additionally, its dense, noxious, and photosynthetic biomass causes a lethal combination of toxic and hypoxic conditions (Brooks and Ganf, 2001; Ma et al., 2013; O'Neil et al., 2011) which favors *M. aeruginosa* dominance by altering the food web competition amongst the phytoplankton community (O'Neil et al., 2011) and lowering biodiversity by poisoning or suffocating sensitive fish species and other local benthic organisms (Montagnolli et al., 2003; Vasconcelos, 2006). The presence of *M. aeruginosa* in estuarine and marine waters can be especially concerning due to its potential to threaten the health and balance of aquatic ecosystems and their wildlife, devastate marine fisheries, and harm human health via seafood vectors through its release of toxic metabolites, which is explored further in Section **2.2: Toxicity of *Microcystis aeruginosa***.

M. aeruginosa blooms are fast becoming a global health concern, increasing in frequency and intensity around the world (Davis et al., 2009; Lehman et al., 2013). Recurrent, toxic *Microcystis* blooms have been reported in Europe, Asia, Africa, Australia, and North and South America (Miller et al., 2010), leaving Antarctica the only continent without reported affliction (O'Neil et al., 2011). *M. aeruginosa* was only recently discovered in Northern California, blooming for the first time in the brackish waters of the

San Francisco Bay estuary in 1999, and in the fresh waters of the Copco and Iron Gate reservoirs of the Klamath River in 2005. Since those years, *M. aeruginosa* has been making consistent, annual returns at levels considered to have moderate probability for adverse health effects to humans in recreational waters by the World Health Organization (Mosiander et al., 2009). With this recent spread to Northern California waters, considerations for the blame of this onset revolve around understanding nutrient interactions with *M. aeruginosa*, local environmental dynamics, and the influential factors associated with climate change

2.2 Toxicity of *Microcystis aeruginosa*

Toxic strains of *M. aeruginosa* release highly potent hepatotoxins, or toxins damaging to the liver, called *microcystins*, which can severely impact the health of aquatic wildlife. *M. aeruginosa* is a high producer of microcystins and is one of the most widespread freshwater hepatotoxic species in the world (Monchamp et al., 2014). Recurrent, microcystin-releasing blooms have been reported on every continent but Antarctica (Miller et. al, 2010; O'Neil et al., 2011), suggesting that the threat of toxic *M. aeruginosa* strains has extended globally. Over 65 structural variants of microcystins exist with various ranges in toxicity (Montagnolli et al., 2004), with microcystin-LR (MC-LR) as one of the most toxic and most commonly associated variants with toxic blooms (Fischer and Dietrich, 2000; Pouria et al., 1998). Other common variants of microcystins include microcystin-RR (MC-RR) (Gan et al., 2012), and microcystin-YR (MC-YR) (Srivastava et al., 2012). While the role of microcystins is still unknown, their impacts on other phytoplankton, invertebrates, and vertebrates—including marine mammals and humans—have been clearly observed. Though microcystins were once considered a freshwater public health issue from recreational and home use of water from contaminated lakes, rivers, and reservoirs, the growing incidences of *M. aeruginosa* in estuarine and marine habitats have created a widespread concern through expanding potential vectors for microcystin exposure to wildlife and humans, and ultimately degrading aquatic ecosystems and their ecosystem services.

2.2.1 Toxicity to Aquatic Invertebrates and Vertebrates

Large-scale, microcystin-producing blooms of *M. aeruginosa* are common along the Pacific Coast of the United States each year during the summer and fall seasons, particularly in various lakes and rivers in the states of Washington, Oregon, and California (Miller et al., 2010). Microcystins released from these blooms have the potential to flow into the Pacific Ocean and contaminate the adjacent marine waters (Miller et al., 2010). As toxic strains of *M. aeruginosa* are consumed by zooplankton, microcystins begin

to biomagnify in their spread through higher trophic levels as they make their way through the food web. Bioaccumulation of microcystins has been observed in the tissues of both freshwater and saltwater invertebrates and vertebrates, including mussels, crustaceans, corals, and fish (Miller et al., 2010).

Aquatic invertebrates and vertebrates serve as significant carriers for microcystins. Bioaccumulation of microcystins in invertebrates was demonstrated in a laboratory study exposing bivalves (clams, oysters, mussels) and snails to microcystins via toxic *M. aeruginosa* presence in seawater tanks of low exposure, at 2,195 parts per billion (ppb) microcystins, and high exposure, at 10,600 ppb microcystins (Miller et al., 2010). One of the highest microcystin concentrations ever recorded from an *in situ* sample was at 2,900 ppb in the freshwaters of Pinto Lake, California, in 2009; the World Health Organization (WHO) limit for microcystin concentrations for drinking water is 1 ppb (Miller et al., 2010). After 96 hours of microcystin exposure, the seawater tanks were continuously flushed out with fresh seawater until the 21st day of the study.

Clams, oysters, and mussels demonstrated the highest microcystin bioaccumulation in their gastrointestinal tissues, reaching up to 1,324 ppb in the high exposure tank after one day of exposure (Miller et al., 2010). Clams contained 183 ppb microcystins in their gastrointestinal tissues up to 14 days after microcystin exposure, and mussels contained 30.5 ppb microcystins in their gastrointestinal tissues 21 days after exposure. Although microcystin concentrations in the bivalve tissues decreased after initial exposure, the presence of microcystins after 17 days of continuous flushing with fresh seawater demonstrates a slow depuration rate, or cleansing ability, of the bivalves (Millet et al., 2010).

Although aquatic invertebrates and vertebrates can be carriers of microcystins, microcystin intoxication has also expressed harmful impacts on their function, including the health of crustaceans, fish, and small aquatic turtles, or terrapin. Uptake of microcystins through consumption has demonstrated changes in the metabolism in microcrustaceans (Montagnolli et al., 2003); hepatopancreas cell death, kidney damage, and mortality (in acute doses) in carp and rainbow trout (Fischer and Dietrich et al., 2000); and mortality of terrapin (Nasri et al., 2008). Even species of bacteria, protozoa, and other phytoplankton, including diatoms and green algae, are susceptible to microcystin poisoning which inhibit their proliferation and can severely alter the structure of the food web, giving dominance to *M. aeruginosa* (Ginn et al., 2010; Montagnolli et al., 2003; Sedmak and Eleršek, 2006).

Most microcystins released by *M. aeruginosa* are particularly harmful to mammals because of their promotion of liver damage and tumors (Montagnolli et al., 2003). This toxic effect poses a serious

concern for marine mammals that feed on shellfish, crustaceans, and juvenile fish and absorb the accumulated toxins in their tissue (Miller et al., 2010; Mosiander et al., 2009).

2.2.2 Toxicity to Marine Mammals

Consumption of microcystins can be fatal to aquatic mammals, having been associated with liver failure in federally threatened southern sea otters along the Pacific Coast who rely on potentially affected mollusks and other invertebrates for food (Lehman et al., 2013; Miller et al., 2010). The first known case of microcystin intoxication and mortality of a marine mammal was reported in 1999 for a southern sea otter off the Pacific Coast in Monterey Bay, California. However, recognition for microcystin-induced fatalities in marine mammals did not begin until 2007, when eleven sea otters were found dead and dying from microcystin poisoning in North America's largest marine sanctuary in Monterey Bay (Miller et al., 2010). Furthermore, these deaths were not documented in published scientific literature until 2010 (Miller et al., 2010). Up until 2012, thirty-one cases of microcystin-induced fatalities for the southern sea otter were recorded along the California coast (USFWS, unpublished). Most of these cases were located in Monterey Bay, although Estero Bay and Big Sur along California's central coast also have confirmed cases of microcystis-induced mortalities in southern sea otters. Each of these cases of dead and dying sea otters was found to have hepatic lesions suggestive of acute liver failure (Miller et al., 2010). Postmortem examination of their bodies by the California Department of Fish and Game Water Pollution Control Laboratory confirmed the presence of microcystins in their damaged liver tissues, with concentrations ranging between 1.36 to 348 ppb wet weight for the first twenty-one mortalities reported between 1999-2008 (Miller et al., 2010). Their livers demonstrated similar damage consistent with microcystin-positive livers in humans and animals of other reports, including necrosis and hemorrhaging (Davis et al., 2009; Miller et al., 2010; Montagnolli et al., 2003; Nasri et al., 2008; Yoshida et al., 2007).

Carcasses of southern sea otters from microcystin poisoning were found in clusters around areas where microcystin-contaminated plumes could have been discharged from freshwater systems, including river mouths, coastal ponds, harbors, and embayments (Miller et al., 2010). Additionally, the locations of sea otter carcasses along the northern central coast of Monterey Bay to the southern central coast of Estero Bay suggests that multiple point-sources of microcystin exposure to marine habitats exist along California's coast. This distribution further supports that many of the *M. aeruginosa* communities and their resultant microcystins present in the brackish waters of California's coast originate from discharged contaminated freshwater outflows, and is indicative of the potential freshwater origins of *M. aeruginosa*

found in other brackish and marine ecosystems around the globe, including the Swan River estuary in Australia, the Patos Lagoon estuary in southern Brazil, and the Baltic Sea in Europe (Belyk et al., 2012; Montagnolli et al., 2003; Robson and Hamilton, 2003).

Microcystins are emerging as a serious health threat to sea otters with the growing trend of microcystin-related poisonings since 2007. It is believed that a combination of trophic transfer through preying on microcystin-positive marine invertebrates feeding on contaminated organisms of lower trophic levels, and the flushing of microcystin-contaminated freshwater into the estuarine waters of the California coast, are the two principal routes of exposure for these sea otters (Miller et al., 2010). The injury and death of sea otters from microcystin poisoning along the California Coast is indicative of similar harm that can happen to other marine mammals as well as humans, as seafood sources are shared from the same region and amongst the same invertebrate species that demonstrated slow depuration of microcystins after absorption. Additionally, human utilization of the same coastal habitat in Monterey Bay is also common for direct-contact water recreation, fishing, and tourism, expanding their risk of harmful exposure (Millet et al., 2010).

2.2.3 Toxicity to Humans

Humans are also at risk of microcystin poisoning from consumption of microcystin-contaminated shellfish and fish, and from direct contact with consumption of microcystin-contaminated waters. Due to the history of *M. aeruginosa* in freshwater habitats, most studies on the effects of microcystins on humans are associated with exposure from recreational use in freshwater habitats or drinking contaminated water, since large populations utilize freshwater and microcystins tend to be in high concentrations in their more stagnant waters (Backer et al., 2009). Human outbreaks of microcystin-related illness have been sporadic and less clear than impacts on animals, as many studies are conducted in laboratories (Backer et al., 2009). However, acute exposure to microcystins has resulted in severe injury and deaths from massive hepatic hemorrhages, liver failure, and neurological disruptions in humans (Davis et al., 2009; Nasri et al., 2008; Pouria et al., 1998; Yoshida et al., 2007).

The first reported and probably most infamous case of fatal microcystin poisoning in humans happened in Carauru, Brazil, in 1996 (Pouria et al., 1998). A haemodialysis unit used inadequately treated water from a local reservoir containing toxic cyanobacteria blooms to treat 126 patients. All 126 patients experienced varying degrees of signs and symptoms consistent with acute neurotoxicity and subacute hepatotoxity, 60 of which resulted in death (Pouria et al., 1998). Although the species of cyanobacteria

was not identified, it was later confirmed that toxins in the tainted water used for dialysis were the same microcystin variants that can be produced by *M. aeruginosa*, which was one of the genera of cyanobacteria commonly known to inhabit the local reservoir from which the contaminated water was extracted (Pouria et al., 1998). In addition, histological examinations of affected livers were found to have characteristics similar to damage observed in animals poisoned by microcystins. Almost immediately after exposure to the contaminated water, neurological symptoms became evident, consisting of vertigo, mild deafness, visual disruptions and blindness, and grand mal convulsions. In addition, massive swelling of the liver, upper abdominal pain, and gastrointestinal bleeding occurred (Pouria et al., 1998).

Though the microcystin poisoning event in Carauru was extreme, it does not necessarily reflect the degree of harm for common human exposure to microcystins. Exposure through recreational activities in afflicted waters has resulted in far less severe symptoms, though they include skin irritation and temporary respiratory problems from aerosolized microcystins (Backer et al., 2009). Healthy individuals likely will suffer no adverse effects from periodic, acute exposure to microcystins through recreational activities (Backer, et al., 2009). Consumption of microcystins, however, through contaminated seafood or accidental ingestion during recreational activities, may lead to more severe hepatotoxic or neurological concerns. There is no specific treatment for microcystin poisoning in humans, and dialysis on animals afflicted with microcystin poisoning has shown to be ineffective (Pouria et al., 1998). Thus, exposure to microcystins still remains a relevant public health concern (Backer et al., 2009).

2.3 Population Dynamics and Physiology

The population dynamics of *M. aeruginosa* and its physiology are still much of a mystery that is being explored, although nutrient and climatic environmental factors likely play a large role in influencing growth, toxicity, and distribution (explored further in **Chapter 3 - Nutrient Influences** and **Chapter 4 - Environmental Influences**). Additionally, it is the mechanisms controlling the physiology of *M. aeruginosa* that often lead to its dominance in aquatic systems. As discussed in Section **2.2: Toxicity of *Microcystis aeruginosa***, *M. aeruginosa* populations can be comprised of numerous genotypes consisting of both toxic and non-toxic strains (Davis et al., 2009; Ma et al., 2013; Srivastava et al., 2012; Yoshida et al., 2007). Toxic strains are believed to contain clusters of microcystin synthetase genes encoded in their DNA, such as *mcyA* or *mcyB*, which allows for the production of microcystins (Ginn et al., 2010; Yoshida et al., 2007). While toxic and non-toxic strains can coexist in dense blooms, how they interact with components of their environment may shift dominance of certain strains over others, thereby

potentially shifting the toxicity of their blooms (explored in **Chapter 3 - Nutrient Influences**) (Davis et al., 2009; Yoshida et al., 2007). Further, *M. aeruginosa* can exist in colonial or non-colonial populations, which may be associated with or influence the release of microcystins (Ma et al., 2014). Both the colonial and non-colonial populations of *M. aeruginosa* can vertically migrate in water columns, thereby giving *M. aeruginosa* a competitive advantage over other phytoplankton for the acclimation of nutrients and sunlight (Brooks and Ganf, 2001). Thus, *M. aeruginosa* is a resilient species with a diverse set of functions that enables its injurious dominance in aquatic ecosystems.

