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Molybdenum and Iron Interactions as Micronutrients for Growth of a Freshwater Cyanobacterium, Microcystis Aeruginosa

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology

MOLYBDENUM AND IRON INTERACTIONS AS MICRONUTRIENTS FOR GROWTH OF A FRESHWATER CYANOBACTERIUM, *MICROCYSTIS AERUGINOSA*

(Thesis format: Monograph)

by

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Abstract

Microcystis aeruginosa is an important bloom forming cyanobacterium, responsible for the degradation of freshwater environments through the production of a potent hepatotoxin or the deoxygenation of waters during the decline of the bloom. I investigated the growth, photosynthesis and toxin contents of two strains of *M. aeruginosa*, CPCC 299 and CPCC 300, grown with different nitrogen supplies (nitrate or ammonium) and depleted or replete Molybdenum (Mo) and/or Iron (Fe) concentrations. When Mo and Fe were supplied at growth-replete levels, *M. aeruginosa* grew equally well with nitrate and ammonium. Reducing Fe decreased growth rate efficiency when the cells were supplied with nitrate, but not with ammonium. In contrast, the removal of Mo from the medium did not impair growth or nitrogen utilization rates regardless of the form of nitrogen available but negatively impacted photosynthetic capacity. Experiments designed to assess if excess Fe could "mask" the Mo-limitation by replacing key physiological processes with Fe revealed the opposite was true: addition of Mo alleviated the stresses associated with Fe-limitation.

Keywords

Freshwater, cyanobacteria, toxins, microcystin, harmful algal blooms, nutrients, nitrogen, iron, molybdenum, limitation, co-limitation.

Co-Authorship Statement

This M.Sc. thesis is part of a research project funded by the CREATE program, provided by Dr. Charles Trick and Dr. Irena Creed. I wrote each chapter, which were subsequently edited by Dr. Charles Trick and Dr. Irena Creed. This thesis will be formatted into one manuscript and will be submitted to the journal *Freshwater Biology*, in which I will be the first author, and the co-authors will include Dr. Charles Trick, Dr. Irena Creed and Beatrix E. Czikkel. Each of the authors contributed to the conceptual design, experiment conduction, synthesis of data interpretation, the writing or editing of the final manuscript.

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Finally, thank you to all my colleagues and friends at Western University. Wherever I am after my M. Sc., I will remember all the talks, laughs and even cries we had together. I appreciate your support and accompany through my graduate study.

Dedication

I would like to dedicate this thesis to my mother, Fuli Dai,

and my mother in law, Baorui Zhang,

for their caring of me and my little boy,

donating their time and energy in helping me out of the busy days

when I was adapting to the role of a new mother during my school.

With all of my love, thank you.

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List of Abbreviations

BG-11	Blue-green medium #11
C	Carbon
Chl-*a*	Chlorophyll-*a*
CPCC	Canadian Phycological Culture Centre
cyanoHAB	Cyanobacterial harmful algal bloom
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
Exp.	Exponential phase of growth
FAD	Flavin adenine dinucleotide
μ	Cellular growth rate
α^*	Initial slope of the photosynthesis vs. energy assessment
Fe	Iron
FL	Fluorescence channel of flow cytometry
M. aeruginosa	*Microcystis aeruginosa*
MC	Microcystin
MGD	Molybdopterin guanine dinucleotide
Mo	Molybdenum
N	Nitrogen
N_2	Nitrogen gas
NAR	Respiratory nitrate reductases
NAS	Assimilatory nitrate reductases
NH_4^+ / NH_4	Ammonium
NO_3 / NO_3^-	Nitrate
O_2	Oxygen
P	Phosphorus
P_m^*	Light saturation point of the photosynthesis vs. energy assessment
rRNA	Ribosomal ribonucleic acid
S	Sulfur
Stat.	Stationary phase of growth
TN	Total nitrogen
TP	Total phosphorus
Y	Cell yield

Chapter 1

1 Introduction

1.1 Problem Statement

Cyanobacteria harmful algal blooms (cyanoHABs) are excessively dense populations of cyanobacteria that can lead to a wide variety of adverse environmental conditions in freshwater ecosystems. These blooms alter water quality by increasing the turbidity of aquatic ecosystems and reducing the distribution of light energy. CyanoHABs increase primary production and compete with eukaryotic phytoplankton and aquatic plants. They also disrupt the food chain and subsequent energy/carbon transfer to the next trophic level, because ultimately the unutilized cyanobacterial biomass will sink and decompose, depriving invertebrate and fish populations of oxygen (Paerl and Huisman 2008). In addition, some cyanobacteria produce toxins, which can have serious side effects for humans, including liver, digestive, neurological, and skin diseases (Falconer 1999).

Historically, the development of cyanoHABs has been attributed to excess levels of phosphorus (P) in the environment (Oliver and Ganf 2002, Schindler and Vallentyne 2008). Phosphorus reduction measures appear to have decreased the frequency of cyanoHAB events from the “eutrophication” period of the 1960s and 1970s (Dillon and Vollenweider 1974). However, recent reports of increases in the occurrence of cyanoHABs (Winter *et al.* 2011) (Figure 1.1) have forced researchers to reconsider our conceptual understanding of the factors that stimulate, prolong or maintain cyanoHABs (Vasconcelos 2006, Huber *et al.* 2012). Molot *et al.* (2014) challenged the notion that blooms are only regulated by total phosphorus (TP) and total nitrogen (TN) concentrations. They point out that cyanoHABs are not limited to nutrient-rich, eutrophic systems and that toxic or economically adverse cyanobacteria manage to dominate waters ranging from oligotrophic to mesotrophic to eutrophic systems (Watson *et al.* 1997, Downing *et al.* 2001). It is still not possible to predict the timing, magnitude or longevity of a cyanoHAB. Since N and P are thought to be critical to the biomass accumulations of

all phytoplankton, the TN and TP model fails to explain why cyanoHABs are expanding in area and frequency, while blooms of other types of potentially bloom-forming eukaryotic phytoplankton, physiological competitors to cyanobacteria, have failed to thrive. Other environmental factors potentially serve as secondary regulators of the physiology of cyanoHAB species include temperature (Jacoby and Kann 2007, Paerl and Huisman 2008, Paerl *et al.* 2011), light (Paerl *et al.* 2011), water column stability (Jacoby and Kann 2007, Paerl 2008), cellular buoyancy (Paerl and Paul 2012), and brownification of waters (increase in colored dissolved organic carbon) (Trigal *et al*. 2013). Most of these factors deal with the longevity of the bloom once it has formed but do not influence the initiation phase or the bloom sequence when cyanoHABs may outcompete ecologically similar eukaryotic species.

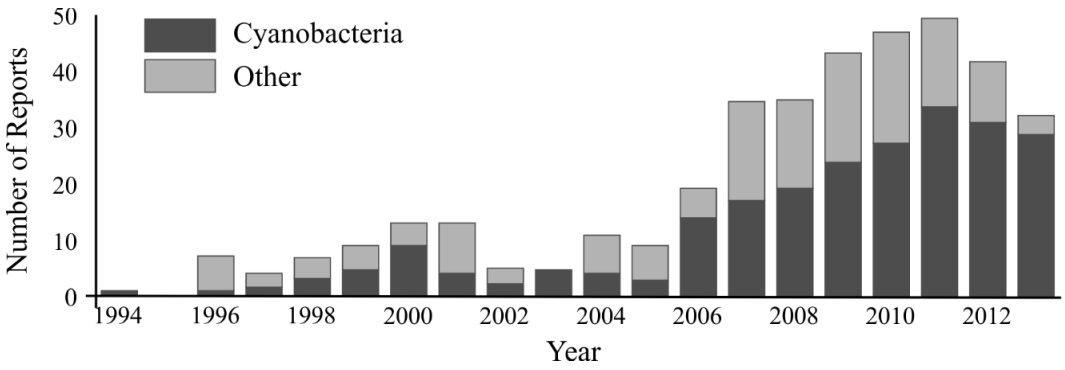

Figure 1.1 Reports of cyanobacterial and other bloom events from 1994 to 2013 in Ontario Lakes (modified from Winter *et al.* 2011).

The effectiveness of cellular use of TN and TP can be regulated by molybdenum (Mo) (Cole *et al.* 1993) and iron (Fe) (Wilhelm 1995), which can contribute to cyanobacterial dominance in blooms over their eukaryotic rivals. As was wonderfully stated in the Molot *et al.* (2014) paper: “Judging by the quantity of literature published in recent years, many scientists must feel that looking at P and N one more time from a different angle could shine enough light to solve the mystery of cyanobacteria dominance in nutrient-enriched waters.” Since cyanobacteria are able to maintain a cellular storage of P, focusing on N alone would be a good start. Here, I hypothesize that limiting levels of these two trace metals, Mo and Fe, modulates the efficiency of the use of the macronutrient N by the globally distributed cyanoHAB species, *Microcystis aeruginosa*.

1.2 The Genus *Microcystis*

Microcystis is one of the most common harmful algal-blooming genera in the world (Moisander *et al.* 2009, Straub *et al.* 2011). While the genus is most often associated with freshwater environments, representative isolates have also been found in estuarine systems and along ocean coasts (Lehman *et al.* 2008, 2013, Davis *et al.* 2009, Miller *et al.* 2010) – although there is some uncertainty whether the "estuarine" and "marine" observations are not simply freshwater cells swept into a saline environment unsuitable for growth, or are only found in low enclaves or embayments of marine seas (i.e., Baltic Sea, Belykh *et al.* 2013). *Microcystis* has a cosmopolitan distribution, yet its distribution within a region is lake specific, so the origin of the genus has drawn some attention (van Gremberghe 2011). Moreira *et al.* (2014) used four molecular markers (16S rRNA, 16S-23S ITS, DNA gyrase ß and *fts*Z) to suggest there are two nodes of origin for *Microcystis*: Africa and Europe (North American strains evolved from the latter).

There is general physical uniformity of species in the *Microcystis* genus. They are generally described as coccoid photosynthetic prokaryotes, with individual cells that range from 1 to 5 µm in diameter and are found either as single cells (less common) or colonies of hundreds of cells in a gelatinous matrix (very common). Initial descriptions were made using microscopic observations by Lemmerman (1907) and Tilden (1910) (see Wehr *et al.* 2015) to generate taxonomic and nomenclatural history, followed by a more pragmatic assessment from Rippka *et al.* (1979). Photosynthetic prokaryotic cells of the primarily unicellular form were placed in the Family Chroococcales. A modern assessment of the 16S rRNA gene sequence data showed that members of this family were widely dispersed and divergent (Turner 1997), and upon reorganization *Microcystis* remained central to the Subsection I of Bergey's Manual of Systematic Bacteriology (Boone and Castenholz 2012). Members of this subsection are defined as unicellular cyanobacteria that divide by binary division, range in size from 0.5 to 30 µm, contain phycobiliproteins in association with chlorophyll-*a* (chl-*a*), and have spheroid or ellipsoid single cell shapes. Reference to colony formation has been removed as this population form varies with environmental conditions.

There are 10 different species commonly listed in the taxonomic genus *Microcystis* (Walsby 1981). *Microcystis aeruginosa* and *M. flos-aquae* are the two most prevalent ecological forms and are often found in the same water samples. These two species are separated from other species of *Microcystis*, because their cells are encased in a mucilage sheath. The two species are distinguished from each other based on the density of cells within a colony: *M. aeruginosa* colonies are loosely formed and easily separated into single cells, and *M. flos-aquae* colonies are densely packed with cells.

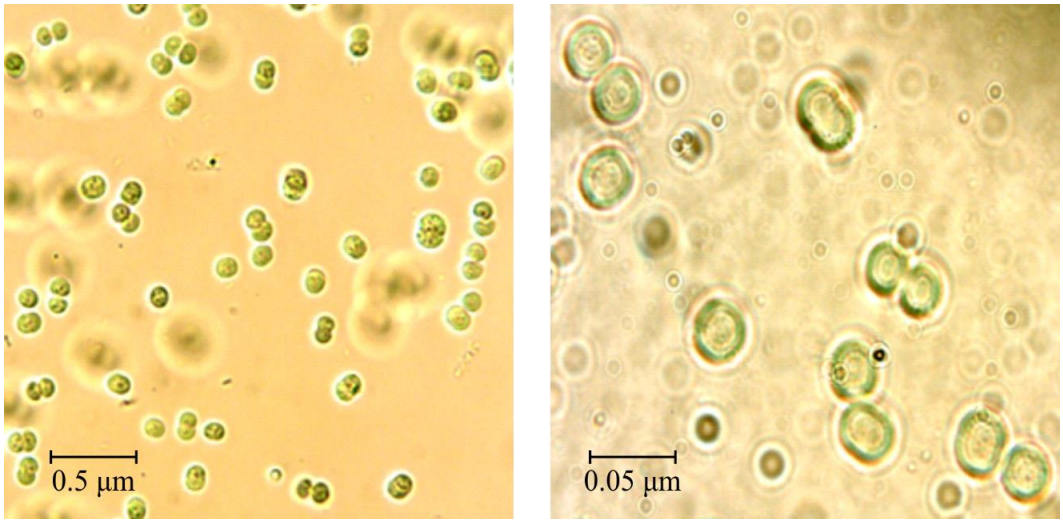

Figure 1.2 *M. aeruginosa* cells under microscope. Figures show one non-toxic strain (CPCC 632) under different microscopic magnifications.

Taxonomic differences aside, the toxic members of the genus *Microcystis* produce a common array of similar toxins: neurotoxins [lipopolysaccharides (LPSs)] and hepatotoxins (microcystins). Microcystin is considered the most dominant cyanotoxin produced due primarily to the ubiquitous distribution of *M. aeruginosa,* the wide diversity of different structural variants of microcystin (~60 variants of this cyclic peptide) and that multiple cyanobacterial species can produce the toxin (van Apeldoorn *et al.* 2007, Neilan *et al.* 2008, Pearson *et al.* 2010).

1.3 Nutrient Requirements

The growth and maintenance of *M. aeruginosa* depends on the supply of macronutrients [Carbon (C), N, P], micronutrients (Mo, Fe, vitamins) and light because it

is a photosynthetic, prokaryotic phytoplankton species. Thus, in a lake system the cyanobacterial growth potential depends strongly on the physical structure of the water column. Cells must be in an area with sufficient and appropriate light levels, while still allowing nutrients generated by decomposition in the sediments of the lake to resupply the euphotic area (light-rich surface) of the water column. Cyanobacterial growth is also strongly regulated by the aquatic ecosystem's relationship with the terrestrial environment (a significant source of both macronutrients and micronutrients). The presence and growth of cyanobacteria are controlled by biotic factors, such as competitors and predators, and abiotic factors, such as temperature, pH and nutrient supply (Moss *et al.* 2013).

Cyanobacteria that make blooms have different physiological and nutritional needs from competing eukaryotic species. The physiology of cyanobacteria gives them an advantage over their eukaryotic competitors: (1) cyanobacteria are generally small and have a higher surface area-to-volume ratio, which influences nutrient scavenging capability when nutrients are low; (2) they are luxury consumers of P, storing it in polyphosphate granules; (3) cyanobacteria are opportunist consumers of N, able to obtain N from a variety of reduced and oxidized forms, with some able to fix dinitrogen (N_2); (4) cyanobacteria can secrete organic molecules to scavenge micro-nutrients when supplies are low (Mo, Fe); (5) they can adjust buoyancy – sinking to achieve access to remineralized nutrients and floating to obtain light energy for photosynthesis; (6) they have a sheath to protect the cell from grazing or desiccation; and (7) they produce secondary metabolites that interfere with grazing or are allelopathic to eukaryotic predators (Fulton and Paerl 2006, Paerl and Fulton 2006). Many of these traits are ancient physiological attributes developed by cyanobacteria in chemical environments dramatically different than modern levels of micronutrient and macronutrients (Gomez-Consarnau and Sunudo-Wilhelmy 2015). As was published in the obituary of Ralph Lewin (1921-2008; phycologist, "discoverer" of *Prochloron*, poet):

"When algae all were bluish-green / and all prokaryotic, / the land (the non-aquatic scene) / was mostly quite chaotic".

From this chaos, arose diversity between cyanobacteria and eukaryotic species.

1.3.1 Macronutrients

Macronutrients are the elements required by a cell that are a major part of the cellular structure (e.g., C is the chemical basis of cell walls, cell membranes, pigments, etc.; C and P form phospholipid membranes etc.) or comprise major cellular biochemicals (e.g., N is required for amino acids, proteins, enzymes, pigments; P is required for the structure of DNA, phospholipids, etc.). As a consequence, these elements make up a substantial proportion of the cell biomass, and cells have a strong requirement for each element to enable the cell biomass to double during binary reproduction.

Since cyanobacteria can take up and accumulate P at rates greater than their cellular needs (luxury uptake) (Paerl and Otten 2013, Ma *et al.* 2014), the logical starting point for understanding variations in the growth and maintenance of cyanobacteria in freshwater systems is understanding N. Nitrogen sources that can be commonly utilized by cyanobacteria include nitrate, nitrite, and ammonium. Some cyanobacteria are also able to assimilate organic forms, such as amino acids (Flores and Herrero 2005) and urea (Valladares *et al.* 2002). Nitrate is a common N source for cyanobacteria and is assimilated by being transported through the cellular membrane and then reduced in a two-step process: reduction of nitrate to nitrite catalyzed by nitrate reductase and reduction of nitrite to ammonium using nitrite reductase (Ohashi *et al.* 2011). The assimilation of nitrate is regulated by the activation of existing cellular nitrate reductase, as synthesizing of new reductase is rare (Avissar 1985). It is also inhibited by the presence of ammonium (Martin-Nieto *et al.* 1989, Ohashi *et al.* 2011). Nitrate assimilation processes are closely related to photosynthesis, as the energy currency adenosine triphosphate and the electron donor ferredoxin are all generated photosynthetically (Flores *et al.* 2005). Thereby, factors influencing photosynthesis may also affect nitrate uptake in cyanobacteria.

Since the utilization of ammonium does not involve enzymes such as nitrate and nitrite reductases, it is the energetically preferred source of N for most algae and cyanobacteria. Ammonia is readily diffusible via the lipid bilayer, while ammonium

requires cationic transporters (mono-component permeases) for uptake, especially at low ambient concentrations (Herrero *et al.* 2001, Ritchie 2013). Urea may represent another natural N source for cyanobacteria. Some species can assimilate urea at very low (less than 1 µM) extracellular concentrations (Valladares *et al.* 2002). There is an adenosine triphosphate-binding cassette type permease urea transporter in the cyanobacterium *Synechocystis* sp. that is similar to those used for ammonium assimilation (Valladares *et al.* 2002), indicating that the assimilations of urea and ammonium share some similarity, but the exact mechanism of urea utilization remains unclear.

Molecular N_2 can be reduced by N-fixing species of cyanobacteria. Biological N_2 fixation is a feature shared by many species of cyanobacteria and is significant for the biosynthesis of cellular nitrogenous compounds, which helps primary productivity in oceans and therefore is important for the biogeochemical history of Earth (Latysheva *et al.* 2012). In aquatic ecosystems, this process supports the growth of phytoplankton not capable of fixing N_2, as well as higher hierarchies of the food web that feed on phytoplankton. In N_2-fixing species, an inducer such as nitrate may be required, but the presence of ammonium can either positively promote N_2 fixation by inhibiting nitrate uptake or negatively affect gaseous N_2 assimilation by repressing nitrate reductase (Martin-Nieto *et al.* 1989).

1.3.2 Micronutrients

In contrast to macronutrients, micronutrients include elements that are required at lower levels by the cell for growth. They regulate the processing of macromolecules in the cell and as such are consumed at low rates or are recycled and reused. As an example, both Fe and magnesium are required in the formation of chl-*a* – Fe is used as a catalyst of heme formation and thus is a micronutrient; magnesium is part of the structure of chlorophyll and is required as a macronutrient (von Wettstein *et al.* 1995). Because of differences in demand for macronutrients and micronutrients, amounts of macronutrients are often discussed based on the availability of elemental mass, while amounts of micronutrients are discussed based on the concept of flux rate. Thus, terms such as "nutrient-limited" growth are reserved for macronutrients (i.e. N-limited vs. P-limited cells). Whereas "nutrient depleted" and "nutrient replete" are employed in the

assessment of micronutrient supply and needs. "Nutrient replete" indicates that a micronutrient is at a level that allows for (near) maximum macronutrient processing, and "nutrient depleted" indicates a low level of available micronutrient that does not allow for the sufficient cell supply to meet the needs of maximum growth.