2.3.1 Colony Formation

Much is unknown about what drives the existence of *M. aeruginosa* into colonial populations or populations of non-colonial, single-cells. Generally, it has been observed that *M. aeruginosa* exists as single or paired cells under laboratory conditions, while it takes on the colonial form under conditions of the natural environment (Gan et al., 2012; Ma et al., 2014). Thus, it has been hypothesized that local environmental conditions stimulate colony formation, perhaps as a defense mechanism, which are not incorporated into the controlled conditions of cultured laboratory studies.

Colonial populations are the cause of dense blooms that can create adverse eutrophic and toxic conditions. Larger colonies have been observed to be associated with more genotypes of toxic *Microcystis* species, including toxic strains of *M. aeruginosa*, while smaller colonies are associated with more non-toxic genotypes (Gan et al., 2012). Thus, the presence of microcystins may be an integral factor in the formation of large colonies. A 2012 study found that exposing *Microcystis* to two common microcystin variants, MC-LR and MC-RR, at environmentally relevant concentrations often found in freshwater systems (0.25–10 μg l^{-1}), increased the size of *Microcystis spp.* colonies up to 2.7 times after six days of exposure (Gan et al., 2012). Reduction of microcystin exposure consequently decreased colony size. This finding supports a previous study that found exposure of MC-LR, MC-RR, and MC-YR at very high concentrations (500 μg l^{-1}) not only significantly increased colony size of *M. aeruginosa*, but that even non-colonial strains of *M. aeruginosa* began to form colonies with high microcystin exposure (Sedmak and Eleršek, 2006). In addition, a study by Beversdorf et al. (2013) also observed high concentrations of microcystins when *M. aeruginosa* reached abundance in a eutrophic lake in Wisconsin during the growing season.

However, dense colonies of *M. aeruginosa* do not always indicate a high concentration of microcystins. Cells of *M. aeruginosa* have been observed to retain microcystins within their structures until

senescence or external stressors rupture the cell membranes, thereby causing the release of microcystins into the water column. Concentrations of microcystins released by *M. aeruginosa* have increased by up to 90% upon exposure to environmental stressors (explored further in **Chapter 4 - Environmental Influences**) (Ross et al., 2006). Nonetheless, *M. aeruginosa* is a high microcystin producer, where it has been observed to release high concentrations of microcystins relative to biomass compared with other cyanobacteria (Monchamp et al., 2014). These findings of colony size growth and toxin release upon stress suggest the potential protective role of microcystins to *M. aeruginosa* from external threats. This protective role is further supported by the positive correlation of mucilage in the presence of microcystins (Gan et al., 2012). Although the role of microcystins is still largely unknown, evidence suggests that while microcystins may be toxic to other aquatic life forms throughout the food web, they are intimately involved in strengthening and protecting microcystin producers, including *M. aeruginosa* (Gan et al., 2012; Ginn et al., 2010).

Mucilage is associated with colonial populations of *M. aeruginosa*, which forms defensive sheaths that protect against zooplankton grazing and viral and bacterial attacks (Ma et al., 2014). Studies have shown that the release of mucilage can be a reactionary mechanism to the presence of heterotrophic bacteria and flagellate grazing, in which extensive buildups of mucilage have been observed on cells of colonial *Microcystis* species (Ma et al., 2014). It is this mucilage that may also help *M. aeruginosa* populations aggregate. Aggregation of *M. aeruginosa* also increases its floating velocity, allowing dense colonies to float to the surface and shade out other phytoplankton from essential sunlight, giving it a competitive advantage over other phytoplankton genera and enabling its dominance in aquatic ecosystems (Lehman et al., 2013; Ma et al., 2014). This floating mechanism is due to intracellular gas vesicles in *M. aeruginosa* cells that allow it to control vertical migration in water columns depending on light and nutrient conditions (Brooks and Ganf, 2001; Miller et al., 2010; Ross et al., 2006). Colonial populations are typically surface dwellers, while single *M. aeruginosa* cells have been observed to be uniformly distributed vertically in water columns (Ma et al, 2014). Nevertheless, both forms of *M. aeruginosa* are able to control their buoyancies for nutrient and sunlight acclimation.

2.3.2 Buoyancy

The ability for *M. aeruginosa* to sink to lower depths of the water column and float to the surface demonstrates a competitive advantage over other phytoplankton for the acquisition of essential resources. During thermal stratification, available nutrients in N and P sink to the hypolimnion of an aquatic ecosystem, or to the coldest, bottom layer. As a result, surface waters become depleted of

available nutrients. *M. aeruginosa* is capable of exploiting thermally stratified conditions and scavenging for separated nutrients in the stratified layers of the water column, as it is equipped with gas vesicles that can fill up or deflate depending on light and nutrient availability. Upon conditions of nutrient limitation in N and P, gas vesicles of *M. aeruginosa* tend to lose volume, and subsequently the cells begin to migrate deeper into the water column where more nutrients have accumulated (Brooks and Ganf, 2001). Conversely, an inverse relationship has been observed with sunlight; under conditions of limited solar irradiance, gas vesicles tend to increase in volume and cells of *M. aeruginosa* begin to rise to the surface as they regain buoyancy (Brooks and Ganf, 2001). Thus, buoyancy is promoted under nutrient-rich and light-limiting conditions, allowing *M. aeruginosa* to float to the surface and shade out other phytoplankton for available sunlight.

2.3.3 Nitrogen Fixation

Most cyanobacteria are diazotrophic, or capable of N fixation (N_2-fixation). Accordingly, diazotrophic cyanobacteria have a competitive advantage over other phytoplankton during N limitation. Under limiting N conditions, diazotrophic cyanobacteria can fix N from the atmosphere and convert it into bioavailable forms of dissolved inorganic N (DIN) for sustaining their populations. Consequently, diazotrophic cyanobacteria can dominate an aquatic ecosystem under N-limited conditions while the non-diazotrophs are outcompeted, as they have access to an unlimited supply of N from the atmosphere.

M. aeruginosa often dominates aquatic ecosystems in the form of toxic blooms, which has been observed particularly under N-limited conditions (Dolman et al., 2012; Fujimoto et al., 1997; Lehman et al., 2013; Monchamp et al., 2014; Paerl et al., 2011, 2012; Schindler et al., 2008; Smith, 1983). However, unlike most cyanobacteria, *M. aeruginosa* is also non-diazotrophic, and it therefore cannot acquire N from the atmosphere when N concentrations are deficient in the water column. Thus, there is much to be understood about *M. aeruginosa* and its interaction with nutrients that enables it to thrive under N deficiency (explored further in **Chapter 3 - Nutrient Influences**).

Several studies have supported theories that non-diazotrophs can use diazotrophic cyanobacteria as sufficient sources of N to promote and sustain blooms during N-deficient conditions (Anderson et al., 2008; Mulholland et al., 2006). Like other phytoplankton, *M. aeruginosa* can coexist with and be just as abundant as N_2-fixing cyanobacteria during N-deficiencies (Smith, 1983; Paerl et al., 2012), where it can potentially thrive off of N introduced to the water column through N_2-fixation (Beversdorf et al., 2013).

A recent study in a eutrophic lake, Lake Mendota, of Wisconsin, examined the relationship between *M. aeruginosa* and the diazotrphic cyanobacteria, *Aphanizomenon*, relative to total dissolved phosphorus (P) and total dissolved N concentrations (Beversdorf et al., 2013). Large N_2-fixation events were led by *Aphanizomenon*. Days where N_2-fixation was high, *Aphanizomenon* was most abundant, and initial N availability in the water column was considered low, near an N:P ratio of 7:2 by weight (discussion N:P ratios is presented in **Chapter 3 - Nutrient Influences**). After the N_2-fixation events, *Aphanizomenon* declined and *M. aeruginosa* reached maximum abundance. Following weeks without N_2-fixation events, N:P ratios reached as high as 100:1 (Beversdorf et al., 2013), which suggests N_2-fixation may be able to replenish an N-deficient water system with enough bioavailable N to support non-diazotrophic cyanobacteria like *M. aeruginosa*.

The theory behind N_2-fixation providing enough bioavailable N to sustain non-diazotrophic phytoplankton has its holes. Beversdorf et al. (2013) also observed three N_2-fixing events where initial N:P ratios were high, at >30:1 by weight, providing a more ambiguous relationship between non-diazotrophs utilizing N inputs from diazotrophs. Additionally, a separate, 15-year study examining the relationship between *Aphanizomenon* and *M. aeruginosa* as part of the North Temperate Lakes Long Term Ecological Research Program observed that *M. aeruginosa* was more closely associated with low N:P ratios, while *Aphanizomenon* was more associated with higher N:P ratios (Beversdorf et al., 2013), suggesting that *M. aeruginosa* can thrive without diazotrophic aid during N-limitation.

The role of N_2-fixation in the production of sufficient, "new" N inputs in aquatic ecosystems has its skeptics (Paerl et al., 2011; Thad and McCarthys, 2010). Diverse studies have observed that N_2-fixation meets far less than 50% of an ecosystem's N demands (Paerl et al., 2011). Rather, it has been proposed that natural N-cycling and subsequent N regeneration in water columns takes precedence over N_2-fixation in sustaining *M. aeruginosa* when N concentrations in bioavailable forms are low (Monchamp et al., 2014; Paerl et al., 2012). Nevertheless, the coexistence of the non-diazotrophic *M. aeruginosa* with diazotrophic cyanobacteria, despite evidence that each should have different nutrient requirements in the water column, suggests a potentially significant relationship *M. aeruginosa* shares with N and diazotrophs. Further studies should be conducted to explore the role of diazotrophs in supporting *M. aeruginosa* during N-deficient conditions.

2.4 Summary

M. aeruginosa blooms are increasing with frequency and intensity on a global scale, showing patterns of expansion in aquatic ecosystems on every continent except for Antarctica. These blooms carry a severe degradation potential, inflicting freshwater, brackish, and marine ecosystems with a lethal combination of anoxic conditions and the release of toxic hepatotoxins known as microcystins that can injure biota in all trophic levels. Research has observed several strategies *M. aeruginosa* has developed to maintain dominance in aquatic ecosystems, giving them a distinct advantage over other phytoplankton. These strategies include dense colony formation and supplemental microcystin release to potentially protect against predators from grazing, vertical migration to hunt for nutrients less accessible to other phytoplankton under nutrient deficient conditions, and potentially utilizing N from diazotrophs during N-deficiencies.

Whether or notN_2-fixation is involved in the mechanism *M. aeruginosa* uses to obtain N, research is indicating that *M. aeruginosa* can dominate aquatic ecosystems under N-limited conditions. While the strategies in colony formation and vertical migration that *M. aeruginosa* has developed may aid in sustaining itself under nutrient stress, **Chapter 3 - Nutrient Influences** explores in depth the specific influences that the principal nutrients in ecosystem productivity, N and P, have on the growth, toxicity, and distribution of *M. aeruginosa.*

Chapter 3 - Nutrient Influences

Nutrients are fundamental in shaping and sustaining phytoplankton communities. Nitrogen (N) and phosphorus (P), and their respective chemical forms, are two of the most influential nutrients in sustaining primary production in aquatic ecosystems. Availability of N and P influences phytoplankton growth, biomass, and species composition (Xu et al., 2010). Seasonal shifts of phytoplankton communities from diatoms to cyanobacteria and dinoflagellates and vice versa have been consistent with shifts in N and P availability in aquatic ecosystems (McCarthy et al, 2009; Paerl et al, 2011). Depending on regulations managing the N and P inputs of nutrient sources, management of rapidly increasing HABs—and, more specifically, *M. aeruginosa*—may be possible through manipulating N and P loads to favor non-harmful phytoplankton growth. N and P have different roles in influencing the presence of *M. aeruginosa*. Therefore, how the availability of N and P influences the growth, distribution, and toxicity of *M. aeruginosa* must be understood first to effectively manage its blooms.

Nutrient influences on *M. aeruginosa* are not simply a matter of N and P availability in aquatic ecosystems. The *forms* of N and P also play a role in phytoplankton community structure, and hence the presence of *M. aeruginosa* in those communities. Thus, the individual role of N and P on the growth, distribution, and toxicity of *M. aeruginosa* must be understood before examining the influence of both nutrients together. Additionally, theories in ecological stoichiometry propose that the influence of N and P availability on phytoplankton communities may go beyond the issue of respective N and P concentrations in the water column. Rather, it has been suggested that N:P ratios have a vital role in controlling the presence of *M. aeruginosa*.

3.1 Role of Nitrogen

Research is showing that cyanobacteria typically dominate aquatic ecosystems in N-limited conditions, or in conditions where availability of N is too insufficient to satisfy phytoplankton nutritional needs, thereby restricting or limiting their growth and ability to sustain themselves. In many cases, cyanobacteria dominance during N-limitation can be explained by their N_2-fixing capabilities, an advantage other phytoplankton are not equipped with, which can theoretically offset the N-deficiencies in the water column and satisfy their nutritional needs (Beversdorf et al., 2013). However, many non-diazotrophic cyanobacteria species, like *M. aeruginosa*, also tend to dominate the phytoplankton community during periods of N-limitation (Dolman et al., 2012; Fujimoto et al., 1997; Lehman et al., 2013; Monchamp et al., 2014; Paerl et al., 2011; Schindler et al., 2008; Smith, 1983). For example, Paerl

et al. (2011) observed the dominance of *Microcystis* species during the summer and fall bloom periods in China's third largest freshwater lake, Lake Taihu, for two years in a row in 2008 and 2009, despite periods of chronic N-limitation. More recently, Lehman et al. (2013) observed peak *Microcystis* abundance in both fresh and brackish waters of the San Francisco Bay estuary occurring at N concentrations considered low for the estuary (0.442-1.77 mg L^{-1} nitrate (NO_{3-}) and 0.0129-0.103 mg L^{-1} ammonium (NH_4+)). This pattern of *M. aeruginosa* dominance during N-limitation suggests a superior ability for it and other non-diazotrophic cyanobacteria to compete for N over other phytoplankton despite the type of aquatic ecosystem (Fujimoto et al., 1997; Paerl et al., 2011; Smith, 1983). Furthermore, Marinho et al. (2007) observed growth trends that demonstrate *M. aeruginosa* can produce more biomass per unit of N than diatoms, thereby rendering it easier to dominate an ecosystem over other phytoplankton that require more N to achieve the same level of biomass. However, it is important to consider N beyond a single unit in controlling phytoplankton communities; the chemical forms of N available as nutrients can play different roles in influencing the growth of *M. aeruginosa*, and it would be a negligent oversight to simplify them as a single factor to manage.

3.1.1 Bioavailable Chemical Forms to *Microcystis aeruginosa*

The chemical forms of N are just as crucial in controlling phytoplankton communities as the availability of the nutrient itself (McCarthy et al., 2009; Monchamp et al., 2014). Part of what makes understanding the influence of nutrients on *M. aeruginosa* difficult is the inconsistent results studies are publishing on their role in stimulating its growth, toxicity, and distribution. While research has supported that *M. aeruginosa* and other cyanobacteria typically demonstrate biomass dominance in phytoplankton communities under N-limitation, other studies are finding an increase in N creates favorable conditions for *M. aeruginosa* and other cyanobacterial HABs (Davis et al., 2009; Ma et al., 2014; McCarthy et al., 2009; Mosiander et al., 2009). For example, Ma et al. (2014) observed that N additions, from as low as 4 times (6.20 mg L^{-1}) to as high as 32 times (49.6 mg L^{-1}) that of ambient concentrations, to samples of phytoplankton communities from Lake Taihu, China, increased the total proportion of *Microcystis* biomass relative to the phytoplankton communities which they comprised. Alternatively, N additions 2 times and below the ambient N concentrations had no significant effect on *Microcystis* proportion within the phytoplankton communities. These inconsistencies can be due to multiple factors, including strain type and the supplementary influence of local environmental forces (explored further in **Chapter 4 - Environmental Influences**). However, some chemical forms of N are more available to *M. aeruginosa*

than others, which can significantly influence its growth response and potentially determine whether *M. aeruginosa* will dominate or be outcompeted by other phytoplankton.

M. aeruginosa is an effective competitor for inorganic, reduced forms of N, showing a particular affinity for ammonium (NH_4+) (Chaffin et al., 2011; Lehman et al., 2013; X. Liu et al., 2011; Mosiander et al., 2009; McCarthy et al., 2009; Paerl et al., 2011). This is because ammonium is more bioavailable to *M. aeruginosa* and other cyanobacteria than the oxidized forms of N, nitrate (NO_3-) and nitrite (NO_2-), because less energy is required to assimilate it (Dugdale et al., 2007; Paerl et al., 2011). Thus, an increase in phytoplankton biomass, despite low N concentrations in the water, can be explained by the availability of various forms of N in the water and which of these forms species of phytoplankton are effective competitors for. Typically, it is the non-diazotrophic cyanobacteria like *M. aeruginosa* that prefer ammonium, while diatoms prefer nitrate (X. Liu et al., 2011; McCarthy et al., 2009; Monchamp et al., 2014). Cyanobacteria, including *M. aeruginosa*, has widely been observed to have positive growth trends with increasing NH_4+:NO_x ratios in eutrophic, freshwater lakes around the world, including in the United States, Canada, and China (Donald et al., 2013; X. Liu et al., 2011; McCarthy et al., 2009). Increasing NH_4+:NO_x ratios indicate that the proportion of ammonium is larger than the proportion of nitrate and nitrite, thereby providing *M. aeruginosa* with the appropriate form of N to outcompete other phytoplankton. Thus, low availability of nitrate coupled with a sufficient supply of ammonium typically creates favorable conditions for *M. aeruginosa* (X. Liu et al., 2011). Collo and Harrison (2014) observed ammonium concentrations of approximately 45 mg L^{-1} (2500 µM) to optimally support *M. aeruginosa* and other cyanobacterial growth. However, as a superior N-competitor in general, ammonium concentrations as low as 0.009 mg L^{-1} have been sufficient enough to support *Microcystis* blooms in the freshwater Steilacoom Lake in Washington (Lehman et al., 2013).

Despite the form of N being critical in influencing phytoplankton composition, many studies observing the role of N in assembling phytoplankton communities often only use nitrate as the source of N, which could under or overestimate the degree of N limitation for species under observation (X. Liu et al., 2011). A high concentration of N could yield different results in phytoplankton growth responses, depending on the amount of oxidized N (NO_x) or ammonium available in that concentration. For example, McCarthy et al. (2009) analyzed the influence of nutrient availabilities on phytoplankton community structure using ten and four-year nutrient datasets for two shallow, subtropical, eutrophic lakes: Lake Okeechobee, of the United States (Florida), and Lake Taihu, of China, respectively. Results demonstrated a trend that the cyanobacteria proportion of the phytoplankton community increased

with increasing total N-to-total P (TN:TP) ratios in Lake Okeechobee, while the cyanobacteria proportion decreased with increasing TN:TP ratios in Lake Taihu (further discussion on nutrient ratios is provided in Section **3.3: Nitrogen-to-Phosphorus Ratios**). *Microcystis* species were only present in, and dominated, the phytoplankton in Lake Taihu at a range from 68-99% of the entire community. Dominance of *Microcystis* species occurred as TN:TP ratios began to decrease, which is consistent with research trends demonstrating *M. aeruginosa* dominance under N-limitation (Dolman et al., 2012; Fujimoto et al., 1997; Lehman et al., 2013; Monchamp et al., 2014; Paerl et al., 2011 2012; Schindler et al., 2008; Smith, 1983). Although the species composition of the cyanobacteria in both lakes were not the same (*Microcystis* species were either not present or negligible in abundance in Lake Okeechobee), McCarthy et al. (2009) acknowledge that the chemical forms of N available in the total N concentrations of both lakes could explain why non-diazotrophic cyanobacteria showed opposite growth responses to increasing TN:TP ratios between the lakes. This could potentially explain why the current realm of scientific literature is publishing different conclusions on the influence of nutrient availabilities on *M. aeruginosa* growth and distribution, particularly for N.

Although *M. aeruginosa* is a better competitor for ammonium over nitrate, it does not necessarily signify a negative response pattern to direct nitrate inputs (Davis et al., 2009; Yoshida et al., 2007). Typically, as the proportion of nitrate increases as part of the total N concentration in an aquatic ecosystem, the species composition of phytoplankton communities shifts from non-diazotrophic cyanobacteria, like *M. aeruginosa*, to other phytoplankton, like diatoms, as a result of the low assimilation rate *M. aeruginosa* has for nitrate (McCarthy et al., 2009). The low assimilation rate for nitrate in *M. aeruginosa* allows phytoplankton with a higher assimilation rate to outcompete *M. aeruginosa* and dominate a phytoplankton community (X. Liu et al., 2011). However, if fed nitrate directly without any competition from other phytoplankton, particularly under N-limited conditions, *M. aeruginosa* has shown significant, positive response patterns demonstrating rapid growth and subsequent biomass abundance (Davis et al., 2009; Ma et al., 2014; Yoshida et al., 2007). Furthermore, while there is generally a positive correlation between N inputs and growth rate, toxic and non-toxic strains of *M. aeruginosa* have expressed different response patterns to inputs of nitrate and other N forms.

3.1.2 Role of Nitrogen on Toxic and Non-Toxic Strains

As discussed in **Chapter 2 - Ecology and Toxicity of *Microcystis aeruginosa*,** toxic and non-toxic strains of *M. aeruginosa* can coexist in the same phytoplankton community (Davis et al., 2009; Ma et al., 2013;

Srivastava et al., 2012; Yoshida et al., 2007). While *M. aeruginosa* typically responds positively to direct N inputs, its toxic and non-toxic strains may have different response patterns to N.

Although *M. aeruginosa* has shown to have a low assimilation rate for nitrate, its toxic strains may be superior competitors for nitrate and other forms of N than its non-toxic counterparts. Yoshida et al. (2007) conducted a study measuring the size of the potentially toxic *M. aeruginosa* populations carrying the microcystin synthetase gene, *mcyA*, relative to total *M. aeruginosa* populations without the synthetase gene during periods of rapid nitrate loadings in the freshwater lake, Lake Mikata, of Japan, from July 2004 to October 2005. Strains of *M. aeruginosa* with the *mcyA* gene responded the most to rapid increases of nitrate during the summer growing seasons. The *mcyA* strains had the fastest growing rate of the strains of *M. aeruginosa*, rapidly increasing its abundance from 0.5% to 6% of the total *M. aeruginosa* populations during accelerated nitrate loadings from 0.00177-2.21 mg L^{-1} in September 2004, and from 8.5% to 35% with accelerated nitrate loadings from 0.0265-0.522 mg L^{-1} in August 2005. No significant correlation was observed between *M. aeruginosa* and temperature, although warmer temperatures combined with higher nitrate levels seemed to generate higher bursts of abundance in general (the role of temperature is discussed further in **Chapter 4 - Environmental Influences**).

Other studies outside of the field have suggested that nitrate uptake is positively correlated with microcystin production. Several lab studies have also shown consistent results that increases in nitrate loads not only increases the growth rate and toxicity of toxic *M. aeruginosa*, but that higher nitrate concentrations favor toxic strains over non-toxic strains (Beversdorf et al., 2013; Davis et al., 2009). Davis et al. (2009) conducted a study in Lake Champlain, a freshwater lake bordering northern Vermont, New York, and Quebec, subjecting *M. aeruginosa* samples to experimental nutrient manipulations in order to examine the dynamics between toxic strains, this time carrying the *mcyD* microcystin synthetase gene, and non-toxic strains during various nutrient conditions. Results demonstrated that direct treatments of 1.24mg L^{-1} (20µM) nitrate significantly increased the growth rate of the *mcyD* strains over non-toxic strains by 40%.

Whether observed *in situ* or in lab culture experiments, toxicity of *M. aeruginosa* has been positively correlated with an increase in nitrate concentrations in many studies (Lee et al., 2000). Although nitrate inputs have demonstrated positive growth responses showing particular favor toward toxic strains of *M. aeruginosa*, it is important to remember that many studies default to nitrate as their only source of N for nutrient experimentation or observation. Thus, nitrate may not be the most influential chemical form in influencing growth of toxic strains, particularly in the field with other phytoplankton competitors

(X. Liu et al., 2011). N inputs with ammonium have also shown similar favor to toxic strains. Ha et al. (2009) observed *M. aeruginosa* populations comprising toxic, *mcyA*-carrying strains, and non-toxic strains, in the freshwaters of Hirosawa-no-Ike pond of Kyoto City, Japan. Mixed concentrations of nitrate, nitrite, and ammonium enhanced overall microcystin concentrations by promoting the growth of toxic *M. aeruginosa* strains carrying the *mcyA* gene over non-toxic strains. Thus, it is perhaps that toxic strains of *M. aeruginosa* outcompete non-toxic strains for N in general, despite the chemical forms available. Microcystin content of *M. aeruginosa* blooms has been reported to be up to two-to-three times higher while growing under non-N-limited conditions than under conditions of N-limitation without regard to specific N form (Lee et al., 2000).

Many studies observing the positive correlation of N on toxic strains of *M. aeruginosa* do not acknowledge how different chemical forms of N may impact the formation of toxic blooms in aquatic ecosystems. Couple studies observing the relationship between N inputs and toxic strains with studies including competitive diatoms, dinoflagellates, and other phytoplankton found in the field, and the probability of dominance of toxic *M. aeruginosa* strains becomes more ambiguous. Nevertheless, research is strongly supporting that rapid and high N loadings play a significant role in favoring the growth of toxic strains within the *M. aeruginosa* communities.

While *M. aeruginosa* typically grows slower under nitrate due to its low assimilation rate, sufficient amounts of nitrate may render an aquatic ecosystem N-sufficient, thereby providing *M. aeruginosa* with enough nitrate to sustain itself without being completely outcompeted by more effective nitrate assimilators in the absence of ammonium. Additionally, N-limited *M. aeruginosa* may respond more positively to nitrate and other N forms than in N-sufficient conditions. For example, Mosiander et al. (2009) observed *M. aeruginosa* increased in abundance to both nitrate and ammonium inputs in the San Francisco Bay estuary and the Copco and Iron Gate Reservoirs in California during N-limited summers. In fact, the relative proportions of nitrate and ammonium in the water column may alter the degree of preference *M. aeruginosa* has for them.

3.1.3 Ammonium Inhibitory Effects

While ammonium is the preferred N source for *M. aeruginosa*, studies in and out of the field have observed that under N-limitation, starved *M. aeruginosa* responds positively to surges in nitrate levels by rapidly increasing its biomass (Davis et al., 2009; Lee et al., 2000; Yoshida et al., 2007). Thus raises the question of whether or not the physiological dependence on ammonium versus nitrate remains the

same under N-sufficient conditions as under N-deficient conditions (SFBRWQCB, unpublished). Yoshiyama and Sharp (2006) observed that phytoplankton growth in the nutrient rich Delaware Estuary does not follow a linear trend. Rather, biomass reached its peak at relatively low nutrient concentrations and decreased as nutrient concentrations became saturated.

This concept of High Nutrient Low Growth (HNLG) is not an isolated phenomenon. While it has been theorized that saturated nutrient levels can exceed the assimilative capacity of phytoplankton (Xie et al., 2002), rich concentrations in ammonium may enable an inhibiting factor in phytoplankton in the uptake of other nutrients (Dugdale et al., 2007; Parker et al., 2012). The San Francisco Bay estuary has shown a declining trend in primary productivity despite heavy ammonium loadings from wastewater treatment plant effluents (Parker et al., 2012). Ammonium concentrations are increasing in many aquatic ecosystems that serve as discharge points for wastewater effluent, as wastewater treatment plants (WTPs) that lack advanced secondary treatment of their effluents primarily discharge N in the form of ammonium (Parker et al., 2012). This is a particular issue in the San Francisco Bay, where 75% of its wastewater effluents are treated to the secondary level, rendering ammonium-rich discharge into the San Francisco Bay estuary.