Cell quota is the content of a chemical element in the cells (Smirnov and Revkova 2002). Cells, particularly cyanobacteria that are steadily dividing, have minimum requirements (cell quotas) to maintain function. As cells initiate cell division, these amounts need to be maintained in daughter cells. Thus, for any division rate there is a corresponding rate of quota resupply needed. Maximum assimilation rates are a function of cell physiology, but the achieved assimilation rates are most likely controlled by the biogeochemistry of the element outside the cell, and it is important to assess if the element is in sufficient concentration to enable saturation of the uptake system (Monod equation growth kinetics, comparable to the more commonly assessed Michaelis-Menten uptake kinetics equation) (Huisman and Hulot 2005). The concentration of the element is determined by both the rate of environmental supply (leaching from catchment, remineralisation from the sediments, atmospheric deposition) and the chemical form of the element since only certain forms of an element can be utilized by cells (also a function of supply, but also impacted by the redox of the water or the complexity of the chemical matrix, particularly the level of complication sites that compete with the cell assimilation site).

1.3.3 Iron, Biological Function and Assimilation

For most micronutrients (boron, zinc, copper, iodine, cobalt), either the cell quota is extremely low or the supply concentrations are high and thus the needs for cyanobacterial growth are usually met. For Mo and Fe, the natural supply rates may not be sufficient to meet the demands for growth of some freshwater cyanobacteria. The possibility that Fe limits the growth rates of phytoplankton has drawn the most attention. Early work on large lakes such as Lake Ontario (Murphy *et al.* 1976) indicated that adding Fe enabled the existing phytoplankton community to grow to a higher density (or at least turn "greener" because the level of chlorophyll in the water is commonly used as a proxy for biomass) (Lean and Pick 1981, Wilhelm 1995). Iron-limitation in lakes has

been clearly demonstrated in a wide variety of lakes of different trophic status (Chang *et al.* 1992, Haraughty and Burks 1996, Coats and Goldman 2001, Shaked *et al.* 2004, Nagai *et al.* 2006, Vrede and Tranvik 2006, Kosakowska *et al.* 2007, Imai *et al.* 2009, Du 2013, Sorichetti *et al.* 2014a, 2014b, Goldberg *et al.* 2015). Thus, low Fe levels may modulate the efficiency of N use in lake systems, and perhaps more frequently than commonly considered (Molot *et al.* 2014).

Iron is one of the most abundant elements in the earth's crust (6% by weight). However, due to variations in the oxidation states of the element (elemental iron Fe°, reduced form ferrous Fe^{2+} and oxidized form ferric Fe^{3+}) and variations in the complexes formed with dissolved organic material, sulfides, carbonates, oxides/hydroxides, the form of Fe that cyanobacteria can transport (non-complexed Fe^{3+} – a.k.a. "free ferric ions") can be exceeding low – particularly in oxic environments where cyanobacteria thrive. In most oxic environments, the highly soluble form of iron (Fe^{2+}) quickly forms $FeCO_3$ and FeS complexes, which in turn are quickly oxidized to form the precipitate $Fe(OH)_3$. Cyanobacteria must intercept the Fe as "free Fe^{3+}" before the oxide is formed. The biogeochemical battle is sometimes referred to as the "ferrous wheel", which indicates the recycling of Fe within the microbial food web. In this case, Fe entering cells regulates N assimilation and photosynthesis. There is a direct link between Fe availability and the key physiological processes of cyanobacteria. Iron is a key component in enzymes and the biochemical constituents of photosynthesis and in nitrate assimilation. Photosynthetic efficiency and apparatus are highly Fe-dependent in both cyanobacteria and plants. In cyanobacteria, electron transportation takes place in the thylakoid membrane. Iron is a key element in the PSI-Cytochrome (b_6f)-PSII electron transport chain (Figure 1.3) and is a part of three major components in photosynthesis: the PSII complex contains two Fe atoms; cytochrome b_6f has four Fe atoms, and PSI contains 16 Fe atoms in the form of FeS clusters, including a [2Fe-2S] cluster that belongs to ferredoxin (Briat *et al.* 2015). In photosystems, 22-23 iron atoms are required in order for the photosynthetic apparatus to function properly (Baulina 2012). While Fe is not a constructional element in chlorophyll, it serves as a synthesis activator of certain enzymes or enzyme cofactors in the biosynthesis of chlorophyll. Chlorophyll contents, as well as the heme enzymes activity, are positively correlated with Fe concentrations (Marsh Jr. *et al.* 1963). When grown

under Fe limitation, phycobilisomes also degrade quickly, with lower binding firmness between chlorophyll to chlorophyll-binding protein. Even though binding ability is reduced, the production of an Fe-deficiency related protein that simulates the chlorophyll-binding protein can enhance the light harvesting of PSI (Boekema *et al.* 2001), which may serve as a protection mechanism.

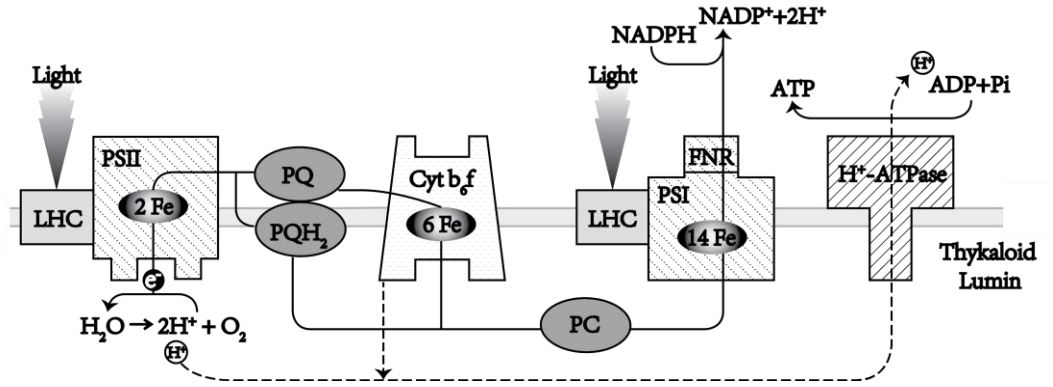

Figure 1.3 Photosynthetic electron transportation between PSII and PSI. Upon light activation, one electron is lost from PSII and transferred to cytochrome b_6f. Plastocyanin carries the electron between b_6f and PSI (LHC: light-harvesting complexes. PQ: plastoquinone pool. PQH_2: reduced PQ. Number of Fe atoms required in each stage/component is shown. Modified from Cavet *et al*. 2003, Briat and Gaymard 2015).

Since Fe levels in nature can be low, cyanobacteria have evolved two responses to maintain growth under Fe deficiency: either decreasing cellular Fe requirements or improving their ability to scavenge ambient Fe (Ferreira and Straus 1994, Wilhelm 1995). Some genes that control the production of Fe-related proteins are tightly regulated by Fe concentration. Cyanobacteria could re-regulate the synthesis of those proteins by reducing the protein biosynthesis and producing substitute proteins (such as the substitution of ferredoxin with flavodoxins) under Fe deficiency (Ferreira and Straus 1994). Secondly, some cyanobacteria employ a high-affinity siderophore system to scavenge Fe when it is limited. Siderophores are ferric-specific chelators with low molecular weight that are synthetized by some cyanobacteria species and can be transported through the cell membrane by transporter proteins (Lange 1974, Murphy *et al.* 1976, Simpson and Neilands 1976, Lammers and Sanders-Loehr 1982, Kerry *et al.* 1988,

Wilhelm and Trick 1994, Wilhelm 1995). There are two types of siderophores: hydroxamate-type siderophores and catechol-type siderophores. Hydroxamate-type siderophores are hydrophilic. They use hydroxamic acid moieties to coordinate Fe molecules (Wilhelm 1995). They are released into the extracellular environment and after binding with Fe, make the Fe-siderophore complexes available for cellular assimilation (Wilhelm and Trick 1994). Catecholate-type siderophores, however, are hydrophobic and after being secreted by the cell, remain at the cell surface. In general, catechol-type siderophores have a higher ferric affinity than hydroxamate ones (Ferreira and Straus 1994, Wilhelm and Trick 1994) (Figure 1.4) Ferrous (Fe (II)) siderophore complexes also exist, but with much weaker binding ability than the ferric specific siderophores (Lewis *et al.* 1995).

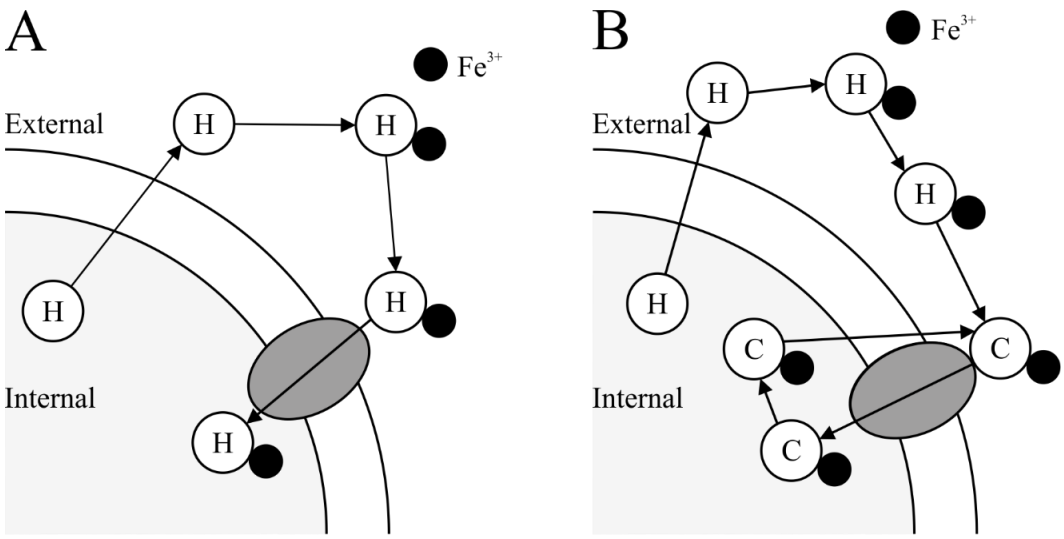

Figure 1.4 Two models of the mechanisms of high-affinity iron acquisition strategies used by cyanobacteria in the aquatic environment. Left: Iron assimilation using hydroxamate siderophores (H) only. Iron that is bound to a siderophore is transferred into the cell through certain transmembrane and intra-membrane proteins. Right: Iron scavenging in presence of both hydroxamate siderophores and catecholate siderophores (C). Iron is transferred from hydroxamate siderophores to catecholate siderophores and into the cell (modified from Wilhelm and Trick 1994).

1.3.4 Molybdenum and Molybdenum Co-factors

Molybdenum has not received the same level of research attention as Fe, but it is also of high cellular need in relation to its low biological availability, controlling the ecology of some cyanobacteria species. The trace metal Mo is essential for numerous N_2-fixation and nitrate reduction systems. In aquatic ecosystems, the primary bioavailable form of Mo is molybdate (MoO_4^{2-}) (Cole *et al.* 1993, Mendel *et al.* 2009). In marine systems, the levels of Mo (well-mixed at about 105 nmol $\cdot$ L^{-1}) are significantly higher than cellular needs relative to the level of nitrate available, but in freshwater systems Mo is usually scarce, typically at 20 nm L^{-1} (Page 1995). In 2012, Mo concentrations ranged from <1 nmol L^{-1} to over 1000 nmol L^{-1} in Ontario lakes (Provincial Water Quality Monitoring Network 2012). Low levels of biologically available Mo limited primary production in Castle Lake, California, and small scale Mo fertilization had positive responses (Goldman 1960).

Molybdenum is important in the catalytic center of essential enzymes such as nitrogenase, nitrate reductase, sulphite oxidase, xanthine oxidoreductases and dimethylsulphoxide reductase (Mendel *et al.* 2009). The latter three are grouped into pterin-based Mo enzymes, which is a big family containing more than 50 enzymes (Mendel *et al.* 2009). Nitrogenase and nitrate reductase are important because they are the key enzymes in N assimilation processes. Most cyanobacteria can carry out N-fixation using the nitrogenase enzyme system. Reduced N is a constructional material in proteins and nucleic acids (Kim and Rees 1994). Therefore, the acquisition of bioavailable N is critical to the growth and survival of all organisms (Kim and Rees 1994, Burgess and Lowe 1996).

Molybdenum-containing prokaryotic nitrate reductases are sub-grouped into three categories: respiratory nitrate reductases (NARs), periplasmic nitrate reductases (NAPs) and assimilatory nitrate reductases (NASs) (González *et al.* 2006). NARs have not been identified in cyanobacteria while NASs are common (Richardson *et al.* 2001). The active site of NASs contain a Mo-bisMGD cofactor, but the electron and molecular properties vary among organisms. Besides the active site, [4Fe-4S] clusters and [2Fe-2S] clusters are contained in the catalytic subunits, and FAD cofactors with flavodoxins and

ferredoxins serve as electron donors (González *et al.* 2006). The roles of Mo and Fe in N assimilation by cyanobacteria are discussed in more detail in Section 1.4.

The level of biologically available Mo affects photosynthesis rates as well. There was an increase in the rate of photosynthesis caused by Mo supplementation in Castle Lake, California (Romero *et al.* 2013), but the role of Mo in photosynthesis remains unclear. Molybdenum levels may shape the community structure of freshwater phytoplankton (Howarth and Cole 1985) and control the growth of cyanobacteria and related blooms by controlling their N and C assimilation.

1.4 Molybdenum, Iron and Nitrogen Assimilation

1.4.1 Nitrate Reductase

Nitrate reduction in cyanobacteria as well as in algae and plants started with the reduction of nitrate to nitrite by the enzyme nitrate reductase. Molybdenum and Fe are both involved in the functioning of this enzyme (Suzuki *et al.* 2000, Schwarz *et al.* 2009). As discussed in section 1.3, Mo is a structural element in the Mo cofactor at the active site (Schwarz *et al.* 2009), and since the reduction of nitrate is in need of large quantities of reducing power, Mo also assists the catalysis of the transfer of electrons (two or multiples of two) to or from the substrate (Cole *et al.* 1993).

Fe plays a redox role as Fe-S clusters, and is responsible for a majority of the electron transportation in nitrate reductase (Timmermans *et al.* 1994). Cyanobacteria use ferredoxin, an Fe-sulfur protein, as an electron donor, with electrons transferred from ferredoxins to Mo cofactors (Flores and Herrero 2005). In Fe-depleted environments some marine phytoplankton (*Emiliania huxleyi*, *Isochrysis galbana* and *Tetraselmis sp.*) reduced their nitrate reductase activities by 15 to 50 percent (Timmermans *et al.* 1994). Despite catalyzing the same chemical reaction, the structure of nitrate reductases as well as synthesizing and catalyzing processes are different in prokaryotes and eukaryotes. In cyanobacteria, Pro-NAS is most common, which contains [4Fe-4S] and [2Fe-2S] clusters in the catalytic subunits. Prokaryotic nitrate reductases belong to the dimethylsulfoxide (DMSO) reductase family, while eukaryotic nitrate reductases are classified into the

sulfite oxidase family and contain a simpler type of cofactor (Moreno-Vivián *et al.* 1999, Stolz and Basu 2002).

1.4.2 Nitrite Reductase and Nitrogen Fixation

Molybdenum and Fe may also be involved in nitrite assimilation and N fixation (Stolz and Basu 2002, Flores and Herrero 2005). The former process is catalyzed by nitrite reductase. Nitrite reductase is similar to nitrate reductase in size and charge, and the Mo active site of nitrate reductase generally favors nitrite binding and reduction as well (Silaghi-Dumitrescu *et al.* 2012). There are several types of nitrite reductase, though. The cytochrome c nitrite reductase is common in bacteria and is ferredoxin-dependent (Flores and Herrero 2005). Cyanobacterial nitrite reductase bears some resemblance with eukaryotic nitrite reductase, both containing [4Fe-4S] clusters (Flores and Herrero 2005). In this enzyme system, electrons are transferred from reduced ferredoxin to the Fe-sulfur cluster, and then to sirohaem, where the reduction of nitrite to ammonium occurs (Flores and Herrero 2005).

Biological N_2 fixation is catalyzed by Mo- and Fe-containing nitrogenase (Romero *et al.* 2013). More than three classes of nitrogenase have been identified based on the heterometal located in the active site (Mo, Fe or Vanadium) (Mendel 2009, Yoshizawa 2009). Molybdenum nitrogenase is the best characterized of these, which consists of a Fe protein and a MoFe cofactor at the active site. Similarly as in nitrate and nitrite reductase, the Fe protein is capable of transferring electrons to the Mo-Fe protein for substrate reductions (Burgess and Lowe 1996, Mendel 2009).

1.4.3 Interchangeability of Molybdenum and Iron

Non-Mo nitrate reductases do exist (Leigh and Jimenez-Tenorio 1991, Stolz and Basu 2002, Romero *et al.* 2013). Many anaerobic archaea and bacteria can utilize tungsten instead of Mo for growth (Schwarz *et al.* 2009). Such systems have been found in *Pseudomonas isachenhovii* and archaeon *Pyrobaculum aerophilum*, which indicates the existence of alternative systems (Stolz and Basu 2002). Stolz and Basu (2002) also described a Heme-C-containing enzyme complex in the bacterium *Geobacter metallireducens* that contains cytochrome c, which has a heme-molecule coordinating a

central Fe atom and can complete nitrate and nitrite reductions without the involvement of Mo (Murillo *et al.* 1999, Stolz and Basu 2002).

Iron may be the only transition metal needed in the assimilation of N. Iron, instead of Mo, may serve as the binding site in the FeMo-cofactor, and Mo is not required in this system (Anderson *et al.* 2013). There is an Fe complex that can catalyze the reduction of N to ammonium without the involvement of Mo (Anderson *et al.* 2013). Fe can be exchangeable with Mo in the functioning of nitrogenase (Leigh and Jimenez-Tenorio 1991). An Fe (II) dinitrogen complex was proposed, and a reductive cycle for N gas was constructed by adjusting hydrogen ion concentrations in the environment (Leigh and Jimenez-Tenorio 1991). Even though this reaction was not observed in real organisms, the system suggested that Fe-only nitrogenase may exist as more ancestral nitrogenase than the ones containing Mo (Leigh and Jimenez-Tenorio 1991).

1.5 Cyanobacteria and Microcystin

The factors that regulate the growth of cyanobacteria are also critical in regulating the negative health impacts of the presence of cyanobacteria in lakes. CyanoHABs have become increasingly common in fresh (lakes, ponds, rivers and reservoirs) and brackish (seas, estuaries, and lakes) waters throughout the world (Carmichael 2001, O'Neil *et al.* 2012). Anthropogenic nutrient input of P, N and organic compounds may increase the frequency and severity of cyanoHABs. A low N:P ratio and low CO_2 availability also contribute to cyanobacterial dominance (Rastogi *et al*. 2014).

CyanoHABs cause harm either due to the production of toxins and toxic metabolites released by HAB species or through the development of high biomass that affects co-occurring organisms and alters food web dynamics (Anderson *et al.* 2002, Havens 2008). Approximately 50% to 75% of reported cyanoHABs were related to toxin production, usually with more than one toxin variant (Malbrouck and Kestemont 2006). Some algal toxins are extremely potent, causing poisonings at concentrations as low as a few hundred cells per litre (Havens 2008). Toxins released by cyanobacteria are collectively called cyanotoxins, which pose risks to ecosystem sustainability and seriously threaten human health by inducing liver, digestive, neurological, and skin

diseases (Falconer 1999). The mechanisms of their toxicity are poorly understood, but they may function as protective compounds like anti-herbivore compounds in vascular plants (Carmichael 1992). Based on the detection method, cyanotoxins can be grouped into two categories: cytotoxins, which can be detected using cultured cells, and biotoxins, which are more lethal and must be detected using whole organisms (Carmichael 1992). Organisms in more than 40 genera are responsible for cyanotoxin poisonings, including *Anabaena*, *Aphanizomenon sp.*, *Cylindrospermopsis sp.*, *Lyngbya sp.*, *Microcystis sp.*, *Nostoc sp.*, and *Oscillatoria sp.* (Carmichael 2001).

Microcystins are a family of monocyclic hepatotoxins produced by several genera of freshwater cyanobacteria, primarily *Anabaena* and *Microcystis sp.* (Dawson 1998, Brittain 2000, Rastogi *et al*. 2014). More than 85 microcystins have been identified from both cultured and natural strains, but microcystin-LR is by far the most common (Rastogi *et al*. 2014). There are over 40 steps in the biosynthesis of microcystins, which are non-ribosomal, built by large multi-enzyme complexes consisting of polyketide synthases (PKS), non-ribosomal peptide synthetases (NRPS) and some tailoring enzymes (Rastogi *et al*. 2014). Orr and Jones (1998) observed a linear correlation between toxin production rate and growth rate. At all times cell division rates were almost equal to microcystin production rates, and microcystin content remained constant or only declined slightly during the stationary and death phases. The highest microcystin cell quota was observed in the late exponential or early stationary phases (Orr and Jones 1998). Similarly, in most *Microcystis*-dominant cyanoHAB events, microcystin levels peak at the collapse of the bloom (Malbrouck and Kestemont 2006).

1.6 Thesis Questions, Hypotheses, and Objectives

The supply rates of N and P are thought to be the predominant drivers of cyanoHAB biomass (Schindler *et al.* 2008, Schindler and Hecky 2009, Lewis *et al.* 2011, Paerl *et al.* 2011, Michalak *et al.* 2013). The relationship between macronutrient loading and climate change parameters (temperature, length of season, hydrologic pattern) are thought to change and perhaps enhance the probability of cyanoHAB formation and maintenance (McQueen and Lean 1987, Molot *et al.* 2014). When macronutrient supplies are high, the availability of trace metals could limit the assimilation of macronutrients by

cyanobacterial cells. Here, I hypothesize that the low availability of two trace metals, Mo and Fe, negatively impact the conversion of macronutrients into cell biomass. The aim of this study is to examine the effects of low levels of available Mo and Fe on the growth, photosynthesis and toxin production in bloom-forming freshwater cyanobacteria. Using the ubiquitous cyanoHAB forming *M. aeruginosa* as a model species, the primary research objectives in this thesis are:

(1) Evaluating the cellular quota for Mo and Fe of *M. aeruginosa* cells grown on different N sources;

(2) Examining the physiological changes in growth and photosynthesis associated with Mo- and Fe-depleted growth conditions;

(3) Exploring if there is a relationship between Mo-limitation and the cellular need for Fe in *M. aeruginosa*.

Five experiments were performed on two toxin-producing strains of *M. aeruginosa* to test the research objectives:

(1) Establishing the optimal growth conditions for *M. aeruginosa* under different forms of N.

(2) Determining the levels of Mo in the medium that reduce the growth and photosynthetic characteristics of *M. aeruginosa*;

(3) Determining the levels of Fe in the medium that reduce the growth and photosynthetic characteristics of *M. aeruginosa*;

(4) Determining if the cellular demands for Mo and Fe are independent or if one element could supply the needs of the limiting nutrient.

(5) Determining what effects replenishing Mo and Fe have on cultured cells.

Chapter 2

2 Materials and Methods

The species tested throughout this project was *Microcystis aeruginosa*, a common freshwater cyanobacterium. *Microcystis aeruginosa* is responsible for a majority of freshwater cyanoHABs, and its production of hepatotoxic microcystins leads to more serious consequences during and after cyanoHABs (Lifshits *et al*. 2012). Therefore, biomass, photosynthetic capability and microcystin production were observed in cell cultures.

2.1 Cultures, Media and Culture Maintenance

For this study, two toxic isolates of *M. aeruginosa*, CPCC 299 and CPCC 300, were tested. They were originally collected from Pretzlaff Pond (Alberta, Canada) by E. Prepas and A. Lam on August 7th, 1990 and have been maintained by the Canadian Phycological Culture Centre (CPCC) at the University of Waterloo, formerly the University of Toronto Culture Collection of Algae and Cyanobacteria (UTCC) (CPCC 2013). They are among the most common bloom-forming strains. They have been proven to be injurious to the liver (hepatotoxic) and produce microcystins at 204 µg per gram dry weight and 415 µg per gram dry weight, respectively (CPCC 2013). I received the isolates from the CPCC in April 2014, and the first inoculation was carried out with 1:5 inoculum: media inoculation ratios.

All experiments and culture maintenance were conducted in batch cultures. Modified BG-11 was used as the culture medium in all experiments (Rippka *et al.* 1979) (Appendix A and Section 2.2, below). Cultures were maintained and the majority of experiments were conducted in 250 mL Erlenmeyer flasks containing 100 mL medium (150 mL Erlenmeyer flasks containing 50 mL medium were used for the trace metal replacement experiment). Flasks were capped with non-absorbent cotton and cheesecloth plugs and aluminum foil caps to eliminate contamination yet allow an intermediate degree of gas exchange (Lee and Michael 1991). Cultivation took place under continuous illumination with light irradiance of ~60 µmol photons m^{-2} s^{-1} that was

supplied by cool white fluorescent light lamps, measured with a Quantum Scalar Laboratory (QSL) 2100 radiometer (Biospherical Instruments Inc.) equipped with a 1.9 cm Teflon® 4 π spherical optical collector.

Stock solutions of media (1000× concentrated) were stored in 250 mL Nalgene™ polycarbonate bottles. For the Mo and Fe replenishment experiments, Mo and Fe stock solutions were microwave-sterilized (1250 watt Panasonic® Nn-sn933w oven) on "high" for a total exposure of 10 minutes. A time sequence of 3 min, 2 min, 3 min and 2 min, with agitations in-between, brought the solutions to near boiling but without bubbling. This method avoids trace metal contamination from the autoclave (Keller *et al.* 1988).

To avoid physiological changes and adaptations, including loss of toxin production, caused by perpetual maintenance over long periods (Andersen *et al.* 2005), inoculations into fresh media were conducted every 7 to 10 days. The inoculation ratios were calculated based on the existing culture density to make the starting cell concentration approximately 1,000,000 cells per litre. Prior to the initiation of experiments, cultures were allowed to acclimate to the experimental conditions for two weeks.

2.2 Experimental Procedures

Five experimental sets were conducted with *M. aeruginosa* culture strains CPCC 299 and CPCC 300. For each experiment, cell density and chl-*a* fluorescence were measured twice from one replicate. These parameters were recorded each day (every 24-30 hours) (except for the replenishment experiment, which were recorded every other day), starting from the day of inoculation (Day 0) and continuing until cells entered the stationary phase of growth (Day 9 for Experiment 1, Day 10 for all other experiments). Photosynthetic activities were monitored during the mid-exponential phase (5^{th} to 6^{th} day after inoculation), determined using one measurement from each of two replicates. Microcystin concentrations were only measured for the cells after Mo and Fe were replaced in early stationary phase, measured once from each of two replicates.

The key variable that defined each experiment was the elemental composition of the medium. The basal medium was modified BG-11 media (Appendix A). Experiment 1 assessed the capacity of the strains to grow on different N sources: nitrate (10 mM), ammonium (10 mM) or urea (5 mM $Co(NH_2)_2$). In Experiment 2 (Mo-limitation), cells were first cultured in Mo-free media with nitrate or ammonia as N sources for five days to remove their cellular Mo stores and to get acclimated. Cultures were then inoculated into media with the following Mo concentrations in the form of $Na_2MoO_4 \cdot 2H_2O$: 0 mol L^{-1}, 1×10^{-16} mol L^{-1}, 1×10^{-14} mol L^{-1}, 1×10^{-12} mol L^{-1}, 1×10^{-10} mol L^{-1}, 1×10^{-8} mol L^{-1} and 1×10^{-6} mol L^{-1} (the Mo-satiated control). In Experiment 3, (Fe-limitation), cells were starved in Fe-free media with nitrate of ammonia for five days prior to inoculating into media with different Fe concentrations (in the form of ferric citrate): 0 mol L^{-1}, 2×10^{-14} mol L^{-1}, 2×10^{-12} mol L^{-1}, 2×10^{-10} mol L^{-1}, 2×10^{-8} mol L^{-1}, 2×10^{-6} mol L^{-1} and 2×10^{-5} mol L^{-1} (the Fe-satiated control). In Experiment 4, (Mo and Fe co-limitation) cells were first grown in media with Mo and Fe omitted so that cells consumed their cellular storages, and then inoculated into fresh Mo-free BG-11 media with various Fe levels (Experiment 4): 0 mol L^{-1}, 2×10^{-14} mol L^{-1}, 2×10^{-12} mol L^{-1}, 2×10^{-10} mol L^{-1}, 2×10^{-8} mol L^{-1}, 2×10^{-6} mol L^{-1} and 2×10^{-5} mol L^{-1} (control). In Experiment 5 (replenishment), inoculums were taken from the Experiment 4 (co-limitation) cultures on Day 7 with the following concentrations: [Fe] = 2×10^{-6} mol L^{-1}, [Fe] = 2×10^{-10} mol L^{-1} and [Fe] = 2×10^{-14} mol L^{-1}, which were then inoculated with (A) an additional 1×10^{-6} mol L^{-1} Mo, (B) an additional 2×10^{-6} Fe, or (C) into media without Mo or Fe. Microcystin was measured using an enzyme linked immunosorbent assay (ELISA) on Day 7 to capture toxicity during the early stationary phase from the three treatments in Experiment 5, plus a fourth treatment that had an additional 1×10^{-6} mol L^{-1} Mo and an additional 2×10^{-6} Fe. The complete experimental procedures are shown in Figure 2.1.

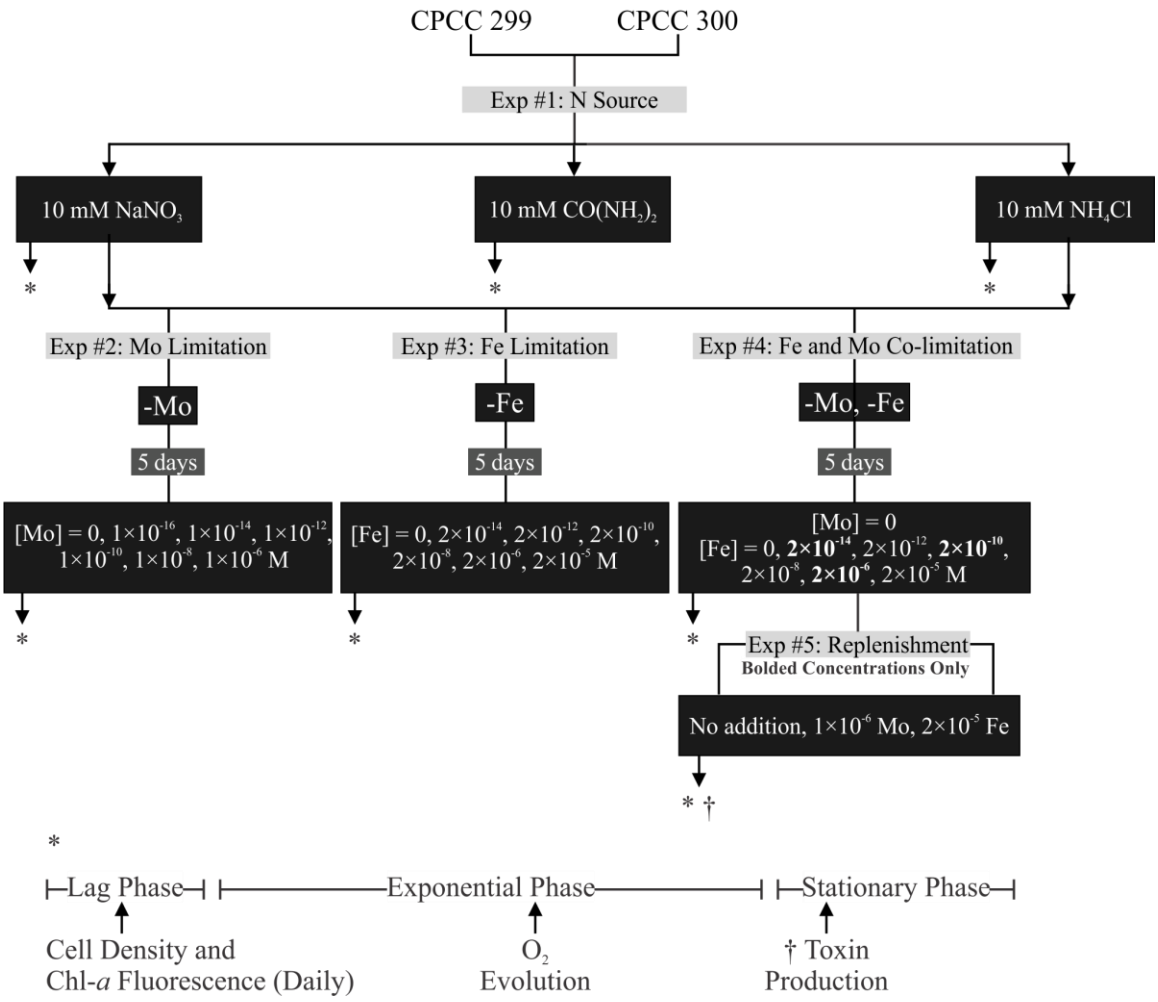

Figure 2.1 Experimental procedures: Experiments started from CPCC 299 and CPCC 300 cultures in complete BG-11 media. For each experiment, the forms of N and concentrations of Mo and/or Fe were manipulated to meet certain limiting conditions. Cells in Experiment 1 (N-limitation) were maintained for nine days with daily observations. Cells in Experiments 2, 3 and 4 (Mo-, Fe- and co-limitation) were maintained for ten days with daily observations. Cells in Experiment 5 (replenishment) were maintained for ten days with observations every other day. Microcystin toxin production was observed in the three Experiment 5 treatments, as well as a fourth treatment that had an additional 1×10^{-6} mol L-1 Mo and an additional 2×10^{-6} Fe

2.3 Methods of Analysis

2.3.1 Flow Cytometry

Cell counts and chl-*a* fluorescence were determined at the start (t=0) and then daily for the duration of the experiment using Turner Designs PhytoCyt™ flow cytometer (now marketed as the BD Accuri C6® flow cytometer). A flow cytometer measures multiple parameters of cells or other particles in a liquid stream moving through a laser-activated detector system. This method is widely used to differentiate algae, cyanobacteria and other populations according to their fluorescence and morphology (Veldhuis and Kraay 2002, Peniuk *et al.* 2015). As a cell passes through the laser beam, light enters the cell and is reflected and refracted by cell structures and/or absorbed by pigments. Fluorescence emitted at different wavelengths is quantified using an array of specific detectors. The refracted or scattered light is measured by a photodiode that is forward but adjacent to the laser beam. From this, the number of particles of a known size and granularity are counted using the forward-scatter method. Similarly, chl-*a* concentrations within a particle can be observed by exciting a particle with a 488 nm laser beam and measuring the resulting red chlorophyll fluorescence emission at > 650 nm (red, FL3 detector) (Rogers *et al.* 2012).

Sampling for flow cytometry involved transferring 1 mL of the culture solution to 12 mm × 75mm VWR® borosilicate glass culture tubes. All sampling was conducted in laminar hood to avoid contamination. Cultures were re-suspended by swirling before sampling. Samples were re-suspended by vortexing prior to flow cytometry. For growth curves, each flask was sampled twice each day.

Samples were analyzed on fast rate (66 µL m^{-1}) for 30 µL in the flow cytometer. Debris was electronically eliminated from the assessment by gating (selecting only particles with a measureable level of chlorophyll). Using a 670 nm long filter (FL3, 488 nm excitation), the chl-*a* fluorescence of cyanobacteria was presented, and the number and mean fluorescence of particles with strong signal were recorded and gated, giving the number of cells ml^{-1} and the average relative fluorescence of each particle due to chl-*a* content.

2.3.2 Photosynthesis Analysis – Light Specific Oxygen Evolution

Photosynthesis measurements were conducted using a Hansatech Chlorolab 2 System. Light was generated by a LH11/2R light source at 650 nm. The light scheme followed a pre-entered illumination pattern (Table 2.1). Experiments, where photon fluxes changed from 0 to 800 µmol photons m^{-2} s^{-1} over a 26-minute exposure were carried out. Measurement procedures followed the operational manual provided by the manufacturer. A liquid-phase calibration was performed each day before measurements.

Table 2.1. Process flow diagram table for photosynthesis measurements.

Incubation time (min)	0	4	7	10	13	16	18	20	22	24
Photon fluxes (µmol m^{-2} s^{-1})	0	20	40	60	80	100	200	400	600	800

Light specific oxygen evolution rates were determined by averaging steady states of photosynthesis and respiration (determined by changes in dissolved O_2 levels) during each period of a defined illumination level. An initial measurement in the dark (lasting for four minutes) provided the respiration rate ($R_{resp.}$) of the sample. In order to calculate cellular photosynthesis, cell counts were conducted using a Turner Designs PhytoCyt™ Flow Cytometer and results were normalized to µmol O_2 per 10^9 cells per hour. The photosynthetic performance of the cells was evaluated using the P vs. E (photosynthesis over irradiance) curve generated for each measurement (see section 2.4).

2.3.3 Toxin Quantification

The concentrations of microcystin ([MC]) in cultures were determined by indirect competitive ELISA using an Enzo Life Sciences® Microcystins (Adda specific) ELISA kit. ELISA methods offer one of the best options for monitoring microcystins, because they are relatively fast, inexpensive and commercially available (Preece *et al.* 2015). In ELISA, microcystins in the sample compete with a microcystin-protein analog for binding sites of the anti-microcystin antibodies. A color signal is generated that is inversely proportional to the microcystin concentration present in a sample. As there are more than 80 variants of microcystin, the ADDA kit is specific for a variant on the C20 of the cyclic heptapeptide allowing for the assessment of the most toxic form (Fisher *et al.* 2001).

Analysis procedures followed the protocol published in the instruction manual from the Enzo Life Sciences® ELISA kit. Samples and standards (50 µL) were seeded in duplicates in a 12 × 8 microtiter plate in which microcystins-protein analog had been immobilized (Figure 2.2). The plate was incubated, washed and color-labeled. Absorbance was read at 450 nm on a Versamax microplate reader (Molecular Devices®). A standard curve was constructed to calibrate each assay ($R^2 > 0.99$) using known standards (0, 0.15, 0.4, 1, 2, 5 ppb). The microcystin concentration of samples was determined using the formula for a standard curve (in the unit of ppb or ng mL^{-1}). Cell density was determined using flow cytometry, and cellular microcystin content was calculated for each sample (in the unit of fg $cell^{-1}$).

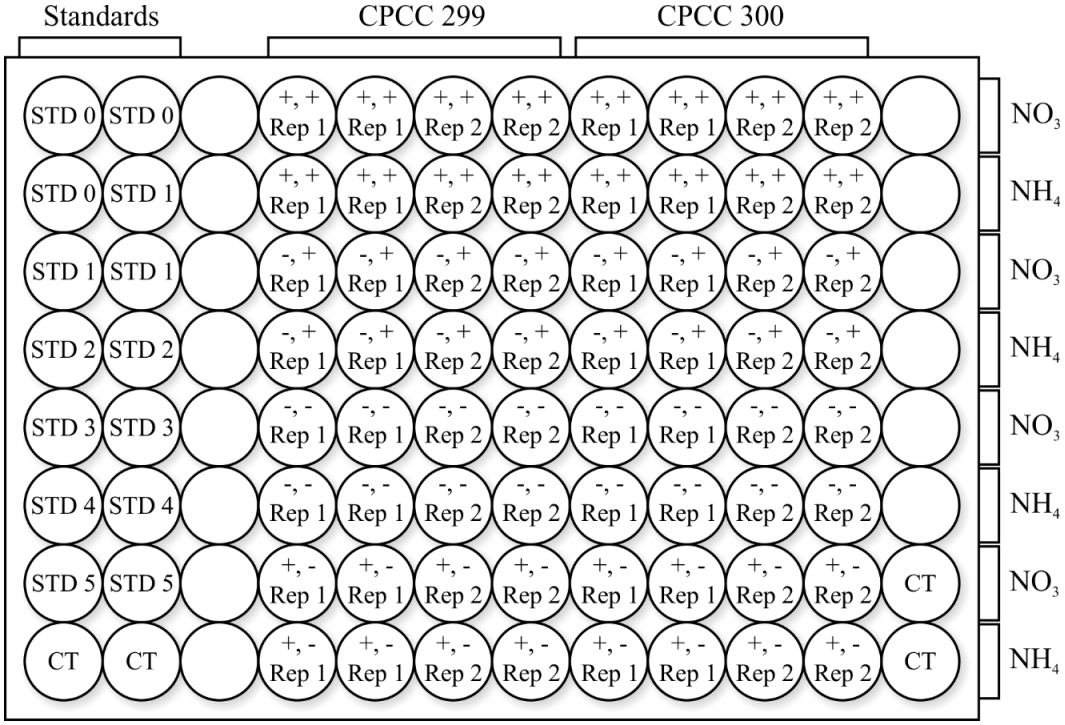

Figure 2.2 ELISA plate design. STD: standard solution. CT: control solution (0.75 ppb). Rep 1 and Rep 2 represent the first and second replicates with each replicate sampled twice. The first +/– sign indicates the addition of Mo. The second +/– sign indicates the addition of Fe. To avoid the interference between standards and samples, wells in between were left empty.

2.4 Data Analysis

2.4.1 Calculating Growth Parameters

Growth rate was determined during the exponential phase using the following equation:

$$\mu = \frac{lnN_2 - lnN_1}{t_2 - t_1}$$

where N_2 and N_1 represented biomass at time t_2 and t_1, which are cell densities (mL^{-1}) in the present study but could also be chl-*a* fluorescence (written as F_2 and F_1) in situations where chlorophyll levels do not change throughout the experiment (Levasseur *et al.* 1993, Mikulec *et al.* 2015). Three growth rates were calculated for each replicate.

Doubling times (T_d) and doublings per day (RT_d, as doublings per day, equal to reciprocal doubling time) were calculated from growth rates using the following equations:

$$T_d = 0.6931/\mu,\ RT_d = \mu/0.6931$$ (Mikulec *et al.* 2015)

Cell yield was calculated using

$$Y = \overline{C_{max}}$$

where $\overline{C_{max}}$ was the average of two or three adjacent maximum cell counts during the stationary phase based on the growth curve, giving two or three values for each nutrient condition.