Parker et al. (2012) observed that the daily discharge of 15 metric tons of primarily ammonium into the Sacramento River from the Sacramento Regional Wastewater Treatment Plant (SRWTP) led to a 60% decrease in primary production. In addition, it was observed that phytoplankton production consistently demonstrated a shift from nitrate uptake upstream of the SRWTP to solely ammonium uptake downstream. While the shift in uptake from nitrate to ammonium could be due to the preference some phytoplankton has for ammonium (Lehman et al., 2013), the uptake rates of ammonium also decreased as ammonium concentrations increased, inhibiting overall primary production. The results of Parker et al. (2012) support an existing hypothesis in scientific literature that elevated concentrations of ammonium not only inhibit the uptake of nitrate, but that higher concentrations of ammonium also suppress its own uptake, leading to an overall decrease in primary productivity.

Many cyanobacteria have been observed to use ammonium preferentially under N-limitation, yet ammonium concentrations have also demonstrated inhibitory or suppressive mechanisms of nitrate uptake and a consequential decrease in aquatic primary productivity in phytoplankton communities (Dugdale et al., 2007; Parker et al., 2012). Dugdale et al. (2007) observed that phytoplankton in the San Francisco Bay estuary do not rapidly uptake nitrate unless ammonium levels are within or below the range of 0.018-0.072 mg L^{-1} (1-4µM). Britto and Kronzucker (2002) refer to ammonium as a paradoxical

nutrient; one which is used preferentially in N-limited conditions, yet is considered a growth hindrance at saturating levels. Thus, high concentrations of ammonium may suppress the uptake of nitrate and ammonium during phytoplankton productivity. However, differences in the physiology of phytoplankton taxa render unique response patterns to ammonium that cannot be generalized across the entirety of phytoplankton communities. Collos and Harrison (2014) observed that *M. aeruginosa* and other cyanobacteria can grow optimally at ammonium concentrations of approximately 45 mg L^{-1} (2,500µM), while being able to tolerate concentrations as high as 234 mg L^{-1} (13,000µM) without showing inhibitory effects, far exceeding the growth and tolerance thresholds of four other classes of microalge: Prymnesiophyceae, Diatomophyceae, Raphidophyceae, and Dinophyceae. While the potential nitrate uptake-inhibiting effects ammonium has on *M. aeruginosa* have not been frequently studied, it is important to be aware of the potential role reversal of ammonium in inhibiting the growth of *M. aeruginosa* when ammonium concentrations are considered high for its physiological tolerance.

3.1.4 Role of Nitrogen Stress on Toxicity

Although there is evidence that toxic strains of *M. aeruginosa* outcompete non-toxic strains with rapid inputs of N during N-limited conditions, it may actually be a lack of N that contributes to more toxic populations of *M. aeruginosa*. Culture-based lab studies have demonstrated that short-term N stress can stimulate microcystin gene expression in *Microcystis* (Beversdorf et al., 2013).

The NtcA protein, also known as the global N regulator factor, is a key regulator for N metabolism and carbon (C) metabolism in cyanobacteria (Ginn et al., 2010; Kuniyoshi et al., 2011). This protein acts by binding to the DNA sequence of *M. aeruginosa* and other cyanobacteria to allow for the assimilation of N. NtcA is produced in the presence of ammonium, which is increased under N stress (Ginn et al., 2010). Regions in the DNA of *M. aeruginosa* containing sequences for the microcystin synthetase genes, *mcyA/D*, have been indentified to have potential binding sites by NtcA (Ginn et al., 2010). When NtcA binds to these microcystin promoter regions, microcystins are synthesized (Kuniyoshi et al., 2011).

2-oxoglutarate (2-OG), an enzyme in *M. aeruginosa* cells, has been observed to increase in concentration during N deficits (Kuniyoshi et al. 2011). Kuniyoshi et al. (2011) found that the binding of NtcA to the *mcyA* promoter region in *M. aeruginosa* DNA is enhanced in the presence of 2-OG. Thus, the accumulation of 2-OG acts as a signal for N-starvation, and subsequently activates the synthesis of microcystins.

The results of Kuniyoshi et al. (2011) support findings by Ginn et al. (2010), who observed that under periods of N stress, NtcA binding sites enable *ntcA* genes to activate in the DNA sequence of *M. aeruginosa*. *ntcA* genes transcribe the NtcA protein, which allow it to fulfill its function in N regulation. In this study, the transcription levels of *ntcA* and the microcystin synthetase gene, *mcyB*, were examined under N-excess, N-limited, and N-starved conditions. The transcription levels for *ntcA* and *mcyB* increased by 4.07 and 14.09 times, respectively, in N-limited cells compared to cells in N-excess conditions. For N-starved conditions, transcription levels for *ntcA* and *mcyB* increased by 2.36 and 9.70 times, respectively, indicating that more NtcA proteins are binding to microcystin promoter regions in *M. aeruginosa* DNA during N-stress.

These results strongly suggest that N plays a key role in the synthesis of microcystins in toxic strains of *M. aeruginosa*. The presence of ammonium assists in the production of NtcA proteins, which have demonstrated to facilitate and augment the synthesis of microcystins under conditions of N stress.

3.1.5 Role of Nitrogen on Colony Formation

Most studies on *Microcystis* have explored the role of N in growth and toxicity, but the role of N in colony formation has been far less explored. Ma et al. (2014) treated samples of phytoplankton communities including *Microcystis* from Lake Taihu, China, with various nutrient manipulations, and compared results against control samples. *M. aeruginosa* was among the main *Microcystis* species comprising the sample phytoplankton communities.

Additions of total N (TN) alone, ranging from concentrations of 3.1-49.6 mg L^{-1}, promoted comparatively large colonies, ranging from approximately 105-210µm in diameter and promoting overall *Microcystis* biomass (Ma et al., 2014). The lower concentrations of TN sustained larger-sized colonies, yet all colony sizes from TN treatments of any concentration were significantly larger than colonies formed by total P (TP) alone (the role of P in colony formation is explored further in Section **3.2.4: Role of Phosphorus in Colony Formation**). Interestingly, when TN was mixed with TP, only concentrations of N lower than 7.75-13.95 mg L^{-1} led to the formation of large *Microcystis* colonies (Ma et al., 2014). Concentrations higher than this range significantly decreased colony size and led to the formation and distribution of single cells rather than colonies. It has been described that under an inadequate nutrient supply, *Microcystis* cells synthesize more extracellular polysaccharides (EPS), which compose the protective sheaths associated with their colonies. Thus, conditions of N-limitation have demonstrated the formation of larger *Microcystis* colonies, and consequently denser *Microcystis* biomass, suggesting that

N saturation has the opposite effect. Additionally, in the absence of P, it appears *Microcystis* colonies can sustain large colonies at higher N concentrations, indicating a potential substitution of P for N when P is deficient.

As discussed in **Chapter 2 - Ecology and Toxicity of *Microcystis aeruginosa***, the formation of *Microcystis* colonies may serve as a defense mechanism under stressed conditions, which protect against grazing and bacterial and viral attacks. With some studies observing a positive correlation between N availability and buoyancy, colony formation of N-limited *Microcystis* cells also increases its buoyancy potential by combining its cells and their gas vesicles into a large mass, thereby increasing its total gas vesicle volume. Additionally, large, buoyant colonies shade out other phytoplankton while being able to store more nutrients (Ma et al., 2014), which could explain why dominance of *M. aeruginosa* is typically observed under conditions of lower N concentrations and overall N-limitation. N-limitation could create conditions stressful enough for dense colony formation, which consequently gives *M. aeruginosa* a competitive advantage over other phytoplankton.

3.1.6 Role of Nitrogen on Buoyancy

It was discussed in **Chapter 2 - Ecology and Toxicity of *Microcystis aeruginosa*** that conditions of nutrient limitation causes the gas vesicles in *M. aeruginosa* cells to lose volume, thereby decreasing their buoyancy. However, as with growth and toxicity, *M. aeruginosa* buoyancy responds differently to different levels of N and P. Brooks and Ganf (2001) subjected *M. aeruginosa* cultures to various treatments of nitrate. At 0 mg L^{-1} (0µM) nitrate, *M. aeruginosa* was severely N-limited and was unable to metabolize carbohydrates in its cells, resulting in a decrease in gas vesicle volume and a loss in buoyancy. At 0.310 mg L^{-1} (10 µM) nitrate, gas vesicles in *M. aeruginosa* cells began to increase with light exposure, but buoyancy could not be sustained due to continued insufficient metabolism of increased carbohydrates. At the highest dose of nitrate, 6.20 mg L^{-1} (100 µM), N was no longer considered limiting, and gas vesicle volumes increased further in *M. aeruginosa* cells with light exposure, and metabolism of carbohydrates was sufficient enough to provide and sustain buoyancy.

Buoyancy capability in *M. aeruginosa* has been positively correlated with nutrient availability in previous studies (Brooks and Ganf, 2001), but the concentrations of each nutrient necessary to provide and sustain buoyancy may differ. Brooks and Ganf (2001) demonstrated that as N concentrations approach N-sufficiency, the capability for buoyancy in *M. aeruginosa* cells increase. The role of P in *M. aeruginosa* buoyancy is discussed in Section **3.2.4: Role of Phosphorus on Buoyancy**.

3.2 The Role of Phosphorus

Phosphorus (P) has been widely implicated as having a dominant role in controlling freshwater primary production, eutrophication, and HAB formation, leading to tight regulation on P inputs from agricultural, urban, and industrial sources (Paerl et al., 2011). However, recent studies have observed that P reductions alone are not enough to stop eutrophication and the formation of HABs (McCarthy et al., 2009). This result can further be attested in the continued expansion of *M. aeruginosa* and other cyanobacterial HABs on the global scale (Miller et. al, 2010; O'Neil et al., 2011; Paerl and Otten, 2013), despite current efforts to control P loadings in aquatic ecosystems in some parts of the world (Paerl et al., 2011). Research is suggesting that N is far more influential in controlling the growth and functions of *M. aeruginosa* than P. Nonetheless, like N, *M. aeruginosa* utilizes P to sustain itself, although the roles of P in the growth, toxicity, and distribution of *M. aeruginosa* are not all similar.

3.2.1 Bioavailable Chemical Forms to *Microcystis aeruginosa*

Unlike N, P does not occur in numerous bioavailable forms in aquatic ecosystems (McCarthy et al., 2009). P is available to phytoplankton as phosphate (PO_4^{3-}), and for the sake of simplicity, will be referred to as "P" throughout this text.

M. aeruginosa has demonstrated a superior ability to compete for P, particularly during P-limited conditions in which it is able to utilize its vertical migration and buoyancy functions to forage for less accessible P in the lower depths of the water column and from sediment release. Further, evidence suggests that *M. aeruginosa* and other cyanobacteria can absorb and store P in external portions of its cells as polyphosphate granules, and can use this stored P to persist through periods of prolonged P deficiency—a competitive advantage other phytoplankton are not equipped with (Lewin et al., 2003; Otten et al., 2012; Paerl et al. 2012). However, this ability to use stored polyphosphate granules as a P source has been debated by Saxton et al., (2012), who found no shift in the ratio of P stored in the surface-intracellular interface of *M. aeruginosa* and P stored intracellularly, despite observing *M. aeruginosa* under a range of P concentrations. Nonetheless, *M. aeruginosa* demonstrated no significant change in growth rate under various P treatments, suggesting its ability to successfully respond to various P concentrations. This ability to adapt to various P concentrations, whether from its ability to use stored P or some other unexplored mechanism, in addition to the more influential role of N in its growth, toxicity, and distribution, may explain why *M. aeruginosa* and other cyanobacterial HABs are expanding despite current management efforts to control P loadings in aquatic ecosystems.

3.2.2 Role of Phosphorus on Toxic and Non-Toxic Strains

Although the presence of N appears to favor toxic strains of *M. aeruginosa* over non-toxic strains, the role of P in influencing the proportion of toxic and non-toxic strains seems much less significant, and much less defined. Recall from Section **3.1.2: Role of Nitrogen on Toxic and Non-Toxic Strains** that Yoshida et al. (2007) observed toxic, *mcyA*-carrying strains of *M. aeruginosa* responding to rapid nitrate loadings in Lake Mikata, Japan, by increasing in abundance by as much as 12 times, more than any non-toxic strain of *M. aeruginosa* in the study. Conversely, Yoshida et al. (2007) observed no correlation between P and the growth of toxic *M. aeruginosa* strains. Similarly, Ha et al. (2009) deemed P irrelevant in promoting the growth of toxic, *mcyA*-carrying *M. aeruginosa* strains over non-toxic strains in Hirosawa-no-Ike pond in Kyoto, Japan, while results from the same study observed N favoring the growth of toxic strains. Even more extreme, Beversdorf et al. (2013) acknowledges that P has been negatively correlated with microcystin production.

While P has a significant function in controlling freshwater primary production, this recognition and subsequent stringent regulation of P (Paerl et al., 2012) may have deemphasized its role in *M. aeruginosa* growth characteristics and emphasized the significance of N. Davis et al. (2009) found that 0.119 mg L^{-1} (1.25 µM) P yielded significantly higher growth rates up to double that of control samples for both toxic, *mcyD*-carrying *Microcystis* strains, and non-toxic strains sampled from Lake Ronkonkoma and Lake Champlain in the Northeast United States, both of which were P-limited at the time of sampling. Interestingly, the same P treatments on two P-rich Northeast U.S. lakes, Lake Agawam and Mill Pond, yielded no change in *Microcystis* growth (Davis et al., 2009). Thus, both toxic and non-toxic *Microcystis* strains appear to have similar growth response patterns to P when under P-limited conditions, suggesting that Lake Mikata of Yoshita et al. (2007) and Hirosawa-no-Ike pond of Ha et al. (2009) may not have been P-limited at the time of their studies. However, when P was coupled with enhanced temperatures greater than 25°C, the growth rates for toxic strains of *Microcystis* increased in Lake Champlain, Mill Pond, and Lake Agawam by 170%, 125%, and 20%, respectively, yielding the highest growth rate for any treatments including N (the role of temperature is discussed further in **Chapter 4 - Environmental Influences**).