For these experiments where the level of the growth-limiting nutrient is known (N at 10 mM), the nutrient use efficiency can be determined and compared amongst all experimental outcomes. Nutrient use efficiency is a simple means to compare how much cell yield (biomass) is generated under the same level of a limiting nutrient. Experimental conditions could require the cell to allocate energy or C differently, and thus achieve a lower end-of-experiment cell yield and, by extension, show reduced nutrient use efficiency.

2.4.2 Light Specific Oxygen Evolution and P-E Curve Analysis

The average gross oxygen evolution rate ($R_{O_2-gross}$) in photosynthesis was calculated using the sum of the oxygen production rate (R_{total}) and the absolute value of the respiration rate:

$$R_{O_2-gross} = R_{total} - |R_{resp}|$$

Oxygen evolution rates were normalized using the following equation:

$$P_{O_2}\ (standardized) = \frac{P_{O_2}\ (sample)}{(Sample\ Volume) \cdot (60min) \cdot (Cell\ Density) \cdot 10^9}$$

where P_{O_2} represents oxygen evolution rates calculated from the photosynthesis-irradiance response (P-E) curve. Normalized O_2 evolution was expressed as micromoles of O_2 released per hour per 10^9 cells in the light chamber. Sample volume in this study was 1.5 mL. $P_{O_2}\ (sample)$ was expressed as micromoles of O_2 released per minute in the light chamber.

Photosynthesis-irradiance response curve analysis was based on Sakshaug *et al.* (1997) (Figure 2.3). Two values were obtained from the P-E curve to represent the photosynthesis capability of cell. First is the initial slope (α*) (slope of the first (PAR=0) and third (PAR=40) points on the O_2 evolution curve); second is the light saturation point (P_m*) (average of two adjacent maximum values). The light absorption rate α* is a key description of light-limited photosynthesis, whereas P_m* is a major definition of light-saturated photosynthesis. Both parameters are broadly correlated to the photosynthetic pigment content (MacIntyre *et al.* 2002).

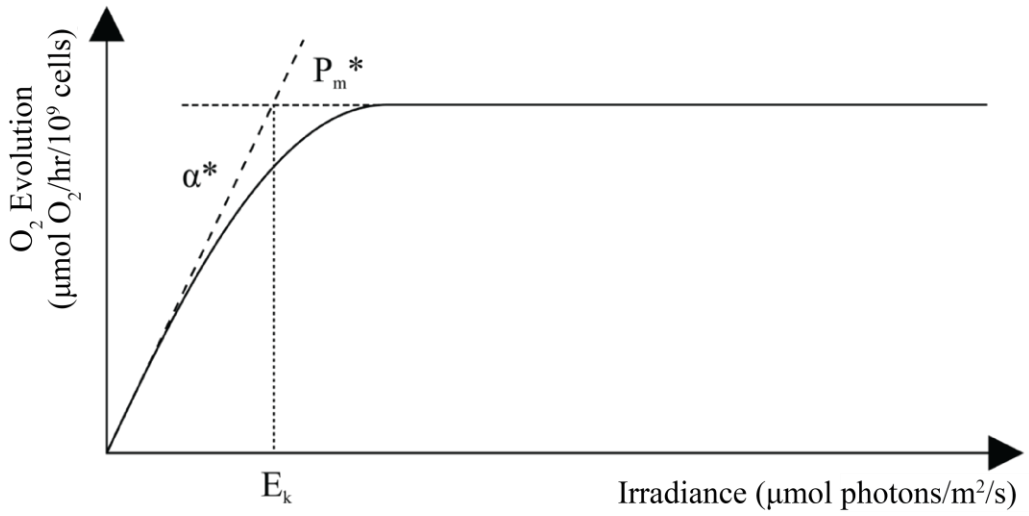

Figure 2.3 P-E curve analysis (photosynthesis (P) versus irradiance). Initial slope (α*) shows the maximum O_2 production rates. Light saturating point (P_m*) represents the maximum achieved rate of O_2 production. E_k is the light-saturation parameter (E_k = P_m*/α*).

2.4.3 Cellular Microcystin Content Analysis

Mean absorbance at ʎ = 450 nm of each standard was calculated. The %B/B_0 value was calculated by dividing the absorbance value of each sample by the zero standard ([MC] = 0 ppb) mean absorbance value of each standard. A standard curve was constructed using the %B/B_0 values of standards and their known microcystin concentrations. The following formula was used to construct the standard curve:

$$A = 0.755[MC]^{-0.369}, R^2 = 0.9917$$

or

$$[MC] = (\frac{A}{0.755})^{-\frac{1}{0.369}}, R^2 = 0.9917$$

The absorbance of samples (A) was then converted to microcystin concentration ([MC], unit: ppb) using the equations above. The microcystin concentration of the positive control ([MC] = 0.75 ppb) gave a value of 0.865 ppb, which was within the acceptable error range of ± 0.185 ppb. The cellular microcystin content was determined

by dividing the microcystin concentration of each well by the corresponding cell concentration measured.

2.4.4 Statistical Analysis

Standard errors were calculated using the following equation:

$$SE_x = \frac{s}{\sqrt{n}}$$

where SE_x stands for the standard error of the samples, *s* stands for the standard deviation of the samples, and *n* represents the number of readings.

Chapter 3

3 Results

3.1 Experiment 1: Nitrogen-Limited Growth and Photosynthetic Capacity

Growth of the two strains of *M. aeruginosa* strains CPCC 299 and CPCC 300 under different forms of N was examined to confirm optimal growth conditions (Figure 3.1). It was critical to assess growth under these otherwise replete nutrient conditions because the achieved growth rates and yields represent the optimal growth against which growth under other experimental conditions will be compared. The medium used (modified BG-11) contained lower levels of N compared to phosphate than the original recipe (10 mM N, 0.175 mM P vs. 17 mM N, 0.175 mM P). By reducing the initial supply of N, the medium contained 10:1 N:P (molar) instead of 17:1 N:P (molar), the latter providing a ratio of nutrients where P could be equally limiting the growth compared with N (Wang and Moore 2014). Thus the lower ratio of 10:1 ensures that N will be used up in the medium (N-limited), leaving unused P (P-replete).

The two strains of *M. aeruginosa* performed similarly when provided with nitrate and ammonium as the N source in the medium. Maximum growth rates for the CPCC 299 and CPCC 300 were 0.411 and 0.401 day^{-1}, respectively, on nitrate and 0.363 and 0.310 day^{-1}, respectively, on ammonium (Table 3.1). The cells provided with ammonium as a N-source had a longer lag phase, even with a period of adaptation to the new nutrient source, and entered the stationary phase after a longer period (Figure 3.1). When provided with urea as the sole N-source, growth rates were 0.062 and 0.066 day^{-1} for strains CPCC 299 and CPCC 300, respectively. There was no indication that N was used by these strains (Figure 3.1). It is possible that urea supplied at 10 mM is inhibitory to *M. aeruginosa*. No further experiments were performed to discern the mechanisms leading to a low growth rate when supplied with urea.

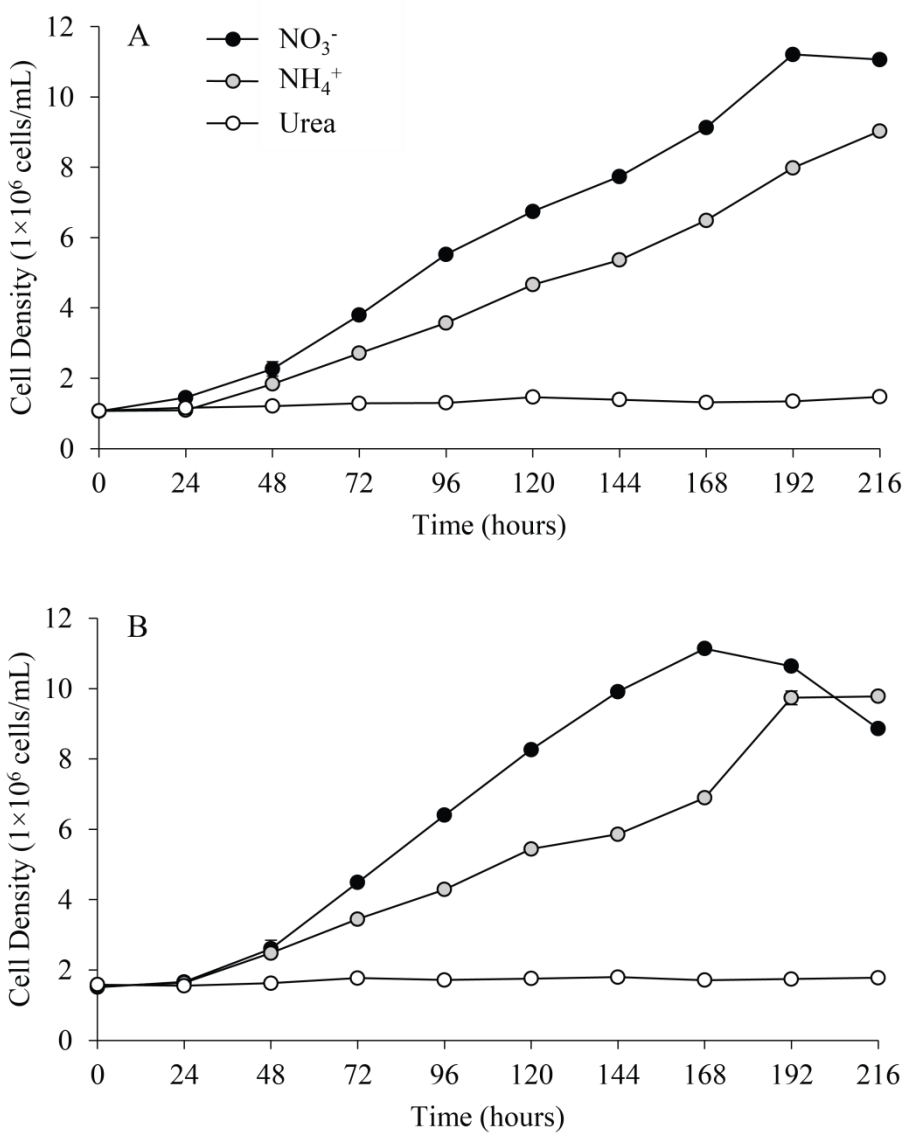

Figure 3.1 Representative growth curves for *M. aeruginosa* culture strains CPCC 299 (A) and CPCC 300 (B) grown in media with nitrate (10 mM NO_3^-), ammonium (10 mM NH_4^+) and urea (10 mM $CO(NH_2)_2$) as N sources. The corresponding growth rates (μ) and yields (Y) are summarized in Table 3.1.

Table 3.1 Growth and photosynthesis parameters of two *M. aeruginosa* strains (CPCC 299 and CPCC 300) grown on 10 mM nitrate, 10 mM ammonium and 10 mM urea [average ± standard error]. Summary of units for each parameter: μ is the growth rate, presented as doublings day^{-1} (three measurements from one replicate); Y is the average concentration of cells mL^{-1} achieved during the stationary phase of growth (two or three measurements from one replicate); chl-*a* $cell^{-1}$ is the relative chlorophyll-*a* fluorescence per particle from the flow cytometer assessment (two measurements from one replicate); Exp. 96h indicates that exponentially growing cells after 96 hours of incubation were assessed; Stat. 192h indicates that cells from the stationary phase of growth (192h) were assessed; α* is the initial slope of the light dependent phase of photosynthesis (μmoles of O_2 evolved 10^9 $cells^{-1}$) (one measurement from each of two replicates); P_m* is the maximum rate of photosynthesis under lights-saturating conditions (μmoles of O_2 evolved h^{-1} 10^9 $cells^{-1}$) (one measurement from each of two replicates).

CPCC 299

N	Growth		Chl-*a* $cell^{-1}$		Light specific O_2 evolution	
	μ	Y	Exp. 96h	Stat. 192h	α*	P_m*
NO_3^-	0.411 ± 0.020	1.11E+07 ± 7.20E+04	6804 ± 24	6901 ± 29	0.224 ± 0.017	28.209 ± 5.886
NH_4^+	0.363 ± 0.053	8.51E+06 ± 5.24E+05	3997 ± 10	3311 ± 6	0.342 ± 0.086	24.746 ± 2.274
Urea	0.062 ± 0.001	1.43E+06 ± 3.71E+04	5900 ± 13	1574 ± 1	0.049 ± 0.005	9.538 ± 1.713

CPCC 300

N	Growth		Chl-*a* $cell^{-1}$		Light specific O_2 evolution	
	μ	Y	Exp. 96h	Stat. 192h	α*	P_m*
NO_3^-	0.401 ± 0.055	1.09E+07 ± 2.51E+05	6032 ± 42	7541 ± 282	0.255 ± 0.107	26.902 ± 8.244
NH_4^+	0.301 ± 0.042	9.76E+06 ± 2.12E+04	3306 ± 13	3003 ± 4	0.170 ± 0.025	19.428 ± 3.432
Urea	0.066 ± 0.012	1.78E+06 ± 1.99E+04	4377 ± 132	1361 ± 4	0.051 ± 0.003	8.643 ± 1.177

Light specific oxygen evolution of the isolates grown on single sources of N (nitrate, ammonium and urea) strongly reflected the growth curves. For strain CPCC 299, the light specific oxygen evolution parameters were indistinguishable from each other between the two positive nutrient sources: nitrate and ammonium. Only strain CPCC 300 grown under ammonium had a reduced photosynthetic rate (Figure 3.2 and Table 3.1). For the two strains, the lowest light specific oxygen evolution rates were observed in cultures grown with urea as the N-source. While urea-grown cells did not grow well, (a reduction of >80% compared to the nitrate-grown cells, for example), there was a positive increase in photosynthesis with increasing light intensities. Cells grown with urea had an approximately 65% reduction in light saturating photosynthetic rates compared to cells grown on nitrate (Table 3.1) – indicating that urea at 10 mM was not toxic but rather inhibitory to the growth of this species. Overall, when the strains of *M. aeruginosa* were grown on either nitrate or ammonium, light saturation occurred at about 200 µmol photons m^{-2} s^{-1}. At the maximum light intensity of 800 µmol photons m^{-2} s^{-1} there was no detectable photoinhibition.

Despite similar growth and light activated photosynthesis parameters, there were discernable differences in the cell biochemistry of strains grown under nitrate and ammonium. The chl-*a* fluorescence was twice as high when grown in nitrate compared to ammonium, and the difference was more pronounced in the stationary phase (Day 8) than in the exponential phase (Day 4) (Table 3.1). In nitrate, chl-*a* decreased initially and increased until Day 7 for CPCC 299 and Day 8 for CPCC 300 (Figure 3.2). In ammonium, fluorescence decreased beginning on Day 3. Cells grown on urea were not able to maintain levels of chlorophyll throughout their growth period, further indicating that the cells were not able to obtain sufficient N to replace the chlorophyll contents of cells as they grew (Figure 3.2).

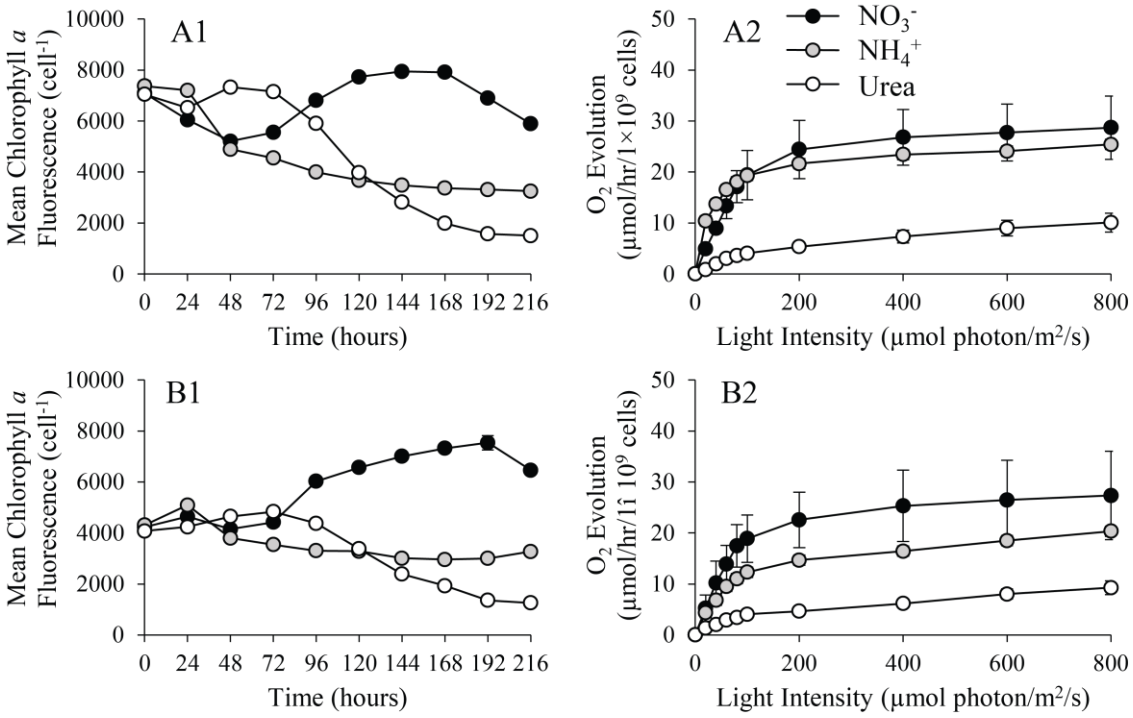

Figure 3.2 The relative chl-*a* content per cell (left) and oxygen evolution versus light intensity (right) of *M. aeruginosa* culture strains CPCC 299 (A1 and A2) and CPCC 300 (B1 and B2) grown in media with nitrate (NO_3^-), ammonium (NH_4^+) and urea ($CO(NH_2)_2$). The corresponding initial slopes of P vs. E curve (α*) and light saturation points (P_m*) were summarized in Table 3.1.

3.2 Experiment 2: Molybdenum-Limited Growth and Photosynthetic Capacity

Removing Mo from the medium had little impact on the growth and fluorescence of the two strains of *M. aeruginosa* (Table 3.2, Figures 3.3, 3.4). Growth rates and cell yields remained constant regardless of the level of Mo supplied to the cells, and cells grown with ammonium did not show distinguishable reductions in growth compared to those with nitrate. While growth was not an informative indicator of Mo-limitation, the metrics associated with light-limited and light-saturating photosynthesis assessment indicated that the CPCC 299 cells were undergoing a reduction in photosynthetic capacity proportional to the level of supplied Mo (Table 3.2). However, given the variation observed among the different N source experiments, this decrease was not significant. None of the strains showed any reduction on the level of relative fluorescence per cell. Therefore, growth under Mo deficiency did not result in significant limitations on growth or photosynthetic capacities for the strains (Figure 3.5).

Table 3.2 Growth and photosynthesis parameters of *M. aeruginosa* strain CPCC 299 (A) and CPCC 300 (B) grown on two different forms of nitrogen and under different Mo concentrations [average ± standard error]. A summary of units for each parameter is provided in legend of Table 3.1.

A

CPCC 299

N	[Mo]	Growth		Chl-*a* cell^{-1}		Light specific O_2 evolution	
		μ	Y	Exp. 92-96h	Stat. 188-192h	α*	P_m*
NO_3^-	1×10^{-6}	0.298 ± 0.049	1.08E+07 ± 4.31E+05	6696 ± 20	6878 ± 89	0.315 ± 0.092	26.726 ± 8.706
	1×10^{-8}	0.318 ± 0.056	1.07E+07 ± 2.18E+05	7227 ± 9	5233 ± 6	0.223 ± 0.065	22.230 ± 6.592
	1×10^{-10}	0.309 ± 0.057	9.55E+06 ±1.54E+05	7375 ± 8	5704 ± 5	-	-
	1×10^{-12}	0.303 ± 0.057	1.01E+07 ± 2.11E+05	7052 ± 52	5250 ± 148	0.140 ± 0.009	20.537 ± 1.800
	1×10^{-14}	0.311 ± 0.056	9.58E+06 ± 1.67E+05	6473 ± 13	4075 ± 5	-	-
	1×10^{-16}	0.309 ± 0.054	9.34E+06 ± 1.25E+05	6903 ± 14	4647 ± 12	0.157 ± 0.137	25.480 ± 17.021
	0	0.320 ± 0.055	9.23E+06 ± 5.94E+05	6475 ± 1	5369 ± 11	-	-
NH_4^+	1×10^{-6}	0.308 ± 0.045	1.07E+07 ± 1.40E+05	5537 ± 10	4382 ± 3	0.152 ± 0.044	15.109 ± 1.714
	1×10^{-8}	0.290 ± 0.050	9.36E+06 ± 2.11E+05	5382 ± 2	4223 ± 2	0.191 ± 0.027	21.528 ± 5.134
	1×10^{-10}	0.314 ± 0.042	9.88E+06 ± 7.09E+05	5101 ± 4	3960 ± 17	-	-
	1×10^{-12}	0.308 ± 0.044	9.72E+06 ± 1.20E+05	5274 ± 6	4133 ± 3	0.085 ± 0.059	12.358 ± 1.183
	1×10^{-14}	0.301 ± 0.044	9.73E+06 ± 1.15E+05	5264 ± 17	3858 ± 1	-	-
	1×10^{-16}	0.285 ± 0.048	9.75E+06 ± 8.70E+04	5185 ± 21	3777 ± 6	0.114 ± 0.020	14.907 ± 1.767
	0	0.300 ± 0.052	1.00E+07 ± 1.67E+05	5111 ± 3	3880 ± 8	-	-

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B

CPCC 300

N	[Mo]	Growth		Chl-*a* cell^{-1}		Light specific O_2 evolution	
		μ	Y	Exp. 92-96h	Stat. 188-192h	α*	P_m*
NO_3^-	1×10^{-6}	0.238 ± 0.010	1.10E+07 ± 6.77E+05	7025 ± 67	6985 ± 11	0.082 ± 0.028	21.336 ± 9.372
	1×10^{-8}	0.220 ± 0.011	1.08E+07 ± 6.84E+05	6710 ± 28	4713 ± 12	0.203 ± 0.042	18.482 ± 2.895
	1×10^{-10}	0.212 ± 0.022	1.03E+07 ± 7.35E+05	7254 ± 16	4991 ± 56	-	-
	1×10^{-12}	0.209 ± 0.025	1.05E+07 ± 7.90E+05	7103 ± 12	4761 ± 6	0.105 ± 0.015	14.588 ± 2.635
	1×10^{-14}	0.212 ± 0.025	1.02E+07 ± 6.87E+05	7410 ± 52	5865 ± 13	-	-
	1×10^{-16}	0.228 ± 0.021	1.02E+07 ± 6.66E+05	7398 ± 5	4649 ± 9	0.224 ± 0.007	15.616 ± 3.735
	0	0.224 ± 0.085	1.01E+07 ± 6.62E+05	6749 ± 33	6260 ± 26	-	-
NH_4^+	1×10^{-6}	0.311 ± 0.051	1.14E+07 ± 5.16E+05	4541 ± 14	3466 ± 6	0.139 ± 0.029	14.987 ± 1.161
	1×10^{-8}	0.298 ± 0.049	1.01E+07 ± 3.76E+05	4153 ± 13	3568 ± 12	0.090 ± 0.038	14.773 ± 1.879
	1×10^{-10}	0.293 ± 0.056	9.64E+06 ± 3.43E+05	4311 ± 26	3113 ± 2	-	-
	1×10^{-12}	0.303 ± 0.052	1.01E+07 ± 3.49E+05	4171 ± 9	3327 ± 21	0.122 ± 0.088	16.083 ± 4.943
	1×10^{-14}	0.291 ± 0.059	9.52E+06 ± 3.72E+05	4038 ± 5	3256 ± 5	-	-
	1×10^{-16}	0.314 ± 0.059	1.10E+07 ± 4.07E+05	4244 ± 4	3406 ± 9	0.100 ± 0.025	13.063 ± 1.122
	0	0.303 ± 0.057	1.06E+07 ± 4.16E+05	4145 ± 5	3194 ± 19	-	-

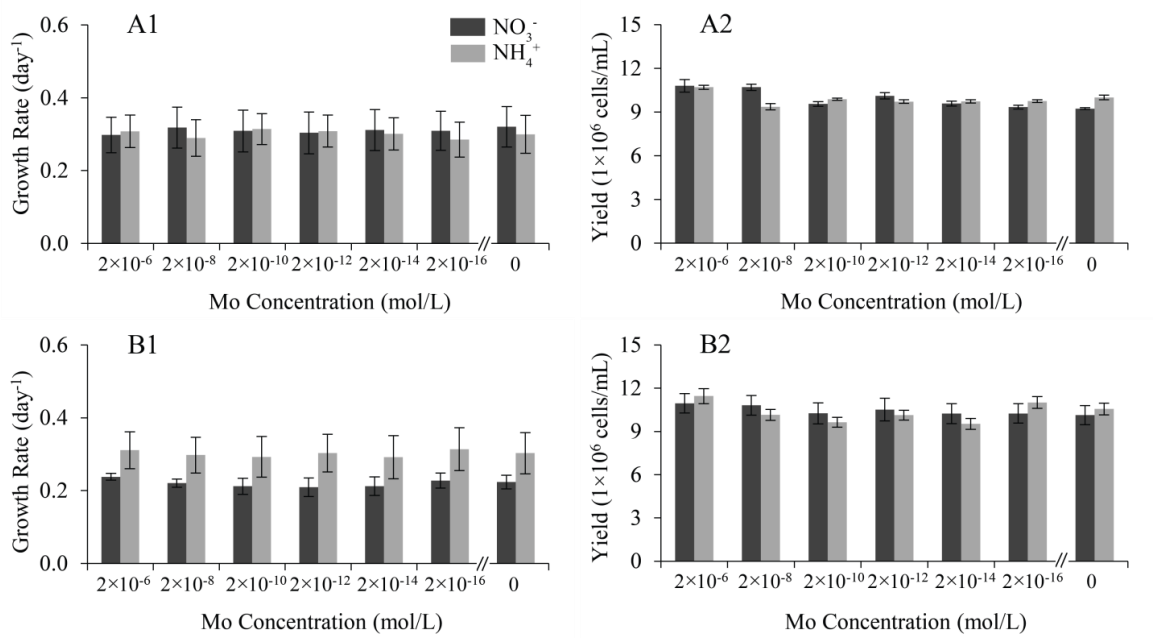

Figure 3.3 Growth rates (left) and achieved cell yield (right) for *M. aeruginosa* culture strains CPCC 299 (A1 and A2) and CPCC 300 (B1 and B2) grown in media with nitrate (NO_3^-) or ammonium (NH_4^+). Mo concentrations ranged from 0 to 10^{-16} M.

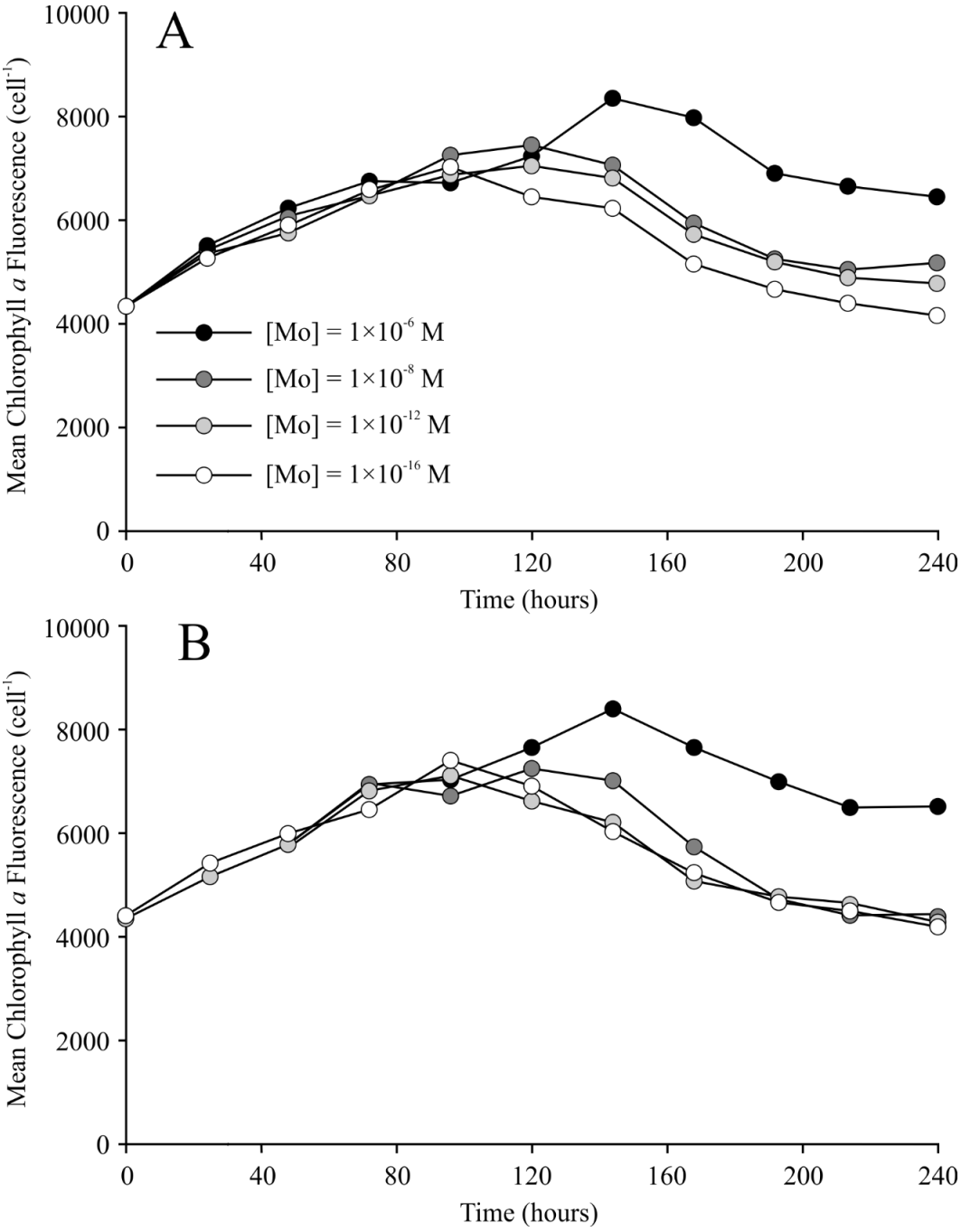

Figure 3.4 The relative chlorophyll-*a* content per cell of *M. aeruginosa* culture strains CPCC 299 (A) and CPCC 300 (B) grown in media with nitrate (NO_3^-) and Mo concentrations ranging from 10^{-6} to 10^{-16} M. The line with the dark solid symbols indicates cell performance when Mo is at the maximum exposed levels.

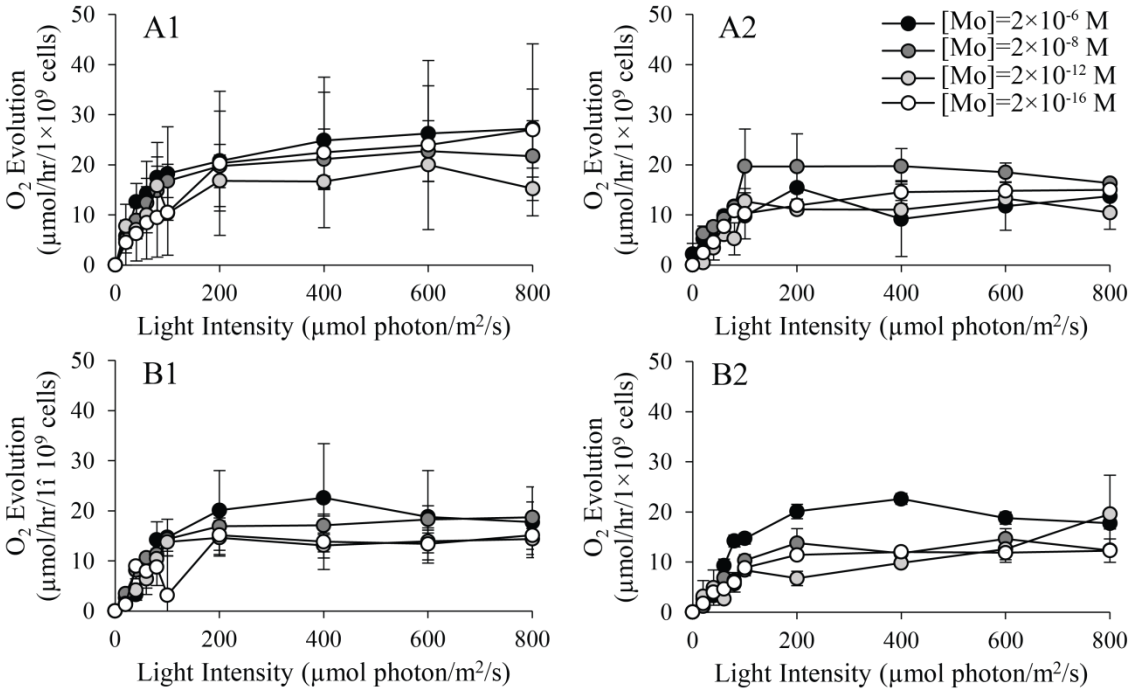

Figure 3.5 An assessment of light saturating photosynthesis of *M. aeruginosa* culture strains (A) CPCC 299 and (B) CPCC 300 grown in media with nitrate (NO_3^-, left) and ammonium (NH_4^+) with Mo concentrations ranging from 10^{-6} to 10^{-16} M. The corresponding initial slopes of the light saturating photosynthetic curve (α*) and light saturation points (P_m*) were summarized in Table 3.1.

3.3 Experiment 3: Iron-Limited Growth and Photosynthetic Capacity

Both *M. aeruginosa* strains showed symptoms of Fe deficiency when provided with medium containing reduced levels of Fe, especially in CPCC 300. The growth rates, yields, cellular chlorophyll fluorescence at exponential cell phases and stationary cell phases, and photosynthetic parameters all indicated a decline in the efficiency of physiological performance with decreasing Fe concentrations (Table 3.3).

Cells grown with nitrate as the N-supply and 2×10^{-5} M added $FeCl_3$, the maximum added Fe level, achieved the highest growth rates of 0.421 day^{-1} and 0.391 day^{-1}, for strain CPCC 299 and CPCC 300, respectively. As supplied Fe was incrementally decreased from 2×10^{-5} to 2×10^{-8} mol L^{-1} the growth rate dropped (~20% in CPCC 299,

~40% in CPCC 300). As the Fe supply was further decreased, there was a slight increase in the growth rates of the cells, followed by a gentle reduction in the growth rate. This "step up" pattern suggests that physiological changes occur when Fe is reduced to the level of 2×10^{-10} M. Thus, either there is a change in the level of available Fe to the cell or the cell physiology is altered to reduce the cellular need for Fe to achieve a specific growth rate or the yield of cells within the culture medium.

The pattern of growth parameters for cells where nitrate was the sole N source was not the same as the pattern when ammonium was the supplied N source, suggesting increased cellular needs of nitrate-grown cells and the added stress on the overall cell physiology. Cells grown with ammonium as the N-supply and 2×10^{-5} M added $FeCl_3$, the maximum added Fe level, achieved the highest growth rates of 0.284 day^{-1} and 0.261 day^{-1}, for strain CPCC 299 and CPCC 300, respectively. Supplying the cultures with incrementally lower levels of $FeCl_3$ reduced the growth rates, such that the growth rate achieved at the lowest level of added Fe (0 M) resulted in a reduction in growth rate of about 15 to 30% (0.238 and 0.180 day^{-1}, for CPCC 299 and CPCC 300, respectively). The reduction in the achieved growth rate of the two cultures was not linearly proportional to Fe concentration but was better represented as a step function: maximum growth was achieved at the two highest levels of added Fe (2×10^{-5} and 2×10^{-6} M) and at a constant reduced growth rate when Fe was added at levels of 2×10^{-8} M or lower. There was a similar pattern between the level of added Fe and the yield of the cells in the Fe-limited medium; however, the yield of the cells in the medium does consistently decline at the lower levels of added Fe (Figure 3.6).

Table 3.3 Growth and photosynthesis parameters of *M. aeruginosa* strains CPCC 299 (A) and CPCC 300 (B) grown on two different forms of nitrogen and under different Fe concentrations [average ± standard error]. Summary of units for each parameter is provided in legend of Table 3.1.

A

CPCC 299

N	[Fe]	Growth		Chl *a* cell^{-1}		Light specific O_2 evolution	
		M	Y	Exp. 90h	Stat. 186h	α*	P_m*
NO_3^-	2×10^{-5}	0.421 ± 0.005	1.14E+07 ± 3.77E+05	5034 ± 45	9972 ± 21	0.217 ± 0.005	35.598 ± 0.886
	2×10^{-6}	0.403 ± 0.002	1.09E+07 ± 2.29E+05	5048 ± 10	6910 ± 1	-	-
	2×10^{-8}	0.344 ± 0.040	1.03E+07 ± 4.60E+05	3089 ± 37	2106 ± 2	0.152 ± 0.004	11.507 ± 0.346
	2×10^{-10}	0.378 ± 0.024	1.32E+07 ± 1.66E+05	3461 ± 4	2535 ± 8	0.081 ± 0.023	11.325 ± 3.015
	2×10^{-12}	0.384 ± 0.022	1.33E+07 ± 4.02E+04	3454 ± 5	2414 ± 4	-	-
	2×10^{-14}	0.385 ± 0.011	1.30E+07 ± 1.59E+05	3531 ± 6	2275 ± 11	0.087 ± 0.058	14.225 ± 4.601
	0	0.298 ± 0.013	7.20E+06 ± 2.84E+05	2421 ± 14	1661 ± 8	-	-
NH_4^+	2×10^{-5}	0.284 ± 0.021	8.58E+06 ± 4.04E+05	4739 ± 21	3039 ± 11	0.475 ± 0.067	26.457 ± 2.341
	2×10^{-6}	0.279 ± 0.014	9.09E+06 ± 2.60E+05	4082 ± 10	2714 ± 8	-	-
	2×10^{-8}	0.204 ± 0.014	5.37E+06 ± 2.88E+05	3617 ± 14	2914 ± 7	0.094 ± 0.093	8.464 ± 8.059
	2×10^{-10}	0.192 ± 0.012	5.35E+06 ± 3.23E+05	3485 ± 4	2834 ± 18	0.058 ± 0.058	8.013 ± 7.546
	2×10^{-12}	0.258 ± 0.014	5.21E+06 ± 4.11E+05	3589 ± 19	2866 ± 14	-	-
	2×10^{-14}	0.244 ± 0.025	4.81E+06 ± 1.89E+05	3303 ± 2	2487 ± 11	0.015 ± 0.015	3.805 ±3.317
	0	0.238 ± 0.029	4.68E+06 ± 2.02E+05	3141 ± 2	2428 ± 8	-	-

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B

CPCC 300

N	[Fe]	Growth		Chl *a* cell^{-1}		Light specific O_2 evolution	
		μ	Y	Exp. 96h	Stat. 192h	α*	P_m*
NO_3^-	2×10^{-5}	0.391 ± 0.027	1.16E+07 ± 8.06E+05	4907 ± 31	8431 ± 76	0.363 ± 0.080	35.241 ± 3.945
	2×10^{-6}	0.386 ± 0.047	1.13E+07 ± 5.12E+05	4761 ± 22	4309 ± 2	-	-
	2×10^{-8}	0.243 ± 0.002	6.99E+06 ± 2.71E+04	2579 ± 21	2045 ± 4	0.117 ± 0.066	14.155 ± 7.100
	2×10^{-10}	0.300 ± 0.039	1.34E+07 ± 2.10E+05	3438 ± 6	2946 ± 6	0.072 ± 0.003	11.587 ± 3.523
	2×10^{-12}	0.276 ± 0.034	1.04E+07 ± 1.92E+05	3038 ± 16	2371 ± 3	-	-
	2×10^{-14}	0.266 ± 0.034	1.00E+07 ± 2.28E+05	3185 ± 21	2226 ± 0	0.135 ± 0.028	10.805 ± 1.972
	0	0.250 ± 0.027	8.67E+06 ± 1.83E+05	2853 ± 1	2137 ± 1	-	-
NH_4^+	2×10^{-5}	0.261 ± 0.035	8.35E+06 ± 2.45E+05	4485 ± 154	3272 ± 8	0.187 ± 0.015	20.857 ± 2.350
	2×10^{-6}	0.258 ± 0.026	8.31E+06 ± 2.17E+05	3837 ± 20	2960 ± 24	-	-
	2×10^{-8}	0.183 ± 0.025	4.22E+06 ± 1.46E+04	3345 ± 2	2556 ± 6	0.059 ± 0.005	11.673 ± 1.383
	2×10^{-10}	0.221 ± 0.023	5.36E+06 ± 1.37E+05	3183 ± 11	2592 ± 70	0.051 ± 0.007	13.431 ± 0.290
	2×10^{-12}	0.183 ± 0.002	4.45E+06 ± 3.17E+05	3089 ± 33	2494 ± 18	-	-
	2×10^{-14}	0.167 ± 0.002	3.82E+06 ± 1.23E+05	2905 ± 24	2255 ± 3	0.094 ± 0.047	13.125 ± 0.000
	2×10^{-5}	0.180 ± 0.009	3.93E+06 ± 2.51E+05	2973 ± 44	2406 ± 1	-	-

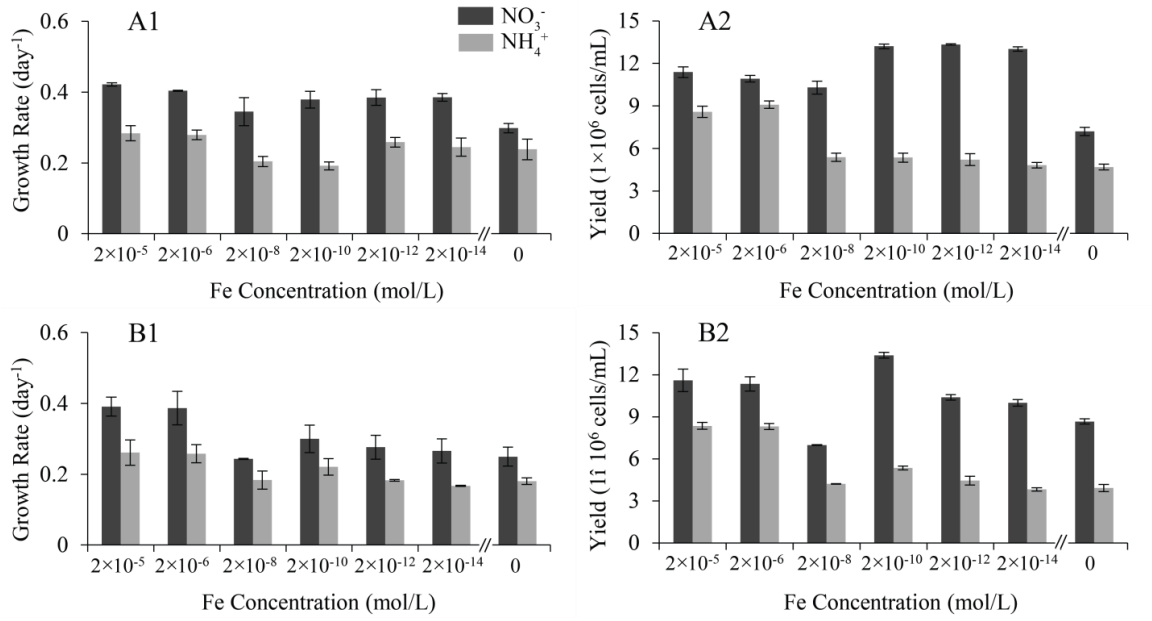

Figure 3.6 Growth rates (left) and achieved cell yield (right) for *M. aeruginosa* culture strains CPCC 299 (A1 and A2) and CPCC 300 (B1 and B2) grown in media with nitrate (NO_3^-) or ammonium (NH_4^+). Fe concentrations ranged from 0 to 2×10^{-5} M.