Past studies have considered P as being the primary influence in increasing microcystin concentrations in aquatic ecosystems (Ha et al., 2009), and the findings of Davis et al. (2009) support that notion when coupled with warmer temperatures above 25°C. While other research is showing that P does not have

the same influential degree as N in favoring the growth of toxic *M. aeruginosa* strains over non-toxic strains, this could be due to various degrees of P-limitation and temperatures in different studies.

3.2.3 The Role of Phosphorus on Colony Formation

Ma et al. (2014) observed that P has a much less significant role in *Microcystis* colony formation than N. While TN was observed to promote and sustain large *Microcystis* colonies, all inputs of TP alone, from 0.164-2.624mg L^{-1}, led to a decrease in colony sizes, resulting in significantly smaller colony sizes than control colonies with no nutrient treatments and colonies subject to N treatments, at ranges from 35-105µm in diameter. The higher concentrations of TP promoted the smaller range of colony sizes. When mixed with TN, TP concentrations higher than 0.41-0.74mg L^{-1} promoted the growth and distribution of single cells, rendering the colonial form of *Microcystis* almost entirely absent.

These results suggest that the less limiting the nutrient availabilities of N and P, the smaller the colony sizes will be. However, when N and P are mixed, the threshold for which *Microcystis* transitions from colony formation to single cell formation is lowered. Since research has suggested that *Microcystis* forms colonies as a defense mechanism to stressed conditions, the lower threshold for transitioning from colony formation to single cell distribution in the presence of both N and P, as opposed to one nutrient over the other, indicates the potential for *Microcystis* to be less nutrient stressed when both nutrients are accessible. Thus, despite evidence supporting the more influential role of N in *Microcystis* growth, toxicity, and distribution, a high concentration of N and a low concentration of P may not fully satisfy the nutritional needs to persist without stress. A combination of N and P together creates optimal nutritional conditions for *M. aeruginosa* to thrive.

3.2.4 Role of Phosphorus on Buoyancy

As with N, P-limitation causes the gas vesicles in *M. aeruginosa* cells to lose volume, with the greater degree of limitation resulting in a greater loss in volume. Brooks and Ganf (2001) observed a similar trend in gas vesicle volumes with additions of P as they observed with N. At 0 mg L^{-1} (0µM) P, *M. aeruginosa* cells demonstrated decreased gas vesicle volume and an increase in carbohydrates. At 0.047 mg L^{-1} (0.5µM) P, both gas vesicle volume and carbohydrates increased, rendering no change in the proportion of cells that could float. The highest dose of P at 0.950 mg L^{-1} (10µM) showed a significant increase in gas vesicle volume and buoyancy, although carbohydrate accumulation continued to render some loss of buoyancy in cells. Like with N, all P treatments required light exposure to demonstrate buoyancy capabilities in the *M. aeruginosa* cells. Though Brooks and Ganf (2001) did not treat *M.*

aeruginosa cells beyond 0.950 mg L^{-1}, it is reasonable to assume that increased P inputs would have led to higher metabolism rates of accumulated carbohydrates, further increasing buoyancy capabilities.

3.3 Role of Combined Nitrogen and Phosphorus

It is important to note that concentrations of N and P included from the field studies in this text are being added relative to N and P already present in the water samples. Depending on how N or P-limited a water sample may be likely renders varying degrees of growth response when new N or P is added. However, examining the individual roles of each nutrient can indicate the potential roles they have in the abundance of *M. aeruginosa* when mixed in various proportions to one another. Various concentrations of N and P can shape phytoplankton communities, and it has commonly been observed that cyanobacteria like *M. aeruginosa* dominate when N concentrations become low enough to be deemed the limiting nutrient. Many times, the proportion of N and P in aquatic ecosystems are observed as N:P ratios, where aquatic ecosystems with low N:P ratios tend to favor dominance by *M. aeruginosa* and other cyanobacteria.

3.3.1 Role of N:P Ratios

Ecological stoichiometry or resource ratio theory suggests that different phytoplankton species dominate under different proportions of nutrients in an aquatic ecosystem. This concept is justified by the idea that organisms like phytoplankton internally allocate critical nutrients like N and P in different proportions depending on the physiological structures that form their biomass (Sterner and Elser, 2002). If the resource ratio theory is an accurate representation of all phytoplankton relationships with the environment, it can presumably predict ecological outcomes – and in the case of *M. aeruginosa* – which ratios would render its toxic blooms so they can be avoided through nutrient management.

The Redfield ratio is the atomic ratio of C:N:P that is often observed in marine seston (both living and non-living particulate matter). Coined after an oceanographer, Alfred Redfield, he noted that the atomic ratios of C:N:P in marine seston were consistently at 106:16:1 after extensive research analyzing marine biomass samples from numerous marine regions (Sterner and Elser, 2002). As focus on this research is on the nutrient influences of N and P, the atomic N:P ratio of 16 would theoretically be the optimum resource ratio for phytoplankton growth. However, despite much evidence that supports the Redfield ratio theory, not all phytoplankton have been observed to grow optimally at the Redfield ratio (Sterner and Elser, 2002).

N:P ratios can be expressed in two forms – by mass (mass:mass) or by moles (mole:mole), otherwise referred to as an *atomic* ratio. Thus, a mass ratio and an atomic ratio can have different numerical values. It is important to realize that N:P ratios do not describe the *amount* of nutrients in an aquatic ecosystem, but only measure the relative concentrations in proportion to one another. Two aquatic ecosystems can have the same ratios, whether expressed in mass or in moles, but one can be nutrient limited while the other can be nutrient rich. Thus, it should be recognized that low N:P ratios do not always refer to N-limited aquatic ecosystems, although this can also be the case.

3.3.2 Low N:P Ratios on Toxic and Non-Toxic Strains

Recall from Section **3.1.2: Role of Nitrogen on Toxic and Non-Toxic Strains** that studies observed that toxic strains of *Microcystis* favor N additions more than non-toxic strains, indicating a superior ability to compete for N. This difference would suggest that high concentrations of N would promote a larger proportion of toxic to non-toxic strains in a *Microcystis* community. Otten et al. (2012) observed that toxic strains of *Microcystis* carrying the *mcyE* gene in Lake Taihu, China, were significantly favored in *Microcystis* communities under atomic N:P ratios less than approximately 61:1 (40:1 by mass), resulting in the toxic strains comprising more than 44% of the *Microcystis* communities. While this ratio exceeds the atomic ratio threshold of 44:1 for *Microcystis* abundance supported by Smith (1983), Fujimoto et al. (1997), Marinho et al. (2007), and Xu et al. (2010), among others, it is comparatively small to the other atomic N:P ratios used in the study, which peaked at 220:1 (140:1 by mass) and rendered only 11% of the toxic, *mcyE*-carrying strain comprising the *Microcystis* communities (Otten et al., 2012).

The results of Otten et al. (2012) do not necessarily contradict the findings in section **3.1.2** supporting N-enrichment favoring toxic *Microcystis* strains. Although atomic N:P ratios on the lower end of the study favored toxic *Microcystis* strains, these ratios were also rendered replete in both N and P, at concentrations of 3.62mg L^{-1} N and 0.23 mg L^{-1} P, associated with the lowest atomic ratio tested at 44:1. In contrast, the highest atomic ratio tested at 220:1 was considered less nutrient rich, at 2.56 mg L^{-1} N and 0.02 mg L^{-1} P. These results support findings by Orihel et al. (2012), who also found that microcystins from toxic cyanobacteria occurred in peak concentrations only under low N:P ratios coupled with nutrient replete concentrations after analyzing 246 Canadian lakes.

3.3.3 Low N:P Ratios on Growth

The dominance of cyanobacteria including *M. aeruginosa* in aquatic ecosystems have been attributed to low N:P ratios (Beversdorf et al., 2013; Chaffin et al., 2011; Fuhimoto et al., 1997; X. Liu et al., 2011; Y. Liu et al., 2011; Marinho et al., 2007; Orihel et al., 2012; Otten et al., 2012; Smith, 1983; Xu et al., 2010). Smith (1983) proposed *M. aeruginosa* and other cyanobacteria do not dominate aquatic ecosystems until the N:P atomic ratio is approximately 44:1 or smaller (29:1 by mass ratio) after analyzing the phytoplankton biomass composition from 17 lakes worldwide during the growing season. Atomic N:P ratios below 44:1 in these lakes were deemed as N-limited and not at a 44:1 proportion of high concentrations considered nutrient rich for these lakes. This dominance of *M. aeruginosa* and other cyanobacteria in these lakes was attributed to their superior competitive ability for N than other phytoplankton and is supported by other studies examining *M. aeruginosa* dominance under N-limited conditions coupled with low N:P ratios.

Marinho et al. (2007) observed the competitive abilities between mixed cultures of *M. aeruginosa* and the diatom species *Aulacoseria distans* under manipulated atomic N:P ratios of 15:1 and 3:1. The reduction of the ratios from 15:1 to 3:1 was accomplished by decreasing the concentrations of N, resulting in smaller N:P atomic ratios that were more N-limited. Biomass production of *M. aeruginosa* per unit N was observed to be more than one order of magnitude than *A. distans*. While *M. aeruginosa* biomass production was proportional to the amount of nitrate available leading to higher biomass yielded at the higher N:P ratios, the exponential growth of *M. aeruginosa* lasted longer than *A. distans* under the lower N:P atomic ratios of 3:1 than 15:1 (Marinho et al., 2007). Xu et al. (2010) observed a similar dominance trend for lower N:P ratios, where *M. aeruginosa* samples from Meiliang Bay, China, dominated the phytoplankton community when conditions were N-limited at atomic N:P ratios less than approximately 30:1. The longer exponential growth and the production of more biomass per unit N for *M. aeruginosa* observed by Marinho et al. (2007) may explain its tendency to dominate under N-limited conditions of low N:P ratios.

While cyanobacteria have typically been observed to dominate under low N:P ratios representing N-limitation, low N:P ratios have also been shown to favor dominance of *M. aeruginosa* over other cyanobacteria. Fujimoto et al. (1997) observed that an increase in the atomic N:P ratio in the freshwater lake, Lake Kasumigaura, Japan, had shifted the algae community from *M. aeruginosa* to the filamentous cyanobacteria, *Phormidium tenue* in the summer growing season. After collecting samples from the Lake, the competitive abilities for N and P between *M. aeruginosa* and *P. tenue* were observed in mixed

cultures under atomic N:P ratios of 11, 22, 44, and 89 along a temperature gradient. Similar to the findings of Smith (1983), *M. aeruginosa* dominated mixed cultures where the atomic N:P ratios were less than 44, however this dominance was only observed when temperatures were between 25°C and 30°C. Temperatures outside of this range did not result in dominance of *M. aeruginosa* despite the N:P ratio (the role of temperature in *M. aeruginosa* growth is further explored in **Chapter 4 - Environmental Influences**). Lower concentrations of N promoted more rapid growth than the higher concentrations, indicating that *M. aeruginosa* can outcompete *P. tenue* for N during N-limitation, rendering its dominance under the lower and more N-limited N:P ratios.

3.3.4 Reliability of N:P Ratios versus N and P Concentrations

The reliability of N:P ratios in predicting phytoplankton community structure over concentration levels is a debated subject in scientific literature. Factors such as the proportion of the chemical forms of N present in an aquatic ecosystem, the ambient concentrations of nutrients already in an aquatic ecosystem, water temperature, and the species of phytoplankton being observed with their respective competitive abilities can determine the role of specific N:P ratios on promoting *Microcystis* growth less static and more dynamic.

An 11-year study in Lake Taihu, China, conducted by X. Liu et al. (2011) observed *Microcystis* species peaked in dominance of phytoplankton communities by over 50% under three parameters: when conditions were N-limited with an atomic N:P ratio of less than approximately 46:1(30:1 by mass), when the NH_4+:NO_3- ratio was less than approximately 5:1 (less than 1 by mass), and when water temperatures were between 25°C to 30°C. While the atomic N:P ratio of 46:1 is slightly higher than the ratio threshold of 44:1 defined by Smith (1983), X. Liu et al. (2011) also considered the relevancy of nutrient forms and temperature at valuing the N:P ratios that generated peak dominance, which could shift the ratio values when those parameters are altered. This hypotheses is supported by Chaffin et al. (2011), who observed *Microcystis* abundance in Lake Erie under both high and low atomic N:P ratios while the presence of ammonium was at a constant concentration above 0.24 mg L^{-1} (1.80 µM), likely due to regeneration from internal cycling. Chaffrin et al. (2011) attribute the shrinking N:P ratios on nitrate depletion from phytoplankton uptake, while the proportion of ammonium in the total N concentration remained at a constant and adequate supply to support *Microcystis* growth. Consequently, the concept of low N:P ratios supporting *Microcystis* growth may become more irrelevant under aquatic ecosystems that are contain adequate supply of ammonium.

While many studies have attributed low N:P ratios under N-limitation on the dominance of *Microcystis*, Y. Liu et al. (2011) observed the influence of N:P ratios on cultured *M. aeruginosa* cells under an abundant supply of N and P. Interestingly, the effect of the N:P ratios on *M. aeruginosa* growth depended on the concentrations of N and P. When N was fixed at a concentration of 10 mg L^{-1}, the optimum atomic N:P ratio that yielded the highest biomass and the longest exponential growth occurred at 16:1, consistent with the Redfield ratio. However, when P became the fixed nutrient concentration at 1 mg L^{-1}, the ratio that yielded the highest biomass and longest exponential growth occurred at an atomic ratio of 40:1. Thus, an ample supply of both N and P within an aquatic ecosystem rendered the most biomass growth. While both of these ratios still fell under the N:P ratio of 44:1 proposed by Smith (1983), these results suggest that concentration of the nutrients alters the nutrient ratio at which *M. aeruginosa* is most favored.