Looking at the photosynthetic parameters of the cells under the different levels of Fe stress, each strain had a "step down" photosynthetic performance as added Fe levels decreased from 2×10^{-5} M (Table 3.3; Figure 3.7), for both nitrate- and ammonium-supplied cells. Only CPCC 300 showed a slight recovery of photosynthesis when Fe was reduced to 2×10^{-14} M. Cellular chlorophyll fluorescence changed similarly in nitrate, with positive Fe effects observed after Day 4 when the Fe level was 2×10^{-5} M (Figure 3.8). This indicates a disconnection between the recovery of growth rate and the recovery of the photosystem in Fe-limited, nitrate grown cells. However, cellular chlorophyll fluorescence did not show differences with variable Fe levels when cultured with ammonium (Figure 3.7).

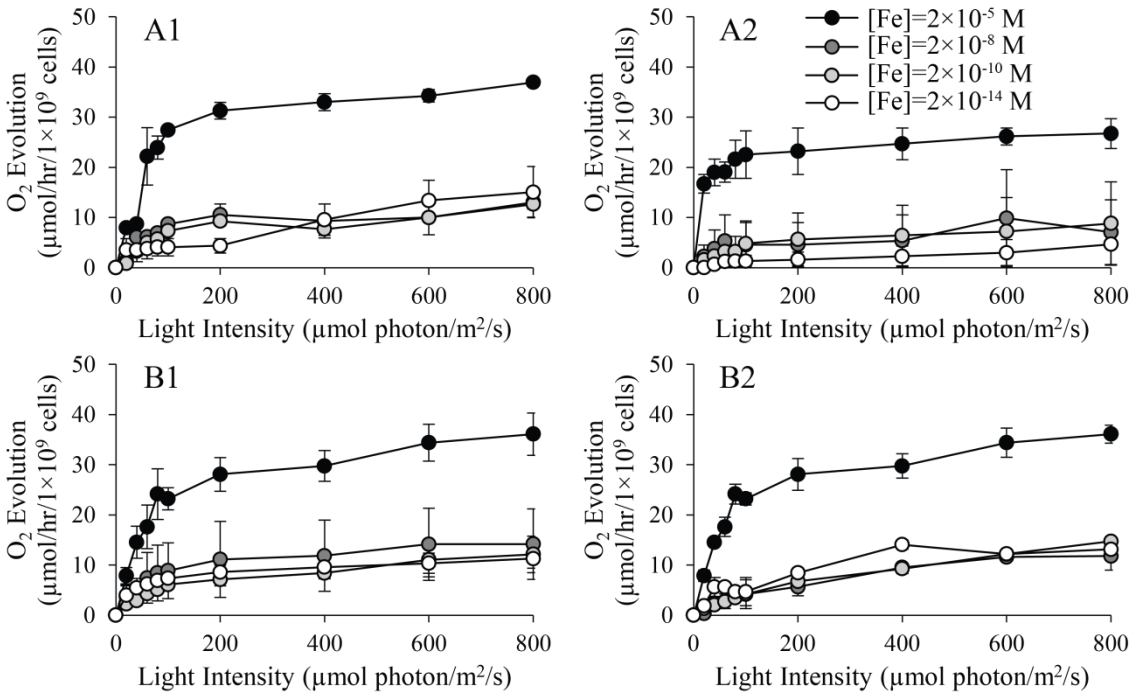

Figure 3.7 An assessment of light saturating photosynthesis of *M. aeruginosa* culture strains (A1 and A2) CPCC 299 and (B1 and B2) CPCC 300 grown in media with nitrate (NO_3^-, left) or with ammonium (NH_4^+, right) and Fe concentrations ranging from 2 × 10^{-14} to 2 × 10^{-5} M. The corresponding initial slopes of the light saturating photosynthetic curve (α*) and light saturation points (P_m*) were summarized in Table 3.3.

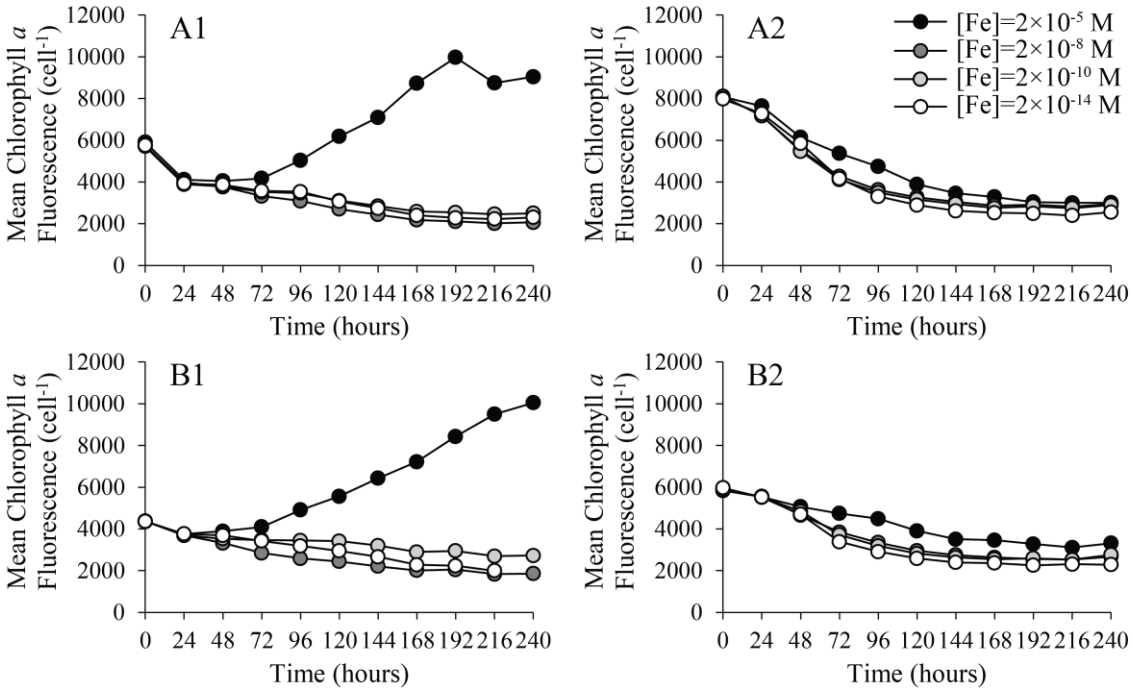

Figure 3.8 The relative chlorophyll-*a* content per cell of *M. aeruginosa* culture strains CPCC 299 (A) and CPCC 300 (B) grown in media with nitrate (NO_3^-, left) or ammonium (NH_4^+, right). Fe concentrations ranged from 2×10^{-14} to 2×10^{-5} M.

3.4 Experiment 4: Growth under Molybdenum and Iron Co-Limitation

Cells grown with no added Mo and a range of Fe levels, from Fe-replete to Fe-depleted, were assessed for growth and photosynthetic capacity (Table 3.4). Cells grown on nitrate had a "step-up" pattern of growth rates and cell yields, with a peak at the 2×10^{-12} M Fe level. In ammonium-grown cells, cell growth and chl-a parameters appeared to be insensitive to the level of added Fe, but light specific O2 evolution was highest with 2×10^{-5} M Fe and dropped considerably with less added Fe (with a minor recovery at 2×10^{-14} M).

Table 3.4 Growth and photosynthesis parameters of *M. aeruginosa* strains CPCC 299 (A) and CPCC 300 (B) grown with different Fe concentrations in the absence of Mo (co-limitation) [average ± standard error]. Summary of units for each parameter is provided in legend of Table 3.1.

A

CPCC 299

N	[Fe]	Growth		Chl-*a* $cell^{-1}$		Light specific O_2 evolution	
		μ	Y	Exp. 96h	Stat. 192h	α*	P_m*
NO_3^-	2×10^{-5}	0.304 ± 0.039	1.14E+07 ± 1.18E+05	6527 ± 96	7142 ± 18	0.441 ± 0.173	34.176 ± 2.024
	2×10^{-6}	0.321 ± 0.020	1.16E+07 ± 5.39E+05	6372 ± 35	3866 ± 18	-	-
	2×10^{-8}	0.308 ± 0.044	1.21E+07 ± 4.73E+05	4752 ± 34	3554 ± 6	0.228 ± 0.048	21.947 ± 1.769
	2×10^{-10}	0.231 ± 0.032	4.92E+06 ± 3.00E+05	2871 ± 9	1969 ± 2	0.194 ± 0.073	23.545 ± 3.197
	2×10^{-12}	0.326 ± 0.052	1.23E+07 ± 9.44E+05	3904 ± 8	3383 ± 8	-	-
	2×10^{-14}	0.253 ± 0.038	6.24E+06 ± 4.21E+05	2901 ± 12	2087 ± 25	0.175 ± 0.007	21.400 ± 1.032
	0	0.278 ± 0.036	7.08E+06 ± 4.87E+05	2993 ± 12	2259 ± 18	-	-
NH_4^+	2×10^{-5}	0.330 ± 0.043	1.12E+07 ± 8.35E+05	2786 ± 54	2893 ± 40	0.170 ± 0.026	15.640 ± 0.864
	2×10^{-6}	0.382 ± 0.052	1.04E+07 ± 9.71E+05	2949 ± 279	2471 ± 213	-	-
	2×10^{-8}	0.401 ± 0.035	1.05E+07 ± 1.06E+06	2594 ± 124	2607 ± 34	0.190 ± 0.045	15.962 ± 3.111
	2×10^{-10}	0.376 ± 0.022	1.03E+07 ± 8.36E+05	2607 ± 62	2737 ± 44	0.017 ± 0.017	7.430 ± 1.279
	2×10^{-12}	0.409 ± 0.040	1.03E+07 ± 4.57E+05	2499 ± 34	2624 ± 43	-	-
	2×10^{-14}	0.344 ± 0.053	1.02E+07 ± 6.17E+05	2470 ± 93	2594 ± 200	0.067 ± 0.017	12.277 ± 0.558
	0	0.328 ± 0.065	1.01E+07 ± 4.46E+05	2435 ± 40	2596 ± 106	-	-

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B

CPCC 300

N	[Fe]	Growth		Chl-*a* cell^{-1}		Light specific O_2 evolution	
		μ	Y	Exp. 96h	Stat. 192h	α*	P_m*
NO_3^-	2×10^{-5}	0.330 ± 0.035	1.22E+07 ± 2.54E+05	5542 ± 2	7201 ± 38	0.562 ± 0.024	43.441 ± 8.870
	2×10^{-6}	0.335 ± 0.011	1.13E+07 ± 2.56E+05	5568 ± 25	3762 ± 12	-	-
	2×10^{-8}	0.350 ± 0.034	1.29E+07 ± 6.05E+05	4378 ± 4	3249 ± 4	0.381 ± 0.010	35.296 ± 4.393
	2×10^{-10}	0.308 ± 0.050	1.01E+07 ± 5.89E+05	3066 ± 6	2316 ± 8	0.341 ± 0.167	29.184 ± 2.945
	2×10^{-12}	0.333 ± 0.050	1.19E+07 ± 8.25E+05	3544 ± 17	2492 ± 6	-	-
	2×10^{-14}	0.278 ± 0.051	7.78E+06 ± 4.22E+05	2699 ± 3	1972 ± 6	0.173 ± 0.089	22.002 ± 3.776
	0	0.281 ± 0.043	9.24E+06 ± 6.87E+05	2935 ± 3	2342 ± 17	-	-
NH_4^+	2×10^{-5}	0.384 ± 0.010	1.23E+07 ± 1.03E+06	2930 ± 45	3017 ± 128	0.239 ± 0.004	17.006 ± 0.307
	2×10^{-6}	0.368 ± 0.028	9.66E+06 ± 5.86E+05	2899 ± 71	2933 ± 96	-	-
	2×10^{-8}	0.333 ± 0.001	9.67E+06 ± 5.58E+05	2715 ± 53	2566 ± 50	0.045 ± 0.029	12.021 ± 2.167
	2×10^{-10}	0.347 ± 0.011	9.62E+06 ± 7.21E+05	2726 ± 43	2659 ± 36	0.018 ± 0.018	8.997 ± 1.316
	2×10^{-12}	0.332 ± 0.001	9.73E+06 ± 4.67E+05	2796 ± 32	2594 ± 42	-	-
	2×10^{-14}	0.330 ± 0.010	9.18E+06 ± 3.48E+05	2653 ± 78	2746 ± 72	0.031 ± 0.031	10.136 ± 4.214
	0	0.293 ± 0.029	8.72E+06 ± 1.83E+05	2627 ± 87	2555 ± 8	-	-

3.5 Experiment 5: Molybdenum and Iron Replenishment

To assess the ability of added trace metals to allow for the recovery of growth and photosynthetic capacity, the two strains CPCC 299 and CPCC 300 were grown under zero Mo and Fe levels ranging from replete (2×10^{-5} M) to depleted (2×10^{-10} M) to growth-stressed (2×10^{-14} M) and then spiked with either sufficient Fe to allow for maximum growth rates or Mo at 10^{-6} M to assess if the level of Mo in the medium could supply missing trace metals in the Fe-depleted cells, or nothing at all. The two strains grown on nitrate (Table 3.5, Figure 3.9) behaved similarly. The growth rate of the cultures was proportional to the initial level of Fe (ranging from 0.172 to 0.503 day^{-1}). Spiking the cultures with additional Fe alleviated the Fe stress, and growth rates of >0.3 day^{-1} were achieved by all cultures. This pattern was also observed when the yield of the culture was assessed, indicating that full replenishment of physiological parameters was met. Adding 10^{-6} M of Mo impacted the growth rates of cells that were initially Fe-stressed, elevating their growth rates by 60 – 70% compared to the control cultures, but it did not stimulate the growth rate of the Fe-replete or Fe-depleted cells. When relatively abundant Fe existed, Mo addition did not play a role in increasing growth. Thus, the growth parameters of cells that are Fe-stressed and growing on nitrate can be relieved by adding Mo.

Cells grown on ammonium medium that were Fe-limited did not respond to additional Mo. With ammonium, Mo addition only increased growth rates and yields in the Fe-depleted cells for CPCC 300, but not under low and high Fe levels (Table 3.5, Figures 3.10). In general, cells grew better in nitrate than in ammonium. For both strains, the final yield obtained was about 30% higher in nitrate than in ammonium. In both N sources, Fe may replace Mo in some key biological functions. Mo and Fe appeared to work collaboratively in increasing cyanobacterial growth, but Mo performed a more obvious compensatory role for Fe under Fe deficiency in nitrate.

Table 3.5 Growth and photosynthesis of *M. aeruginosa* strains CPCC 299 (A) and CPCC 300 (B) after Fe or Mo replenishment [average ± standard error]. Summary of units for each parameter is provided in legend of Table 3.1.

A

CPCC 299 in NO_3^-

Previous [Fe]	N	Growth		Chl-*a* $cell^{-1}$		Light specific O_2 evolution	
		M	Y	Exp. 90h	Stat. 186h	α*	P_m*
Fe+	2×10^{-6}	0.503 ± 0.043	1.11E+07 ± 8.58E+05	5704 ± 26	6787 ± 46	0.596 ± 0.239	41.485 ± 6.726
	2×10^{-10}	0.457 ± 0.062	1.07E+07 ± 1.91E+06	5358 ± 9	7159 ± 16	0.598 ± 0.129	44.439 ± 7.790
	2×10^{-14}	0.454 ± 0.021	1.24E+07 ± 1.70E+06	4927 ± 72	6575 ± 2	0.219 ± 0.173	18.758 ± 14.684
Mo+	2×10^{-6}	0.380 ± 0.109	9.87E+06 ± 4.29E+05	5448 ± 65	3103 ± 31	0.702 ± 0.063	49.004 ± 2.232
	2×10^{-10}	0.313 ± 0.007	3.98E+06 ± 8.76E+05	2473 ± 10	2516 ± 19	0.194 ± 0.194	17.451 ± 12.190
	2×10^{-14}	0.234 ± 0.007	3.56E+06 ± 5.70E+05	2188 ± 6	1982 ± 1	0.275 ± 0.126	20.015 ± 6.075
-Mo/-Fe	2×10^{-6}	0.381 ± 0.093	1.07E+07 ± 5.56E+05	5497 ± 3	2852 ± 8	0.808 ± 0.080	49.979 ± 5.318
	2×10^{-10}	0.343 ± 0.043	4.54E+06 ± 9.18E+05	2533 ± 2	2547 ± 0	0.320 ± 0.019	26.678 ± 4.127
	2×10^{-14}	0.172 ± 0.051	1.41E+06 ± 8.40E+04	1846 ± 9	1633 ± 1	0.163 ± 0.070	17.601 ± 3.690

CPCC 299 in NH_4^+

Previous [Fe]	N	Growth		Chl-*a* $cell^{-1}$		Light specific O_2 evolution	
		M	Y	Exp. 90h	Stat. 186h	α*	P_m*
Fe+	2×10^{-6}	0.295 ± 0.060	7.56E+06 ± 1.12E+05	3231 ± 21	3540 ± 9	0.217 ± 0.017	17.907 ± 0.142
	2×10^{-10}	0.266 ± 0.049	7.37E+06 ± 3.87E+05	3604 ± 21	3646 ± 38	0.217 ± 0.022	15.927 ± 2.934
	2×10^{-14}	0.290 ± 0.034	7.88E+06 ± 3.26E+05	3120 ± 12	3762 ± 3	0.192 ± 0.006	8.969 ± 0.116
Mo+	2×10^{-6}	0.292 ± 0.058	7.57E+06 ± 1.10E+05	2918 ± 6	3485 ± 26	0.193 ± 0.051	15.477 ± 2.484
	2×10^{-10}	0.177 ± 0.033	5.10E+06 ± 5.52E+05	2833 ± 2	2550 ± 3	0.086 ± 0.027	8.658 ± 1.758
	2×10^{-14}	0.198 ± 0.016	4.71E+06 ± 4.94E+05	2757 ± 54	2191 ± 8	0.161 ± 0.013	8.211 ± 0.307
-Mo/-Fe	2×10^{-6}	0.291 ± 0.062	7.88E+06 ± 3.01E+05	2773 ± 26	3182 ± 7	0.192 ± 0.015	19.534 ± 0.866
	2×10^{-10}	0.211 ± 0.022	5.19E+06 ± 5.31E+05	2887 ± 19	2557 ± 2	0.045 ± 0.045	9.596 ± 2.004
	2×10^{-14}	0.198 ± 0.022	4.82E+06 ± 3.46E+05	2866 ± 74	2383 ± 45	0.131 ± 0.023	10.921 ± 1.065