In addition to the findings by Y. Liu et al. (2011), Xu et al. (2010) also observed that the growth rates of *M. aeruginosa* did not depend on specific N:P ratios, but rather on the concentrations of N and P in aquatic ecosystems. At a fixed P concentration of 0.02mg L^{-1}, cultured *M. aeruginosa* demonstrated slow growth at atomic N:P ratios ranging from 4:1-32:1. However, at a fixed P concentration of 0.20 mg L^{-1}, the optimum atomic N:P ratio for *M. aeruginosa* growth was 32:1. P fixed at 2.00 mg L^{1} showed maximal growth of *M. aeruginosa* at various atomic N:P ratios ranging from 4:1-64:1, suggesting that at a high starting concentration of P, the concentrations of N resulting in even a low N:P ratio of 4:1 were sufficient enough for *M. aeruginosa* to reach optimal growth rates (Xu et al., 2010). Thus, N and P concentrations may play a more significant role in controlling *M. aeruginosa* blooms than mere ratios.

If it is true that N and P concentrations are a more accurate predictor in shaping phytoplankton communities than N:P ratios, the consistent results of *M. aeruginosa* dominating under low N:P ratios would counter that claim. Yet, a different perspective considers the possibility that low N:P ratios are actually a *result* of *M. aeruginosa* dominance. It is a prevalent notion in scientific literature that *M. aeruginosa* is a superior competitor for N, particularly ammonium. *M. aeruginosa* can uptake N in larger proportions than P (Paerl et al., 2012), and at significantly faster rates than other phytoplankton. Recalling from Section **3.3.3: Low N:P Ratios on the Growth**, Marinho et al. (2007) further observed that the N:P ratios that *M. aeruginosa* were subjected to reduced on their own, significantly more than the N:P ratios that *A. distans* were subjected to. Marinho et al. (2007) owed this natural reduction in the N:P ratio to the uptake of nutrients, particularly to the rapid uptake of N from *M. aeruginosa*. Although nutrient uptake was faster and higher in *M. aeruginosa* cultures than in *A. distans* cultures, the

proportion of N and P uptake did not change in the culture mediums despite various N:P ratio treatments, as intracellular N:P ratios did not vary significantly within *M. aeruginosa.*

Xie et al. (2002) also observed a natural reduction in the N:P ratio after observing *M. aeruginosa* blooms in Lake Donghu, China, although this reduction is blamed on *M. aeruginosa* scavenging for stored P in lake sediments, releasing P into the water column and ultimately bringing P concentrations nearer to N concentrations. Dense blooms of *M. aeruginosa* were positively correlated with P concentrations in the water column. Additionally, *M. aeruginosa* was observed under ratios both over and under the atomic N:P ratio of 44:1 defined by Smith (1983), thereby deeming the resource ratio theory inapplicable to predicting blooms in this study.

Whether or not N:P ratios play a role in promoting *M. aeruginosa* or if it is the other way around, the fact remains in scientific literature that *M. aeruginosa* is often observed to dominate under low N:P ratios, particularly when conditions are N-limited. A clear link between nutrient ratios and the growth, toxicity, and distribution of HABs is difficult to establish due to a number of other factors potentially overriding the influence of static N:P ratios, including the availability of N forms and local environmental factors that could influence the phytoplankton community more than nutrient availability (Davidson et al., 2012). Furthermore, as of yet, no molecular mechanism has been discovered in which phytoplankton can perceive and react to specific nutrient ratios (Marinho et al., 2007). Thus, no universal N:P ratio for optimal *M. aeruginosa* growth and sustainment definitively exists, and nutrient management for *M. aeruginosa* control should focus on the concentration availabilities of N and P as a more accurate approach for controlling blooms than ratios.

3.4 Summary

N and P are critical in shaping and sustaining phytoplankton communities. The chemical form of N and P may be just as influential in controlling the growth, toxicity, and distribution of *M. aeruginosa* as the availability of the nutrients themselves. *M. aeruginosa* has a particular affinity for ammonium as it requires less energy to assimilate, suggesting that higher concentrations of ammonium in an aquatic ecosystem could favor its dominance in phytoplankton communities. Additionally, research is demonstrating that N has a more influential role in the functions of *M. aeruginosa* than P. Toxic strains of *M. aeruginosa* appear to outcompete non-toxic strains for N, resulting in a larger proportion of toxic strains in *Microcystis* communities under the presence of higher N concentrations. However, *M. aeruginosa* in general is a better competitor for N than other phytoplankton, where it can uptake higher

proportions of N than P over other phytoplankton. This superior ability to compete for N allows *M. aeruginosa* to thrive under N-limited conditions by not only outcompeting other phytoplankton for N, but also by growing more biomass per unit N available. Further, N-limitation to the point of *M. aeruginosa* being N-stressed can lead to dense colony formation and the production of microcystins as a potential defense mechanism to survive under harsher conditions.

While *M. aeruginosa* depends on P for growth, it appears far less influential in controlling its functions. Evidence has suggested that P favors toxic strains over non-toxic strains when coupled with warmer temperatures, while other studies have shown no relationship in the favoring of toxic strains or production of microcystins. These discrepancies could be due to the degree of P limitation and temperatures in these studies. Under P-limitation, both toxic and non-toxic strains of *M. aeruginosa* have demonstrated significant growth response patterns to P additions, although P inputs also appear negatively correlated with colony size and positively correlated with the distribution of *M. aeruginosa* as single cells. Further, research suggests that *M. aeruginosa* can store P in its external portions of its cells for later use under P-limited conditions, a potential competitive advantage over other phytoplankton.

In nature, N and P are found in proportions relative to one another, dissolved in the water of aquatic ecosystems. Some evidence suggests that *M. aeruginosa* can substitute P for N under P limitation, as demonstrated by the lower N required to transition *M. aeruginosa* from colony formation under nutrient stressed conditions to single cell distribution under nutrient sufficiency when in the presence of P. While *M. aeruginosa* has demonstrated dominance under N-limitation, the resource ratio theory suggests that it is the specific ratios of N and P that influence presence of *M. aeruginosa*, based on the idea that phytoplankton internally allocate N and P in different proportions based on their physiological structures. Additionally, the Redfield ratio theory suggests that most phytoplankton require optimal growth at an atomic N:P ratio of 16:1. Neither the resource ratio theory or the Redfield ratio theory appear to support the growth of *M. aeruginosa*, although *M. aeruginosa* commonly dominates phytoplankton communities under low N:P ratios coupled with N-limited conditions. However, this dominance could largely depend on other phytoplankton species and chemical forms of N present, which can potentially shift dominance to other phytoplankton species that can better compete for the chemical N forms available. Different concentrations of N and P have been shown to shift the N:P ratio values at which optimal growth is achieved, and some evidence supports the notion that rapid uptake of N and P by *M. aeruginosa* is actually the cause of low N:P ratios being linked with *M. aeruginosa* presence. Thus, current, albeit limited, research has not identified a specific and universal N:P ratio

under which *M. aeruginosa* thrives best. Rather, it appears that concentrations of nutrients is a more accurate predictor of the growth, toxicity, and distribution patterns of *M. aeruginosa* and should be focused on more over N:P ratios for nutrient management.

To make predicting and managing the presence of *M. aeruginosa* even more complex, nutrients may not even be the most influential factors in the growth, toxicity, and distribution of *M. aeruginosa* in aquatic ecosystems. Depending on the nutrient availability and the setting of the aquatic ecosystems, environmental factors such as temperature, precipitation, and salinity may be more influential in controlling the presence of *M. aeruginosa*.

Chapter 4 - Environmental Influences

The unfortunate truth in understanding the growth, toxicity, and distribution of *M. aeruginosa* is that it is a multidimensional issue controlled by a simultaneous mixture of influential forces. It is not a simple matter of a magical nutrient ratio to avoid, but rather an amalgamation of nutrient availability and the local environmental forces that define the conditions of an aquatic ecosystem. Climatic forces including changes in temperature, precipitation, and salinity can be exacerbated by the overarching threat of climate change, creating a time of more extreme conditions in which *M. aeruginosa* can thrive more than before. Even more concerning is the evidence of *M. aeruginosa* demonstrating adaptive capabilities to changes in the local environment in spite of harsher, more stressful conditions.

4.1 Role of Temperature in Regulating *Microcystis aeruginosa*

Of all the potentially influential factors controlling the presence of *M. aeruginosa*, temperature is the least debated. In general, cyanobacteria prefer warmer temperatures, often blooming during the warmest periods of the year, usually in the summer and early fall months, deemed as the summer growing season (Davis et al., 2009; Lehman et al., 2008; Xiu et al., 2011). This pattern of cyanobacterial preference to warmer temperatures can be observed often when the phytoplankton community in many freshwater systems shifts from diatoms to cyanobacteria during the warmer seasons (Davis et al., 2009). In fact, *Microcystis* specifically has been observed to outcompete other phytoplankton as temperatures become warmer (Davis et al., 2009; Fujimoto et al., 1997). One potential reason for being able to outcompete other phytoplankton is higher water temperatures increase thermal stratification in aquatic ecosystems, thereby reducing vertical mixing and causing nutrient limitation near the water surface. Buoyant phytoplankton, like *Microcystis*, can use its vertical migration techniques to hunt for less accessible nutrients within the water column, giving it an advantage over other phytoplankton during under warmer and stratified conditions (Lehman et al, 2013). Additionally, *Microcystis* has a high Q_{10}, or temperature coefficient that measures the change in growth from increasing temperature by 10°C (Lehman et al., 2013). Many studies have concluded that the optimal temperature for growth and photosynthesis for harmful cyanobacteria like *Microcystis* is at or above 25°C (Davis et al., 2009). This optimal temperature value is consistent with the findings by Davis et al. (2009), Fujimoto et al. (1997), and X. Liu et al. (2011), discussed in Sections **3.2.2: Role of Phosphorus on Toxic and Non-Toxic Strains** and **3.3.4: Reliability of N:P Ratios versus N and P Concentrations**, where *Microcystis* abundance, dominance, and growth peaked at temperatures between 25°C and 30°C, given the appropriate nutrient dynamics.

4.1.1 Role of Temperature on Toxic and Non-Toxic Strains

Similar to Nitrogen (N), warmer temperatures have also demonstrated favorable selection for toxic strains of *Microcystis* over non-toxic strains. Davis et al. (2009) in Section **3.3.2: Role of Phosphorus on Toxic and Non-Toxic Strains** affirmed that elevated temperatures coupled with phosphorus (P) loadings led to the highest growth rate and abundance for toxic *Microcystis* strains than any other nutrient and temperature treatment applied in their study of four Northeastern U.S. freshwater lakes. Elevating only the ambient surface water temperatures by approximately 4°C led to significantly higher growth rates for *mcyD*-carrying *Microcystis* cells than non-toxic cells, in five out of six (83%) of the experiments conducted (two lake experiments were observed twice in two years). In contrast, elevated temperatures only increased the growth rates of non-toxic strains in two of the experiments, while even decreasing the growth rate of non-toxic strains in three of the experiments. Additionally, from Section **3.1.2: Role of Nitrogen on Toxic and Non-Toxic Strains**, Yoshida et al. (2007) observed growth bursts of toxic *mcyA*-carrying strains of *M. aeruginosa* during warmer, summer months in Lake Mikata, Japan, when temperatures were between 27°C and 30°C.

Much like the concept of nutrient ratios, there are exceptions to the optimal temperature range for toxic *Microcystis* growth and abundance. Davis et al. (2009) observed in one experiment that toxic *Microcystis* strains doubled in growth rate at temperatures less than 20°C, and Yoshida et al. (2007) observed significant subpopulations of toxic *M. aeruginosa* at temperatures as low as 13.8°C. Further, Tas et al. (2006) observed toxic *M. aeruginosa* blooms in the Golden Horn Estuary in Turkey between December 1998 and February 1999, a period of cooler temperatures outside of the typical summer growing period. These deviations from the understood optimal temperature range can potentially be explained by the additive roles of other influential factors, including nutrients and other environmental forces.

Nonetheless, there is generally a positive correlation between cyanobacteria blooms and warmer temperatures. The predicted global increase in temperatures of 1.8°C by the end of this century due to climatic warming (Xiu et al., 2011) is especially concerning, as *Microcystis* blooms including *M. aeruginosa* can show up more prevalently. With studies supporting the notion that warmer temperatures favor toxic strains of *Microcystis*, a global increase in temperature would theoretically shift a larger proportion of *Microcystis* blooms to its toxic form, while sustaining these blooms for longer periods (Xiu et al., 2011). This upward trend in global temperatures could explain the seemingly simultaneous expansion of *Microcystis* on the global scale. A global increase in temperatures goes

beyond heating surface water temperatures, where it can lead to more extreme weather events, such as extended dry and wet seasons, exacerbating other environmental forces associated with drought and heavy precipitation that can create more suitable conditions for *M. aeruginosa* blooms.

4.2 Role of Precipitation in Regulating *Microcystis aeruginosa*

The frequency and abundance of harmful cyanobacterial blooms are expected to increase with the environmental forces associated with climate change (Lehman et al., 2013). This increase has already been observed over the past decade, and interestingly, cyanobacterial HABs like *Microcystis* are expanding into harsher aquatic environments which were considered uninhabitable (Lehman et al., 2005, 2013; Miller et al., 2010; Mosiander et al., 2009; Robson and Hamilton, 2003; Ross et al., 2006). Typically, freshwater cyanobacteria are not associated with estuarine environments due to limited tolerance of salinity (Robson and Hamilton, 2003). However, extended rainfall can function not only as a transporter of cyanobacterial HABs into more brackish waters, but it can also act as a saline diluter, creating estuarine conditions more inhabitable for a species prone to freshwater. Though generally associated with freshwater lakes, *Microcystis* blooms have been observed in the Golden Horn Estuary in Turkey (Tas et al., 2006), the St. Lucie River Estuary in Florida (Ross et al., 2006); the San Francisco Bay Estuary and Monterey Bay California (Lehman et al., 2005, 2013; Miller et al., 2010), and the Swan River Estuary of Western Australia (Robson and Hamilton, 2003), among others.

Robson and Hamilton (2003) observed that *M. aeruginosa* bloomed for the first time in the Swan River Estuary in February 2000, following a record amount of rainfall the previous month. While cyanobacteria had generally never exceeded 5,000 cells mL^{-1}, toxic *M. aeruginosa* peaked in density at over 1,000,000 cells mL^{-1} after heavy rainfall and freshwater river inflow transported *M. aeruginosa* from adjoining wetlands and flushed much of the brackish water out of the estuary, reducing salinity from 35% to as low as 4% in some areas (Robson and Hamilton, 2003). While salinity levels were eventually replenished to levels of the pre-storm event, more frequent and intense summer storms induced by the global temperature rise associated with climate change can create suitable conditions for *M. aeruginosa* blooms to recur in habitats atypical of hosting HABs.

It is believed freshwater flushing from heavy precipitation events also served as a transport mechanism for *M. aeruginosa* to reach the San Francisco Bay Estuary and Monterey Bay (Miller et al., 2010). However, while heavy precipitation can transport *M. aeruginosa* into and temporarily dilute a brackish aquatic ecosystem, it can also create a less suitable habitat for it and other HABs to form. Lehman et al.

(2013) observed that toxic *Microcystis* and its respective microcystins were less prevalent in the San Francisco Bay Estuary in the wet years of 2004 and 2005 than in the dry years of 2007 and 2008. Wet years were characterized by heavy precipitation, which in turn increased streamflow, water velocity, vertical mixing, and turbidity (suspended solids) in the San Francisco Bay Estuary. Increased water velocity and vertical mixing not only disaggregates existing *Microcystis* colonies, but they also do not allow *Microcystis* the residence time it needs to colonize (Lehman et al., 2013). Although the wet years in this study demonstrated lower *M. aeruginosa* densities and microcystin concentrations due to flushing , Ross et al. (2006) observed that physical injury to *Microcystis* cells can spur the release of microcystins, after observing a 95% increase in microcystin concentrations after subjecting *mcyB*-carrying *M. aeruginosa* to ultrasonification. Thus, increased water velocity and mixing, while having a flushing effect, can also create more toxic conditions in areas where *Microcystis* blooms already exist, even if only temporarily. Dry years were characterized by low streamflow, low turbidity, and warmer water temperatures, peaking at 25.6°C, within the optimal temperature range for *Microcystis* growth (Davis et al., 2009). With little precipitation in the dry years, the decrease in water velocity and vertical mixing allows light to penetrate deeper through the water surface, providing ample residence time and sunlight for *Microcystis* to colonize. Further, decreased water velocity and flushing elevates inorganic nutrient concentrations, particularly ammonium, the preferred *Microcystis* nutrient source, from reduced dilution of wastewater discharge.

Both extreme and extended wet and dry seasons influenced by global climate change can enable conditions more suitable for *Microcystis* growth and abundance than before. Seasonal variation in *Microcystis* densities in aquatic ecosystems renders nutrient concentrations secondary in importance to local environmental forces, particularly if the ecosystem is already rich in nutrients (Lehman et al., 2008).

4.3 Role of Salinity in Regulating *Microcystis aeruginosa*

It was discussed in Sections **3.1.2: Role of Nitrogen on Toxic and Non-Toxic Strains** and **3.2.2: Role of Phosphorus on Toxic and Non-Toxic Strains** that the degree of N or P-limitation influences the degree of the growth response of *Microcystis* when exposed to those nutrients. Research suggests that salinity may play a role in the tendency of aquatic ecosystems to be more N or P-limited. Blomqvist et al. (2004) made the observation that marine ecosystems tend to be more N-limited, while freshwater ecosystems tend to be more P-limited. In marine ecosystems, more iron (Fe) is sequestered by the higher sulfide content from sea salt, which reduces the number of Fe atoms to precipitate P. Two Fe atoms are needed

for the precipitation of one P molecule, but the Fe:P ratio is typically less than 2 in anoxic marine waters. Freshwater ecosystems typically have a Fe:P ratio greater than 2, which facilitates a much higher rate of P removal. Thus, N-limitation is more typical in more saline ecosystems, while P-limitation is more typical in freshwater ecosystems. However, there are exceptions to this scenario: In the summer growing season of 2007, Mosiander et al. (2009) observed that the brackish waters of the San Francisco Bay Estuary were richer in N (both NO_x and ammonium), while the freshwaters of the Klamath River Reservoir were richer in P. Even so, as *M. aeruginosa* has demonstrated successful establishment in both freshwater and saline conditions, management efforts for nutrient enrichment may require different approaches between coastal marine and freshwater ecosystems.

4.3.1 Salinity Stress on Toxicity

Although *Microcystis* blooms have been present in both saline and freshwater ecosystems, higher salinity concentrations create more stressful conditions for cyanobacteria like *M. aeruginosa* to survive (Lehman et al., 2013; Mosiander et al., 2009, Ross et al., 2006; Tonk et al., 2007). This could explain why, in most cases, *Microcystis* is theorized to have been transported from freshwater influxes into coastal marine and estuarine ecosystems instead of originating *in situ*.

Stressful conditions created by higher salinity content have been observed to spur the release of microcystins from *M. aeruginosa*. Ross et al. (2006) sampled *mcyB*-carrying *M. aeruginosa* from St. Lucie River Estuary in Florida, subjecting various samples of approximately 1.05×10^8 cells to 50 ml of seawater treatment, or water with 32% salinity. While 1.05×10^8 cells in 50 ml of *in situ* estuarine water yielded approximately 3.5 $\mu g\ L^{-1}$ microcystins, treating 1.05×10^8 cells to 50 ml of seawater increased microcystin concentrations by 80% to approximately 6.3 $\mu g\ L^{-1}$ after 5 hours of exposure. Similarly, Tonk et al. (2007) observed toxic *M. aeruginosa* began to release microcystins when salinity exceeded 10 $g\ L^{-1}$ as a result of cell leakage or lysis, indicating a reaction from saline-induced stress. These results suggest that even when *Microcystis* is transported from freshwaters into estuarine ecosystems, brackish water stress can stimulate microcystin release.

4.3.2 Salt Tolerance of *Microcystis aeruginosa*

While wet seasons can temporarily dilute a brackish water system into a more suitable habitat for *Microcystis* growth, *M. aeruginosa* has been observed keep its residence in brackish water systems at their typical salinity levels. For example, *M. aeruginosa* has made consistent, annual returns in the San Francisco Bay Estuary ever since its discovery in 1999, where salinity varies between 0.1 $g\ L^{-1}$ and 9.1 $g\ L^{-}$

^{1} (Mosiander et al., 2009), even reaching as high as 18 g L^{-1} in parts of the western delta (Lehman et al., 2013). *M. aeruginosa* has demonstrated salt tolerance from 9.8 g L^{-1} (Orr et al., 2004) to 15 g L^{-1} (Mosiander et al., 2009), with Tonk et al. (2007) observing continued growth at levels as high as 17.5 g L^{-1} for up to nine days. Freshwater cyanobacteria typically have low tolerance for salinity, however, the increasing presence of *M. aeruginosa* in more estuarine systems suggests *Microcystis* should no longer be considered solely a freshwater species. Lehman et al. (2013) and Mosiander (2009) suggest that *M. aeruginosa* could have very well evolved to tolerate higher levels of salinity. The concept of *M. aeruginosa* adaptation to local environmental factors is gaining traction in scientific literature, which could explain the complexity in pinpointing common parameters suitable for *Microcystis* abundance.

4.4 Adaptations to the Local Environment

Studies on the genetic diversity of *M. aeruginosa* are not as prevalent as studies focusing on nutrient and environmental influences. In fact, only one published study exists on the genetic diversity of *M. aeruginosa* for brackish water ecosystems (Mosiander et al., 2009). Comparisons of strains from different ecosystems are important because they could provide information on the routes of cyanobacterial HAB introductions and whether or not different ecosystems select for different ecotypes. For example, *M. aeruginosa* strains have been observed to have unique genetic "fingerprints" in different aquatic ecosystems, giving rise to the question of whether the strains observed are introduced into the ecosystems, or if local environmental factors selected for those strains to prevail over a mixture of other strains.

Mosiander et al. (2009) compared strains of *M. aeruginosa* in the brackish waters of the San Francisco Bay Estuary and the freshwaters of the Klamath River Reservoir. The San Francisco Bay Estuary and the Klamath River Reservoir are comprised of different and almost contrasting characteristics, yet both of these ecosystems have consistently supported *M. aeruginosa* blooms since their discoveries in 1999 and 2005, respectively. In the San Francisco Bay Estuary, N concentrations (NO_x and ammonium) were lower than in the Klamath River Reservoir, while pH and light visibility were lower. In the Klamath River Reservoir, P, pH, and light visibility were greater (Mosiander et al., 2009).

Consequently, distinct differences in the genotypes of *M. aeruginosa*, both toxic and non-toxic, were found in each ecosystem, suggesting that unique subpopulations of *M. aeruginosa* occupy the San Francisco Bay estuary and the Klamath River Reservoir. Environmental differences between the these ecosystems suggest environmental regulation may play a role in the selection of different strains of *M.*

aeruginosa, perhaps even leading to the development of different ecotypes (Mosiander et al., 2009). It is possible that the increase in *M. aeruginosa* on the global scale could be due to adaptations to the local conditions, where strains of *M. aeruginosa* are becoming more suited to their environment. Thus, while the concept of locally adapting to the environment complicates the identification of trends that influence *Microcystis* growth, toxicity, and distribution, it also implies that toxic *Microcystis* blooms are resilient to changes in local environmental conditions, allowing it to persist and expand spatially and temporally.

4.5 Summary

Environmental factors may regulate the presence of *M. aeruginosa* more than the availability of nutrients, particularly if an ecosystem is already nutrient rich. Temperature has significant influence in promoting and sustaining *Microcystis* abundance. Increased surface water temperatures between 25°C and 30°C have been deemed the optimal temperature range to maximize *Microcystis* growth and abundance, likely due to advantageous nutrient-harvesting capabilities in thermally stratified waters, and the high Q_{10} of *Microcystis*. Additionally, temperatures near and within this range have also been shown to favor toxic strains of *M. aeruginosa* over non-toxic strains. Although temperature may be principal in importance in influencing toxic *Microcystis* blooms in many cases, studies have supported that a combination of both nutrients and elevated temperatures provides maximum growth responses, and therefore management efforts for *Microcystis* cannot separate these two components.

Precipitation has also demonstrated significant control over the distribution and intensity of *Microcystis* blooms. Extreme precipitation events can temporarily dilute and flush the brackish waters of estuarine and coastal marine ecosystems, creating conditions more suitable for the once-deemed freshwater cyanobacteria. In addition, inflated freshwater streamflows into these ecosystems from precipitation events can serve as a transporting mechanism for *Microcystis* into brackish water ecosystems, where *in situ* origination would not be typical. In contrast, heavy precipitation events can also serve as a detriment to *Microcystis* colonization. Increased precipitation leads to increased water velocity and vertical mixing by means of flushing, which not only can disaggregate existing *Microcystis* colonies, but it can also prevent the ample residence time needed for *Microcystis* to colonize.

It has been observed that dry weather conditions are more suitable for *M. aeruginosa* inhabitation in aquatic ecosystems. A lack of precipitation reduces water velocity and vertical mixing, allowing deeper sunlight penetration through the water surface, providing sufficient residence time, sunlight, and

temperature ranges for *Microcystis* to colonize. Moreover, the lack of vertical mixing and flushing elevates the inorganic nutrient concentrations, making conditions more nutritionally favorable for primary production.

The fact that both extreme wet and dry weather conditions can positively correlate with increased abundance and distribution of *M. aeruginosa* is especially concerning due to the impending issue of climate change. Global temperature is expected to increase by 1.8°C by the end of the century, which can significantly influence the magnitude of environmental forces. Environmental forces associated with climate change can lead to more extreme weather events, including warmer temperatures and extended wet and dry seasons, and must be accounted for when considering management options for *Microcystis* blooms.

While *M. aeruginosa* is typically deemed as a freshwater cyanobacterium, its ever-growing presence in brackish and coastal marine ecosystems demonstrates a tolerance for higher salinity levels. While salinity can induce stress and subsequent microcystin release in *Microcystis* cells, *M. aeruginosa* has been observed to tolerate salinities as high as 15 g L^{-1} –even maintaining temporary growth at acute levels as high as 17.5 g L^{-1}.

To make matters even more complex, *M. aeruginosa* has demonstrated local adaptive capabilities to its environment. Because *M. aeruginosa* can grow in abundance in ecosystems comprised of unique and sometimes contrasting characteristics, different genotypes have been discovered in subpopulations occupying different ecosystems. This discovery suggests that *Microcystis* can consist of a diverse mixture of genotypic strains, where the local environmental conditions can select for particular strains to thrive. Thus, the influential factors in nutrients and environmental forces may vary depending on the strain of *M. aeruginosa* occupying an ecosystem.

Chapter 5 - Conclusions and Recommendations

Understanding the influential factors behind the growth, toxicity, and distribution of *M. aeruginosa* is a complex undertaking that is still in the process of being clarified. While research has demonstrated some trends that can identify more influential nutrient and environmental conditions in regulating *Microcystis* blooms, a considerable amount of research still needs to be done in more refined areas. Nonetheless, what can be concluded about the nutrient and environmental regulation of *M. aeruginosa* derived from existing research can generate recommendations for more precise plans of action in learning about—and controlling—toxic *Microcystis* blooms.

5.1 Conclusions

M. aeruginosa is a species of cyanobacteria that can exist in both toxic and non-toxic form. While both forms threaten aquatic ecosystems with dense biomass blooms that result in eutrophication, toxic strains of *M. aeruginosa* also expel potent hepatotoxins, also known as microcystins, into the aquatic environment. Microcystins are emerging as a serious health threat to aquatic ecosystems and organisms who benefit from their services, having been linked to liver failure and death in the federally endangered southern sea otter—and, in extreme cases—in humans.