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B

CPCC 300 in NO_3^-

Previous [Fe]	N	Growth		Chl-*a* cell^{-1}		Light specific O_2 evolution	
		M	Y	Exp. 96h	Stat. 180h	α*	P_m*
Fe+	2×10^{-6}	0.369 ± 0.045	1.22E+07 ± 6.20E+05	5617 ± 16	9097 ± 5	0.581 ± 0.051	36.947 ± 7.604
	2×10^{-10}	0.453 ± 0.008	1.20E+07 ± 1.15E+06	5553 ± 8	8547 ± 12	0.569 ± 0.050	47.782 ± 3.398
	2×10^{-14}	0.519 ± 0.009	1.14E+07 ± 8.76E+04	5133 ± 33	7671 ± 0	0.164 ± 0.116	26.828 ± 4.123
Mo+	2×10^{-6}	0.398 ± 0.069	1.19E+07 ± 6.67E+05	5066 ± 3	4183 ± 5	0.542 ± 0.092	42.027 ± 3.005
	2×10^{-10}	0.293 ± 0.039	7.85E+06 ± 1.10E+06	3073 ± 26	2643 ± 34	0.118 ± 0.007	14.379 ± 1.896
	2×10^{-14}	0.267 ± 0.043	3.74E+06 ± 4.49E+05	2139 ± 7	2199 ± 7	0.096 ± 0.023	13.157 ± 1.569
-Mo/-Fe	2×10^{-5}	0.339 ± 0.114	1.21E+07 ± 9.28E+05	5314 ± 26	3813 ± 5	0.543 ± 0.043	45.540 ± 3.049
	2×10^{-10}	0.369 ± 0.027	7.56E+06 ± 8.23E+05	2837 ± 22	2264 ± 4	0.162 ± 0.037	17.422 ± 0.993
	2×10^{-14}	0.170 ± 0.016	2.06E+06 ± 1.38E+05	2506 ± 4	1707 ± 2	0.101 ± 0.035	15.818 ± 1.080

CPCC 300 in NH_4^+

Previous [Fe]	N	Growth		Chl-*a* cell^{-1}		Light specific O_2 evolution	
		M	Y	Exp. 96h	Stat. 182h	α*	P_m*
Fe+	2×10^{-6}	0.305 ± 0.055	7.55E+06 ± 1.92E+05	3670 ± 65	3857 ± 1	0.187 ± 0.072	15.912 ± 1.311
	2×10^{-10}	0.509 ± 0.053	7.60E+06 ± 1.03E+05	3306 ± 26	3112 ± 1	0.095 ± 0.048	10.823 ± 1.561
	2×10^{-14}	0.316 ± 0.057	7.47E+06 ± 2.23E+04	3035 ± 8	3020 ± 26	0.092 ± 0.040	10.588 ± 0.309
Mo+	2×10^{-6}	0.315 ± 0.052	7.33E+06 ±1.81E+05	3029 ± 7	3018 ± 3	0.096 ± 0.028	15.524 ± 0.392
	2×10^{-10}	0.234 ± 0.015	5.20E+06 ± 4.45E+05	2691 ± 25	2301 ± 2	0.079 ± 0.026	7.955 ± 0.057
	2×10^{-14}	0.153 ± 0.041	3.08E+06 ± 4.90E+05	2212 ± 53	1949 ± 4	0.038 ± 0.038	7.050 ± 1.494
-Mo/-Fe	2×10^{-6}	0.314 ± 0.067	7.74E+06 ± 2.14E+05	3084 ± 6	3172 ± 55	0.167 ± 0.081	16.229 ± 0.196
	2×10^{-10}	0.166 ± 0.025	3.62E+06 ± 3.59E+05	2812 ± 14	2740 ± 2	0.104 ± 0.052	10.608 ±0.3987
	2×10^{-14}	0.162 ± 0.025	3.28E+06 ± 4.40E+05	2554 ± 1	2368 ± 3	0.082 ± 0.053	9.736 ± 1.301

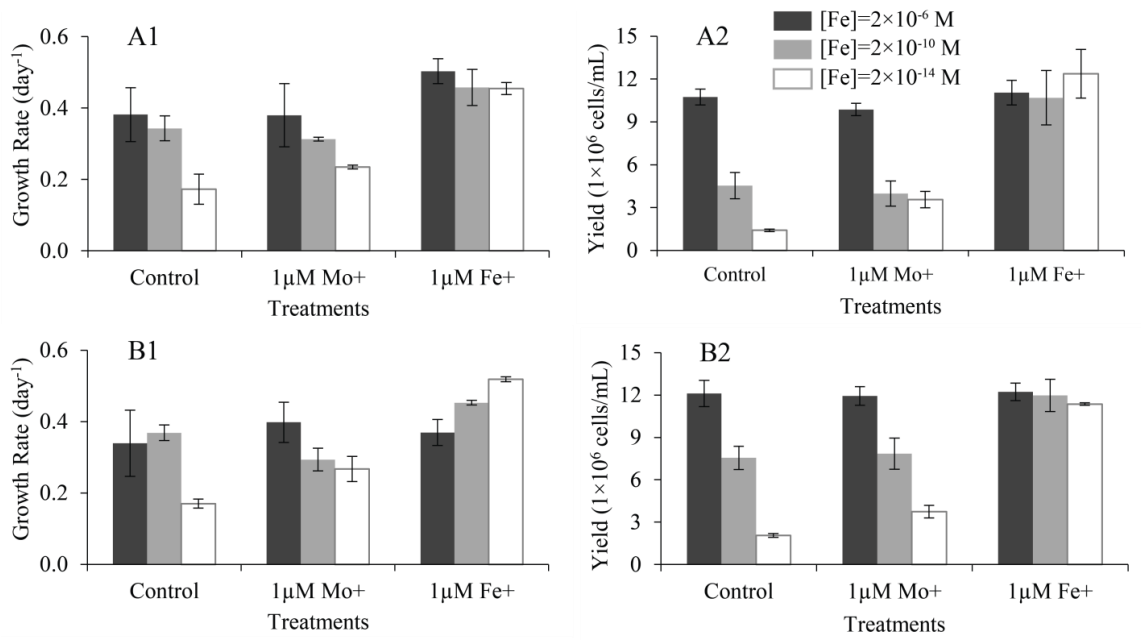

Figure 3.9 The effects of Mo/Fe replenishment on growth rate and yield of *M. aeruginosa* culture strain CPCC 299 (A1 and A2) and CPCC 300 (B1 and B2) grown in media with nitrate as the nitrogen source under three conditions: Mo starved cultures without Mo or Fe (control); Mo starved with Mo addition (1 μM Mo+) (Mo control) and Mo starved with Fe addition (20 μM Fe+) (Fe treatment).

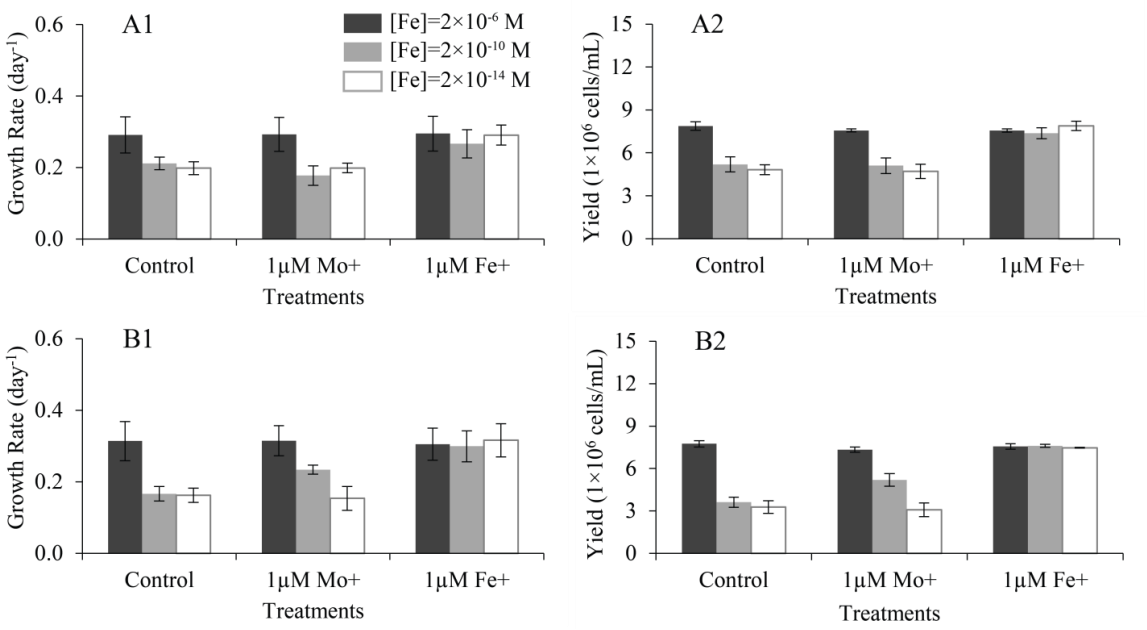

Figure 3.10 The effects of Mo/Fe replenishment on growth rate and yield of *M. aeruginosa* culture strain CPCC 299 (A1 and A2) and CPCC 300 (B1 and B2) grown with ammonium as the nitrogen source under three conditions: Mo starved cultures without Mo or Fe (control); Mo starved with Mo addition (1 μM Mo+) (Mo control) and Mo starved with Fe addition (20 μM Fe+) (Fe treatment).

Replenishing Fe allowed cells to generate higher fluorescence during the stationary phase in nitrate medium; Chl-*a* increased for at least eight days after Fe replenishment (Figure 3.11). In ammonium grown cells, Fe replenishment increased chl-*a* fluorescence to a much lesser extent than in nitrate (Figures 3.11). Cell chlorophyll fluorescence did not increase over time or among initial Fe treatments by adding Mo (Figure 3.11). Similar trends were seen for CPCC 300 in photosynthetic activity over light availability, where Fe plays crucial roles in enhancing photosynthesis. In cells grown on ammonium, addition of Fe increased the photosynthetic capability for CPCC 300, but did not show a positive effect for CPCC 299 (Figure 3.12).

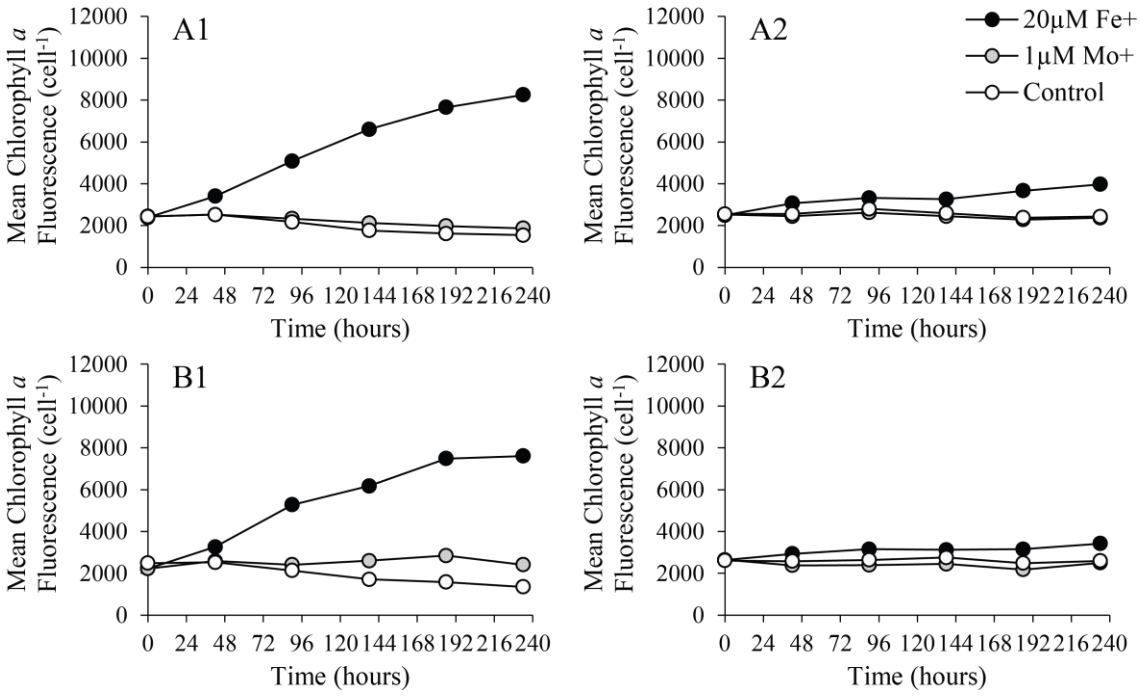

Figure 3.11 Mean chlorophyll-*a* fluorescence per cell of *M. aeruginosa* culture strains CPCC 299 (A1 and A2) and CPCC 300 (B1 and B2) grown in media with nitrate (left) or ammonium (right) as the N source under 3 conditions: Mo starved cultures without Mo or Fe (control); Mo starved with Mo addition (1 µM Mo+) (Mo control) and Mo starved with Fe addition (20 µM Fe+) (Fe treatment).

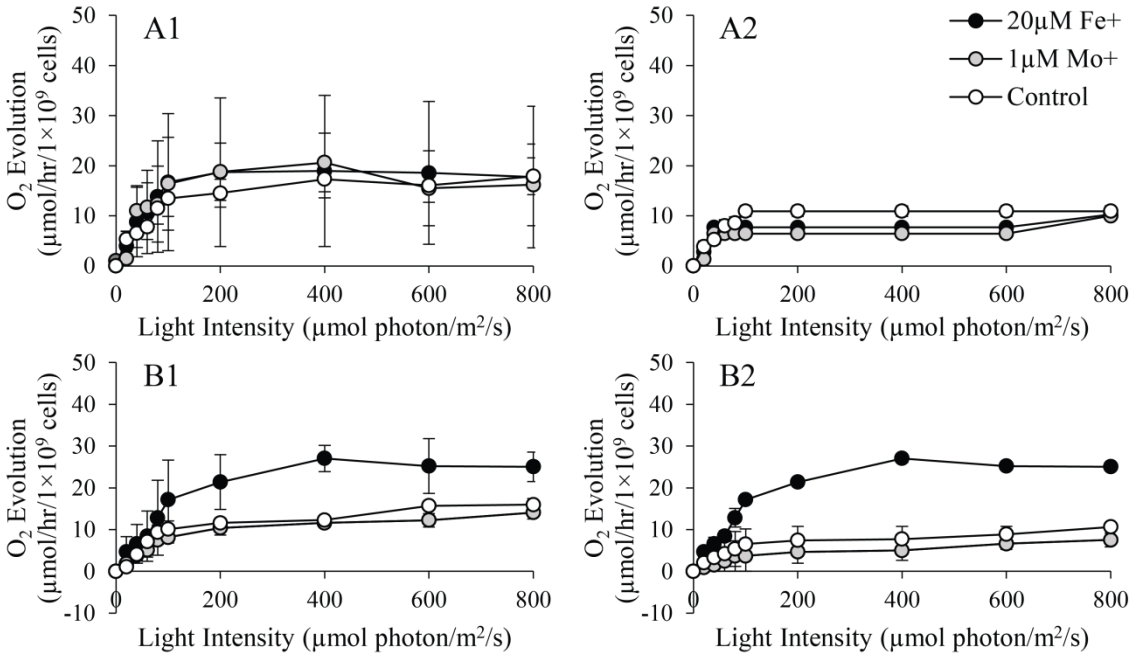

Figure 3.12 Oxygen evolution of *M. aeruginosa* culture strains CPCC 299 (A1 and A2) and CPCC 300 (B1 and B2) grown in media with nitrate (left) or ammonium (right) as the N source under 3 conditions: Mo starved cultures without Mo or Fe (control); Mo starved with Mo addition (1 µM Mo+) (Mo control) and Mo starved with Fe addition (20 µM Fe+) (Fe treatment).

3.6 Molybdenum and Iron Regulation of Microcystin Production

When grown with nitrate, trace metal replete cells (+Mo/+Fe) had the highest levels of cellular microcystin in CPCC 299, while the Mo-limited, Fe-replete (-Mo/+Fe) cells had the highest levels for CPCC 300 (Figure 3.13). Cells grown with ammonium as the sole N source generally produced less microcystin, though the Mo-replete, Fe-limited (+Mo/-Fe) CPCC 299 cells produced the highest microcystin levels (~20 fg $cell^{-1}$). In general, Fe-limited cells produced the lowest level of microcystin, regardless of N source or the level of supplied Mo. In nitrate, cellular microcystin content increased from 6.43 fg $cell^{-1}$ to 16.23 fg $cell^{-1}$ for CPCC 299 and from 6.32 fg $cell^{-1}$ to 11.82 fg $cell^{-1}$ for CPCC 300 after Fe addition.

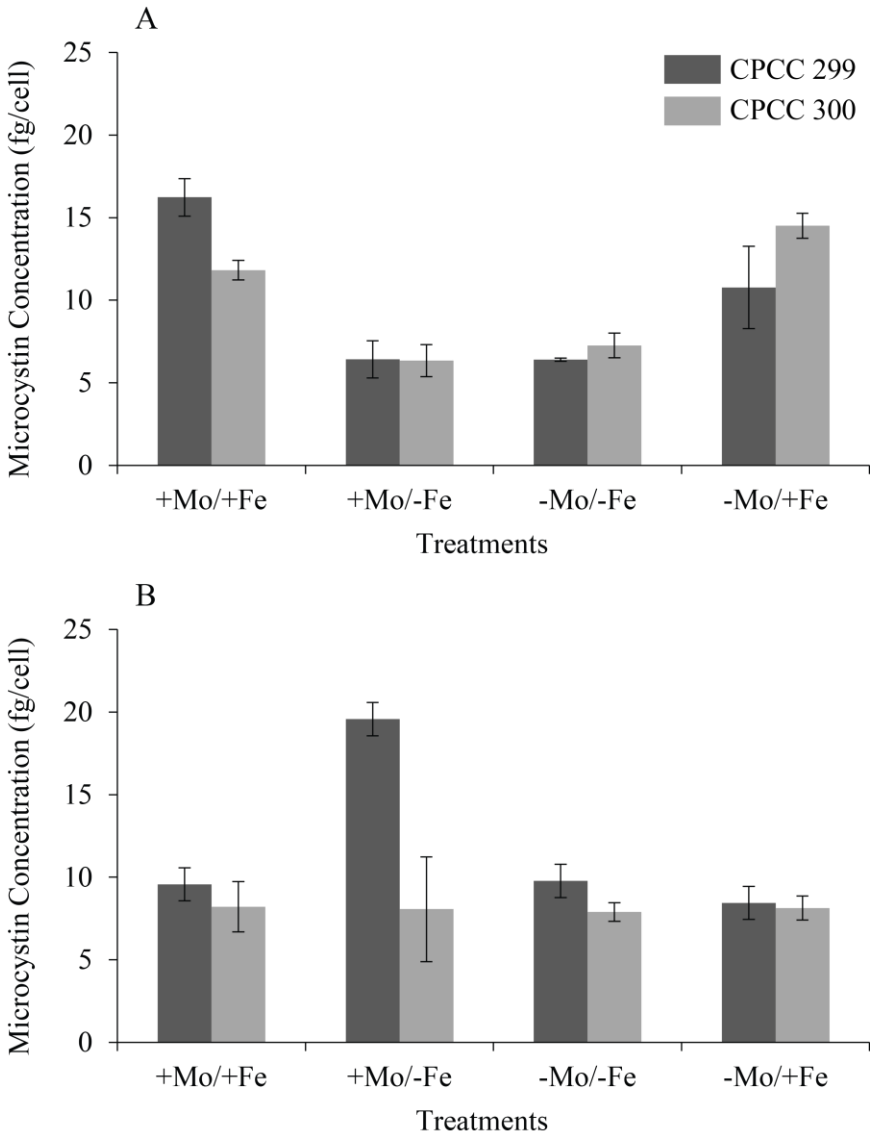

Figure 3.13 Microcystin measurement (ELISA) in *M. aeruginosa* culture strains CPCC 299 and CPCC 300 in each of exponential and stationary phase of cells grown in media with NO_3^- (A) and NH_4^+ (B) being the nitrogen under 3 conditions: Mo starved cultures without Mo or Fe (control); Mo starved with Mo addition (1 µM Mo+) (Mo control) and Mo starved with Fe addition (20 µM Fe+) (Fe treatment).