Although blooms from *M. aeruginosa* are commonly characterized as a freshwater problem, *M. aeruginosa* has extended into brackish and marine coast ecosystems, expanding simultaneously in all three ecosystems on a global scale while making consistent, annual returns. As of yet, Antarctica is the only continent to not report *M. aeruginosa* blooms. This global expansion and recurrence of *M. aeruginosa* brings attention to the nutrient and climatic dynamics that influence its blooms; identifying the most influential factors will help define management strategies to control *M. aeruginosa* blooms and protect the aquatic ecosystems they infest.

5.1.1 Conclusions on Nutrient Influences

M. aeruginosa, like other phytoplankton, depends on nitrogen (N) and phosphorus (P) as nutrients for growth. N has demonstrated a significantly more influential role in regulating *M. aeruginosa* growth, toxicity, and distribution in aquatic ecosystems than P. While cyanobacteria are typically a superior competitor for N than other phytoplankton, *M. aeruginosa* has also been shown to outcompete other genera of cyanobacteria for N under laboratory experiments. Accordingly, *M. aeruginosa* consumes a larger proportion of N than P during nutrient uptake over other phytoplankton species. Furthermore, it

has demonstrated growth characteristics that suggest it grows more biomass per unit N consumed than diatoms. As a result of its superior competitive ability for N and its rapid growth upon N consumption, *M. aeruginosa* is typically dominant under N-limited conditions, persisting under N-limitation while other phytoplankton species starve.

The chemical forms of N are critical in determining the presence of *M. aeruginosa* in phytoplankton communities. *M. aeruginosa* has an affinity for the ammonium N form, whereas it grows slower under oxidized forms of N, particularly demonstrated in nitrate. Accordingly, phytoplankton communities have demonstrated a shift from diatoms to cyanobacteria comprised of *M. aeruginosa* when the proportion of NH_4+:NO_x increases. Ammonium also has growth-inhibiting effects on phytoplankton, although cyanobacteria including *M. aeruginosa* have exhibited a much higher tolerance for ammonium growth-inhibition than other phytoplankton classes, including Prymnesiophyceae, Diatomophyceae, Raphidophyceae, and Dinophyceae. Thus, ammonium is likely the most influential nutrient form that favors *M. aeruginosa* abundance and dominance.

Toxic strains of *M. aeruginosa* have also demonstrated a superior ability to compete for N over its non-toxic counterparts, which suggests that the more N-rich an aquatic ecosystem is, the larger the proportion of toxic *M. aeruginosa* strains comprising the phytoplankton community than non-toxic strains. However, N-limitation to the point of N-stress has demonstrated the ability to stimulate the synthesis of microcystin by *M. aeruginosa*, suggesting that N-stress can lead to higher microcystin concentrations.

Despite the more influential role of N in regulating growth and toxicity of *M. aeruginosa*, both N and P play vital roles in its vertical migration abilities. Under nutrient stress for both N and P, *M. aeruginosa* loses its buoyancy and sinks to lower depths of the water column. While this vertical sinking allows *M. aeruginosa* to forage for less accessible nutrients, it also results in less sunlight exposure necessary for it to survive. Additionally, nutrient-stress conditions rouse *M. aeruginosa* to form dense colonies, whereas under nutrient enrichment, *M. aeruginosa* distributes itself as single cells. Thus, colony formation of *M. aeruginosa* may be a defense mechanism under conditions of nutrient stress, where it can gain more buoyancy and shade out other phytoplankton lacking vertical migration capabilities by colonizing itself into dense blooms. However, even in colony formation, N demonstrates a more influential role. Studies have demonstrated that higher concentrations of N are needed than P to fulfill the nutrient quota for *M. aeruginosa* to cross the threshold from nutrient-starved colony formation to nutrient-satisfied single-cell distribution.

The prevalent theory on resource ratios for the optimal growth and abundance of *M. aeruginosa* suggests that *M. aeruginosa* exhibits dominance in an aquatic ecosystem under atomic ratios lower than 44:1. The more influential role of N than P in regulation of *M. aeruginosa* growth and abundance can justify its dominance under low N:P ratios, implying conditions of N-limitation. However, as N:P ratios are merely the proportion of N to P in an aquatic ecosystem regardless of concentration value, conditions with low N:P ratios can still be replete in both N and P. While it is true *M. aeruginosa* dominance is typically observed under low N:P ratios under 44:1, studies have shown that the N:P ratios for optimal growth can change given different starting concentrations of N or P already dissolved in the water, ranging from as low as 4:1 to as high as 64:1. Optimal growth for *M. aeruginosa* has been demonstrated under a larger range of N:P ratios when nutrient concentrations of N and P are higher, where its growth can peak under lower ratios than under nutrient-deficient conditions, suggesting that concentration values of N and P and the chemical forms of N should be the focus of nutrient management rather than attempting to identify a universal N:P ratio or range of N:P ratios that favor *M. aeruginosa*. Further, no molecular mechanism has been identified in which phytoplankton can react to specific nutrient ratios. The association of low N:P ratios with *M. aeruginosa* has instead been theorized to be due to its observed ability to uptake larger proportions of N to P and its consequential lowering of N:P ratios under laboratory studies, indicating a preferential uptake for N.

5.1.2 Conclusions on Environmental Influences

Local environmental forces on aquatic ecosystems can be more influential on *M. aeruginosa* regulation than nutrients. In particular, conditions most favorable for the growth of *M. aeruginosa* include warm surface water temperatures ranging from 25°C to 30°C, stagnant water conditions with low flushing and turbidity, and ample sunlight exposure. These climatic factors are most associated with extended dry-weather seasons, which are projected to intensify with climate change. To complicate the regulation of *M. aeruginosa* blooms even further, *M. aeruginosa* has exhibited adaptive qualities to its local environment, becoming more tolerant of conditions once considered harsher for survival, particularly in the more saline waters of brackish and coastal marine ecosystems. Thus, management of *M. aeruginosa* will require a multi-faceted approach that may have to be adapted to different locations.

5.2 Management Recommendations

The influential forces regulating *M. aeruginosa* represent a complex interaction between nutrient availability and local environmental forces. Even within the sole realm of nutrients lies a complex

chemistry of N forms that have different roles in influencing phytoplankton communities. Consequently, many research studies attempting to understand the nutrient dynamics behind *M. aeruginosa* growth and abundance result in unclear and sometimes conflicting conclusions about the role of N and P in *M. aeruginosa* regulation. The conclusions summarized in Sections **5.1.1: Conclusions on Nutrient Influences** and **5.1.2: Conclusions on Environmental Influences** derived from an extensive review of scientific literature define significant trends in nutrient dynamics that influence *M. aeruginosa* regulation and are the basis for the following recommendations:

5.2.1 Refine Research Areas

Management of *M. aeruginosa* must consider nutrient dynamics, including chemical form and their individual concentrations, with respect to the local environmental forces within the ecosystems *M. aeruginosa* resides. Therefore, researchers observing the relationship between N, P, and *M. aeruginosa* should refine their focus on chemical N forms, concentrations, and local environmental factors in their studies when making conclusions about N and P influences on *M. aeruginosa* growth, toxicity, and distribution.

Many studies only use nitrate to observe nutrient influences on *M. aeruginosa*, which can oversimplify and create misleading conclusions about the role of N in *M. aeruginosa* regulation. As *M. aeruginosa* has a particular affinity and high tolerance for ammonium and a slow assimilation rate for NOx, more studies observing trends in the concentrations of these N forms that spur optimal growth would further define distinct roles N, P, and their concentration values have in influencing the presence of *M. aeruginosa* blooms. More published support in observing influential trends of nutrient forms would inform decision makers of which chemical forms to prioritize regulation for and offer potentially more feasible management solutions than regulating for all forms of N.

Researchers should also be mindful of the local environmental conditions present in their studies when observing the nutrient roles on *M. aeruginosa* regulation. Environmental variables can potentially influence the presence of *M. aeruginosa* more so than the presence of available N and P and their respective chemical forms. Thus, it is important that local environmental variables, including water temperature, sunlight exposure, salinity, and competition from local phytoplankton species are measured in conjunction with nutrient forms and their concentrations. Aquatic ecosystems in locations prone to environmental conditions favorable for *M. aeruginosa* growth likely increase the role of N and P in *M. aeruginosa* regulation and may require more stringent nutrient management plans. However, it

is possible that *M. aeruginosa* can adapt to the local conditions from which they originate, requiring specialized management approaches for different aquatic ecosystem types and their locations.

Steering future studies on the role of N and P in the growth, toxicity, and distribution of *M. aeruginosa* to incorporate measurements on chemical forms and local environmental conditions can reinforce or further define the most influential nutrient trends in regulating *M. aeruginosa* blooms. Consequently, management decisions for nutrient regulation in controlling *M. aeruginosa* blooms can be streamlined and made with more confidence.

5.2.2 Regulate Ammonium

Nutrient regulation has primarily been focused on P removal (Paerl et al., 2011), largely ignoring N and its chemical forms. Wastewater effluent is a major source of nutrient loading into aquatic ecosystems; in the San Francisco Bay, publicly owned treatment works (POTWs) account for two-thirds of nutrient loading, and the only N constituent regulated is ammonia (NH_3) (SFBRWQCB, unpublished), a toxic compound lethal to fish (Randall and Tsui, 2002).

Ammonium being the preferred N source for cyanobacteria like *M. aeruginosa* necessitates a focus on removing ammonium from aquatic ecosystems. In addition to the preferential uptake of ammonium for *M. aeruginosa*, the inhibitory effects of ammonium on growth and nutrient uptake for other classes of phytoplankton create a dual advantage for the dominance of *M. aeruginosa* and other cyanobacteria with high tolerance levels to the inhibitory effects of ammonium. Furthermore, as ammonium is the ionic form of ammonia (together forming what is called *total ammonia*), removal of both ammonium and ammonia requires the same process of nitrification. Thus, POTWs should focus nutrient reduction efforts on reducing total ammonia concentrations in wastewater before being discharged into aquatic ecosystems, rendering conditions less favorable for *M. aeruginosa* dominance while simultaneously reducing toxic ammonia concentrations.

Regulating N in addition to P is an expensive process in wastewater treatment. However, efforts focused on reducing total ammonia through nitrification can be significantly cheaper than reducing total N. Nitrification is the biological oxidation of ammonium to nitrite and from nitrite to nitrate via ammonia oxidizing bacteria (AOB). Conventional nitrification processes require much more energy in the aeration process and larger facilities due to longer solids retention times for nitrification to occur than removing biological oxygen demand (BOD) for the breakdown of biosolids and the subsequent removal of P. However, novel technologies are being piloted in treatment involving optimization of current POTW

infrastructure to reduce the energy for nitrification to occur, such as using enhanced screenings to remove biosolids, allowing more aeration energy for nitrification than BOD removal for the breakdown of the biosolids (SFBRWQCB, unpublished). While technologies in total ammonia removal may be unique to different treatment plants and their current infrastructure and capacity for optimization, POTWs should review developments in nitrification technologies that not only reduce total ammonia, but also reduce the expenses in capital and operations and maintenance.

5.2.3 Regulate Total Nitrogen

While ammonium is the preferred N constituent for *M. aeruginosa* growth, *M. aeruginosa* has nonetheless demonstrated positive growth responses to nitrate loadings under N-limitation. Further, when ammonium is scarce, nitrite and nitrate may be the only sources of N for *M. aeruginosa.* Although *M. aeruginosa* can dominate aquatic ecosystems under N-limitation, its toxic strains respond particularly well to N additions, including nitrate, suggesting that higher total N concentrations can shift *Microcystis* communities from non-toxic to toxic strains. The influence of N along with P on *M. aeruginosa* regulation has brought forth recommendations in scientific literature for stricter nutrient regulation on N and P (Otten et al., 2012; Paerl et al., 2012; SFBRWQCB, unpublished). Beyond *M. aeruginosa* and other cyanobacteria, increases in total N concentrations contribute to other harmful algal blooms (HABs), such as diatoms, that degrade aquatic ecosystems. Thus, removal of total N is a more definitive approach to preventing toxic *M. aeruginosa* blooms and other HABs.

Many novel technologies being piloted for nutrient removal in wastewater effluents go beyond total ammonia removal to the removal of total N with more cost-effective potential than conventional total N removal methods. Total N is removed through deammonification, which utilizes biological nitrification to convert roughly half of the total ammonia concentration to nitrite using AOB in an aerobic process, followed by the denitrification of the remaining ammonia and nitrite to dinitrogen gas (N_2) in an unaerated process that sometimes requires expensive and dangerous carbon inputs (SFBRWQCB, unpublished). However, promising technology using anaerobic ammonia oxidizing bacteria, or Anammox, to denitrify ammonia to N_2 gas (Montalvo et al., 2012) can significantly reduce oxygen and energy inputs by 50% and 60%, respectively, and completely eliminate the need for external carbon requirements (SFBRWQCB, unpublished).

Anammox technology can also be combined with the use of zeolites, an abundant, natural silicate material that adsorbs ammonium, allowing AOB and Anammox to colonize zeolites and create a biofilm

that deammonifies N to N_2 gas (Montalvo et al., 2012; SFBRWQCB, unpublished). This method would allow a passive, downward-flow reactor in which zeolites pass vertically through an aerated section for nitrification of total ammonia through AOB, followed by the passage through an unaerated section allowing Anammox to denitrify the N into N_2 gas, minimizing the need for expansive facility space that may not be available for POTWs.

Anammox are slow growing bacteria, with a doubling time of 11 days, requiring more space for longer retention times (SFBRWQCB, unpublished). Consequently, Anammox technology might not be applicable to most POTWs and their current infrastructure. Nevertheless, POTWs should shift priorities on regulating total N along with P in their wastewater effluents, while monitoring deammonification technologies as they develop for future application.

5.3 Summary

A comprehensive examination of the influences of N, P, and the environmental factors associated with climate change has identified trends behind the drivers of the growth, toxicity, and distribution of *M. aeruginosa*. Effective management strategies in controlling the growth, toxicity and distribution of *M. aeruginosa* should start with refined areas in research focusing on the chemical N forms, concentrations of N and P, and the local environmental factors present during *M. aeruginosa* blooms. Additionally, management efforts in nutrient regulation from POTWs should focus on monitoring developing technologies for efficiently removing ammonium or total N from wastewater effluents.

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