Chapter 4

4 Discussion

There have been numerous reports indicating that the frequency, magnitude and duration of cyanoHABs are on the rise. For many of these reports the increased incidence has been related to a combination of increased water usage, world population growth and urban or agricultural waste (Smolders *et al.* 2010, Howarth *et al.* 2011) – similar to factors that caused the cyanoHABs in the Great Lakes in the 1960s and 1970s (Schindler and Valentyne 2008). The addition of primary nutrients such as phosphate and N to lakes is beneficial to photosynthetic phytoplankton populations, particularly those with both a high affinity to absorb and store macronutrients and a low rate of grazing consumption (Smayda 1997). For most lakes, the species with these two physiological traits are cyanobacteria (Reynolds *et al.* 1981, Paerl and Millie 1996, Dokulil and Teubner 2000). Thus, the explosion of cyanoHABs in highly populated areas with altered landscapes and incomplete wastewater nutrient filtering systems has dominated the reports of cyanobacterial infested lake systems (Chen and Hong 2012). The modern classic cyanobacterial lakes are temperate or tropical lakes that are hypereutrophic with defined anthropogenic disturbances. For example, a number of lakes in China are undergoing increasing cyanoHABs, such as Lake Taihu (a.k.a. Lake Tai or Tai Hu), Lake Caohu, Lake Dianchi or the reservoirs of Guangdong region (Qin *et al.* 2007, 2010, Lei *et al.* 2011). However, the problem of cyanobacteria in China is just a modern representation of a long list of urban-agricultural-cyanoHAB connections that have occurred globally as humans have influenced the nutrient flux and water flows of surface waters.

The connections between population, land use, and agriculture predominate intellectual thought on cyanoHABs. There is little debate that excessive levels of N (nitrate, ammonium and urea) and P result in cyanoHABs (Lewis and Wurtsbaugh 2008, Paerl 2008, Schindler and Vallentyne 2008, Schindler 2012, Moss *et al.* 2013). CyanoHABs are most common in eutrophic and hypereutrophic lakes worldwide, but oligotrophic lakes, with low or moderate nutrient status, are developing periodic

cyanoHABs without a change in trophic status (Molot *et al.* 2014) and the rising of such blooms has drawn public concern (Winter *et al*. 2011, Beaulieu *et al.* 2014, Sorichetti *et al.* 2014b, Persaud *et al.* 2015, Taranu *et al.* 2015). The nutrient-cyanobacterial response relationship in oligotrophic lakes is not fully understood, but it appears to involve the stimulation of cyanobacteria to utilize the natural levels of macronutrients differently than the competing eukaryotic species. The result is that natural levels of nutrients are now converted to cyanobacterial biomass, which remains in the water column because these cells are poorly grazed. At the completion of the seasonal cycle in temperate lakes the un-grazed cells decompose at the sediment surface, releasing the elements below the thermocline.

There are two explanations for the proliferation of cyanoHABs: (1) major modifications in macronutrients result in an increase in cyanobacteria biomass, or (2) alterations in the natural nutrient chemistries now select for cyanobacteria. It is this latter paradigm that serves as the focus for this dissertation. Molot *et al.* (2014) argued that the supply of micronutrients – particularly Fe, but also Mo and copper – may be sufficiently limiting to lower macronutrient assimilation rates and slow down the growth of phytoplankton that compete against cyanobacteria. Thus, if cyanobacteria can obtain or use micronutrients more efficiently than eukaryotic species, then the pool of macronutrients will be effectively transferred to cyanobacteria. This model is described as the "nutrient use efficiency approach" and is analogous to the large scale Fe-limited, nutrient rich zones in marine systems that have drawn research attention over the last few decades (Sunda and Hunstman 1995, Coale *et al.* 1996, Pitchford and Brindley 1999), where the low availability of Fe influences the consumption of nitrate and as a consequence shapes the phytoplankton community structure. Molot *et al.* (2014) postulate that a similar regulation of N or P use occurs in lakes, depending on the availability of trace metals and that cyanobacteria dominate in the phytoplankton community by controlling the use of trace metals.

In this study, the growth and photosynthetic capacity of the dominant bloom-forming cyanobacterium, *M. aeruginosa*, were assessed over a range of Fe, Mo and Fe-Mo co-limitations. The hypothesis was that this dominant strain of cyanobacteria could

utilize both Mo and Fe at exceptionally low levels. Thus, the dominance of this species could be explained by this unique affinity for trace metals that regulate the use of the N source. Generally, nitrate is the predominant form of N in freshwater systems, followed by ammonium (Morris and Simon 2012). The level of urea in freshwater systems is highly variable depending on the proximity of agriculture activities and may represent as much as 50% of the available N in a lake system (Bogard *et al.* 2012). Levels of urea in areas without direct anthropogenic impact are typically negligible, because as inputs are low, the chemical transformation to ammonia is rapid, and ammonia is not a suitable growth source for phytoplankton.

The ability of *M. aeruginosa* to grow on three different sources of N – nitrate, ammonium and urea – has previously been assessed (Erratt, Trick, unpubl. Data). After presenting cells with the selected N-source at growth-saturating light levels, both nitrate- and ammonium-supported maximum growth rate, but urea did not support growth. Additional studies revealed that the level of urea chosen was inhibitory and that the *M. aeruginosa* strains chosen for this work can grow on urea at lower concentrations.

Cyanobacteria cells typically grow equally efficiently in nitrate and ammonium (reviewed in Oliver and Ganf 2002 and confirmed here), although efficiency of growth on nitrate is light dependent. When light levels are between 24 and 29 μmol photons$\cdot m^{-2}\cdot s^{-1}$, nitrate is a preferred N-source, but in waters with reduced light availability (i.e., range between 2 and 3 μmol photons$\cdot m^{-2}\cdot s^{-1}$), ammonium becomes the preferred N-source (Dortch 1990). This illustrates the strong link between the N-fixation and C-fixation, as the cells compete for the same energy and electrons in order to process and incorporate elements. The resulting biomolecules after assimilation have C:N of 10:1 to 15:1 (mol:mol) (Turpin 1991).

The link between N-source supply and the photosystem was evident in the analysis of chlorophyll fluorescence. *Microcystis aeruginosa* cells grown with nitrate as the sole N-source expressed chlorophyll levels two times the cellular level of ammonium-grown cells. The difference was strongest in the stationary phase of growth compared with cells in the exponential phase. The link between N-source and photosynthesis is best

represented in photosynthesis vs. light intensity curves (P vs. E). The highest rates of photosynthesis and light use efficiencies were observed on nitrate-grown cells. However, in one strain of *M. aeruginosa* there was no difference in the photosynthetic efficiency for ammonium-grown cells, and in the second isolate, the photosystem was reduced when the cells were grown on ammonium, corresponding to the slightly lower growth rate on ammonium-grown cells of strain CPCC 300. In these studies, the photosystem of the urea grown cells was functional but highly reduced compared with the other sources of N, indicating a suppression of the photosystem, rather than toxicity to the cells.

There has been increased speculation that Mo may be a growth-limiting factor in oligotrophic lakes (Glass *et al.* 2012), in part due to reports of highly variable levels of Mo in temperate Ontario lakes (Provincial Water Quality Monitoring Network 2011). I hypothesized that reducing Mo concentrations would indeed reduce the efficiency of nitrate use by bloom-forming cyanobacteria. Strains of cyanobacteria may regulate the acquisition of low levels of Mo, or alter the cell demands for Mo, allowing them to outcompete other phytoplankton for the common pool of nitrate. Removing Mo from the medium had little impact on the growth rates and cell yields and only decreased the photosynthesis of CPCC 299 to a small extent. Repeated transfers at the lower levels of added Mo did not result in a further decrease in growth parameters, indicating that the cells were not thriving on residual or stored Mo, either because the cellular requirement for Mo was too low to be detected, or another element replaced Mo and satisfied cellular needs. Ter Steeg *et al.* (1986) observed that low levels of Mo did not impact growth in an *Anabaena* isolate, perhaps due to an inducible transport system that ensures sufficient supply of Mo. This study did not use trace metal free culturing and used a highly enriched culture medium, so there may have been sufficient residual Mo to supply the cells even at very low levels of added Mo. Ter Steeg *et al.* (1986) documented an inducible Mo transport system, a scavenging system where the transport rate was inverse to the level of added Mo. Control Mo concentrations in the cells were ~ 20-25-fold the concentration outside of the cells; whereas, when Mo levels were low, the concentration ratio was greater than 3000-fold. As a result of increased transport and a lower Mo pool, the cells with the greatest Mo-limitation only showed a ten-fold reduction in the level of Mo in the cells. Thiel *et al.* (2002), recognizing that Mo was an essential co-factor for

both nitrate assimilation and N-fixation, reported that after several generations and transfers of *Anabaena variabilis*, a filament forming N_2-fixing cyanobacterium, Mo starvation could not be achieved or at least recognized using growth characteristics. They suggested that there was a substantial Mo storage system linked to the nitrate transport system and a Mo scavenging transport system that was repressed by Mo. This combination of physiological controls would create cells that modulated the consumption and storage of Mo in a strong homeostatic balance with N transport and consumption (Glass *et al.* 2010). Cells exposed to long-term levels of low Mo adjust their needs, alter the transport affinity to scavenge Mo, and tap a generally un-described Mo storage system. The strong feedback linkage with nitrate transport results in Mo-limited cells that express N-limited characteristics. *Anabaena variabilis* is an N_2-fixing species with a higher cell quota for Mo compared with non-N_2-fixing counterparts (Ungerer *et al.* 2010). Cells without the added need for Mo to act as a cofactor in N_2-fixation would be even more difficult to stress for Mo.

Consistent with the above reports, *M. aeruginosa* maintained growth rates close to the control when presented with increasing lower levels of supplied Mo. Given the evidence that cells scavenge Mo to meet their N needs, alterations in photosynthetic capacity of CPCC 299 are likely due primarily to the change from N-replete to N-depleted cell conditions. There is a strong parallel between the inducible Mo transport system and the inducible Fe transport systems in cyanobacteria. Both systems have likely evolved over biogeochemical time because of the changes in availability of each element as the earth's systems shifted from reducing to oxidizing conditions (Zerkle *et al.* 2005, Glass *et al.* 2009, Planavsky *et al.* 2014). The ecological consequences of the coevolution of two element scavenging systems and the fact that Mo and Fe are cofactors in the N-acquisition systems have led researchers to consider both transport systems in unison. Evans and Prepas (1997) and Tuit *et al.* (2004) have provided linked studies of Mo and Fe transport and scavenging in freshwater and marine systems, respectively.

In this study, Fe-limited growth and photosynthetic characteristics in *M. aeruginosa* were evident. When grown on nitrate (with higher Fe demands than ammonium) there were stepped declines in Fe, resulting in declines in growth rate, yield,

cellular chlorophyll fluorescence and light specific oxygen evolution in both exponential and stationary phases indicating a decline in the efficiency of physiological performance. When grown on nitrate growth rates and yields provided a non-linear response. Under Fe-reduced conditions, growth rate reduced from 10^{-5} M to 10^{-8} M, but then increased from 10^{-8} M to 10^{-10} M, and then slightly reduced from 10^{-10} to 10^{-14} M, but still remained above the low point at 10^{-8} M. This concentration-dependent pattern is dramatically different than the Mo-limited growth experiment. In the Mo experiment, the transport system appears to meet the demands of cell growth, but changes in the Fe transport system do not seem to meet the growth needs of the cells but does improve access to a new pool of Fe. The pattern observed is comparable to the induction of an Fe scavenging system that involves the production of an extracellular Fe-binding ligand (referred to as a siderophore), providing access to otherwise unavailable pools of Fe in the medium. The siderophore system in cyanobacteria has been known for some time (Murphy *et al.* 1976, Simpson and Neilands 1976, Kerry *et al.* 1988, Brown and Trick 1992, Wilhelm and Trick 1994). The key observations are that this species has an inducible active Fe transport system. Iron scavenged from this system is sufficient to meet the growth needs of the cells but not sufficient to fully replace the Fe required to achieve a fully functional photosystem. As a consequence, Fe-limited cells remain "unbalanced" in growth even when supplying new Fe to the cells – with less C fixed per N than fully replete cells.

The ease of access of Mo may alleviate some of the cellular needs for Fe (Evans and Prepas 1997), rebalancing the "unbalanced" growth and allowing the cells to scavenge enough Mo and Fe to satiate both growth and photosynthetic capacity. Extending the thoughts of Thiel *et al.* (2002), if both the Mo and Fe scavenging systems function, then the needs of the nitrate assimilation and the photosystem can be met under micronutrient-limiting conditions. In the present study, Fe-limited cells, resupplied with growth-saturating levels of Mo, expressed a physiology between Fe-depleted and Fe-replete cell. Presumably, Mo can replace some the cellular demands for Fe, allowing the cells to restore the reduced growth rates. Given that adding Mo to low-Fe grown cells did not repair the photosystem, the addition of Mo is probably sufficient to maintain nitrate transport and assimilation. The compensatory role of Mo on Fe-limitation has significant impact on our understanding of the biogeochemical control of cyanoHABs. Recent work

has established that Fe-levels have a significant influence on the possibility and the longevity of the cyanoHABs (Du 2013, Molot *et al.* 2014, Sorichetti *et al.* 2014b, Orihel *et al.* 2015), but it is important to consider the possible influence that Mo concentrations have on shaping the cyanobacterial community.

Chapter 5

5 Conclusions

5.1 Main Findings

This dissertation examined the interaction between the supply of N and the alterations in growth and photosynthetic characteristics of two unialgal cultures (CPCC 299 and CPCC 300) of the freshwater cyanobacterium *M. aeruginosa* due to low levels of two trace metals, Mo and Fe. At growth saturating light levels (60-80 μmol photons·m^{-2}·s^{-1}), unialgal laboratory cultures of *M. aeruginosa* were capable of exponential growth on nitrate and ammonium substrates, whereas urea proved not to be an effective N source for toxic *M. aeruginosa* at the concentration tested (10 μM).

Mo depletion did not significantly limit the growth of *M. aeruginosa* cells. Cell growth rates and the achieved cell yield under defined levels of supplied N were not diminished at lower levels of supplied Mo as predicted from our hypotheses. Only CPCC 299 cells were stressed at the lower levels of Mo, based on the reduction in the kinetic measurements of light–driven photosynthesis (α* – the light limited initial slope of the P-E curve, and P_m* – the light saturating photosynthetic rate). Molybdenum did not reach growth-limiting levels under laboratory conditions, either because the cells had an extremely low cellular requirement for this micronutrient or another element replaced Mo to satisfy cellular needs.

In contrast, reducing the amount of added Fe lowered growth rates and yields as well as photosynthetic capacity in *M. aeruginosa* cells. The reduced growth and photosynthetic potential was strongly evident in nitrate-grown cells, compared with ammonium grown cells. This may be due to the higher Fe quota for cells that depend on nitrate reductase (a major Fe-containing transport and conversion enzyme). Cells grown at low to moderately-low Fe concentrations showed a significant recovery of growth rates and yields, but not a concomitant recovery in the low-Fe depressed photosystem, reflective of a change in the external supply of available Fe. This recovery was evident only in nitrate-grown cells. These forensic criteria are similar to cyanobacteria that

produce siderophores to scavenge low levels of Fe from the matrix of organic-inorganic complexes in the medium (Kerry *et al.* 1988, Wilhelm and Trick 1994).

Experiments on possible co-limitation by Mo and Fe indicated that Fe may replace Mo in some key biological functions during cell reproduction. Molybdenum and Fe are linked in key physiological processes in the cell – notably nitrate reductase. When Fe-limited cells were presented with additional Mo, recovery of growth parameters was evident – but not siginificant recovery of photosynthetic parameters. This indicates a possible trace metal replacement of Mo with Fe, presumably in the functioning of nitrate reductase.

Cellular microcystin concentrations were examined during the stationary phase of growth to evaluate different nutrient stressors on microcystin production. Both strains of *M. aeruginosa* produced microcystin at concentrations between 10 and 20 fg $cell^{-1}$. Cellular microcystin concentrations did not vary substantially with N-source – levels were similar in range for nitrate-grown cells, compared with ammonium-grown cells. Cells grown in Fe- or Mo-limited media produced less microcystin than N-limited cells, in contrast to other published research (Amé and Wunderlin 2005, Li *et al.* 2009, Long 2010).

5.2 Scientific Significance

The potential growth-limiting role of Mo in freshwater cyanobacteria has not been considered for some time. While initially proposed a half-century ago (Goldman 1960, Howarth and Cole 1985) as a credible limiting factor for growth in oligotrophic lakes, the concept of Mo limitation has only recently reemerged (Glass *et al.* 2012). This is the first study to consider Mo and Fe co-limitation in the ubiquitous bloom-forming cyanobacterium *Microcystis*. Co-limitation of Mo and Fe has been considered elsewhere but is usually limited to marine N_2-fixing cyanobacteria (Tuit *et al.* 2004, Zerkle *et al.* 2006), as Mo plays a major role in nitrogenase. However, this study focused more on the role Mo plays in nitrate reductase and its possibility to be involved in photosynthesis.

This study also provides evidence for the hypothesis that trace metals, such as Mo and Fe, which directly affect the stoichiometry of cellular elements by restricting the uptake of N and C, stimulate cellular microcystin production. The stoichiometry – or unbalanced growth – model for toxin production has garnered considerable support recently (van de Waal *et al.* 2014). The findings in this dissertation do not support this hypothesis.

5.3 Practical Significance

The practical significance of this study lies in societal need for knowledge of the fundamental controlling factors for cyanoHABs and toxin levels associated with cyanoHABs. For example, a poorly constructed but highly cited correlational argument links the global increase of urea use with the increased frequency of cyanoHABs. Urea is a commonly used N fertilizer in farmland and has increased 100-fold in the past four decades (Gilbert *et al.* 2004). However, this study suggests that urea may not be a significant N source for cyanobacteria and, in fact, would be inhibitory at comparably low concentrations. Work similar in design to this study may provide a better understanding of physiological needs of cyanobacteria and assist researchers, legislatures, and environmentalists to make regulations and strategies.

In natural freshwaters, the two elements most closely associated with cyanoHAB initiation and biomass are N and P, and there is substantial debate about the importance of each of these elements even after 50 years of study (Schindler *et al.* 2008, Schindler and Hecky 2009). These macronutrients control the level of potential biomass, but minor elements such as Mo and Fe can be key elements in regulating the effective processing of macronutrients and, therefore, the speciation of the bloom (Glass *et al.* 2012, Molot *et al.* 2014). Thus, understanding the physiological needs for macronutrients as well as minor elements and their natural levels in bloom waters is critical in understanding the control of bloom maintenance and longevity.

During a cyanoHAB, microcystin can be produced in large quantities, threatening ecological and human health. Measuring microcystin concentrations in natural waters is

important in order to raise public awareness of the need to mitigate toxin-producing cyanoHABs and to give a better understanding of the factors affecting toxin formation.

5.4 Future Research

The biggest challenge of this study lies in inducing a Mo-limiting condition for cyanobacteria. It was very hard to obtain the Mo cell quota and to understand the exact mechanism of Mo assimilation. Starving cells in Mo-free medium for five days was long enough to remove the Mo from the photophysiology of the cell but may not have been long enough to use up cellular Mo storage. Trace elements that depress the energy acquisition of the cell are difficult to study as cell health declines precipitously. However, in this case, the cell health was depressed but not depleted entirely, suggesting that research should be directed into the areas of co-limitation and element replacement.

Most experiments were conducted with only one replicate, though oxygen measurements were done in duplicate. Therefore, statistical analyses were not carried out. In future research, all experiments should be conducted with at least three biological replicates, multiple readings for each replicate and possibly experimental repeats.

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Appendix

Appendix A: Formula for BG-11 medium used in this study (from Andersen 2005, based on Rippka *et al.* 1979)

Component	Stock Solution ($g \cdot L^{-1}$ dH_2O)	Quantity Used	Concentration in Final Medium (M)
Fe Citrate solution		1 mL	
Citric acid	6	1 mL	3.12×10^{-5}
Ferric ammonium citrate	6	1 mL	$\sim 3 \times 10^{-5}$
$NaNO_3$	-	1.5g	1.76×10^{-2}
$K_2HPO_4 \cdot 3H_2O$	40	1 mL	1.75×10^{-4}
$MgSO_4 \cdot 7H_2O$	75	1 mL	3.04×10^{-4}
$CaCl_2 \cdot 2H_2O$	36	1 mL	2.45×10^{-4}
Na_2CO_3	20	1 mL	1.89×10^{-4}
$MgNa_2EDTA \cdot H_2O$	1	1 mL	2.79×10^{-6}
Trace metals solution	(See following recipe)	1 mL	-

Trace metals Solution

Component	Stock Solution ($g \cdot L^{-1}$ dH_2O)	Quantity Used	Concentration in Final Medium (M)
H_3BO_3	-	2.860 g	4.63×10^{-5}
$MnCl_2 \cdot 4H_2O$	-	1.810 g	9.15×10^{-6}
$ZnSO_4 \cdot 7H_2O$	-	0.220 g	7.65×10^{-7}
$CuSO_4 \cdot 5H_2O$	79	1 mL	3.16×10^{-7}
$Na_2MoO_4 \cdot 2H_2O$	-	0.391 g	1.61×10^{-6}
$Co(NO_3)_2 \cdot 6H_2O$	49.4	1 mL	1.70×10^{-7}

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