

To the Graduate Council:

I am submitting herewith a thesis written by Shannon Pedigo Efteland entitled “The effects of iron on growth and physiology of the cyanobacterium *Microcystis aeruginosa*.” I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

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The effects of iron on the growth and physiology of the cyanobacterium *Microcystis
aeruginosa*

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Shannon Pedigo Efteland
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Dedication:

This work is dedicated to the people who pushed me to strive for more, simply because they believed in me. To name a few: Sid Cahn, Buzz Cahn, and C.W. Minkel

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Thank you, Eric for being my anchor and everything I ever hoped for.

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Abstract:

To determine the effects of iron on the growth of the cyanobacterium *Microcystis aeruginosa*, a series of growth and iron uptake experiments were conducted. Studies included the effects of iron and organic iron-binding chelators on batch cultures of known bacteria and on the natural community of Lake Erie, where blooms of *M. aeruginosa* have been documented since 1995. Results of growth rate studies under iron limitation suggest that *M. aeruginosa* uses an active, high-affinity transport system to acquire iron after a lag time at initial limitation. Further studies indicate that cells continue to take up nutrients and possibly metals, while their growth rate is slowed prior to activation of the high-affinity transport system. Studies to observe iron effects on the natural community of Lake Erie demonstrate that 60% of the iron uptake occurs in the picoplankton (0.2 - 2 μm size fraction) and that DFB is effective at sequestering iron in natural communities.

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1 Introduction

1.1 Basis of study

One of the most researched topics in aquatic systems is the role that nutrient limitation plays in structuring aquatic communities and specifically the determination of "the" limiting nutrient. This thesis will focus on the bloom-forming, potentially toxic cyanobacterium, *Microcystis aeruginosa*, and its response to limitation by the micronutrient iron. Moreover, this study will be carried out in the context of the potential for iron to act as a growth-limiting agent on *M. aeruginosa* in the Laurentian Great Lakes (Lake Erie). Growth rate estimates for *M. aeruginosa* have been determined at varying concentrations of iron as well as for cultures maintained with static concentrations of iron in the presence of a series of iron specific chelators. These results, coupled with *in situ* experiments concerning the natural planktonic community in Lake Erie, are combined to provide some insight as to the potential role iron may play in the reoccurring blooms of *M. aeruginosa* that have been documented in Lake Erie since 1995 (Brittan et al. 2000).

1.2 Cyanobacteria

Cyanobacteria are photosynthetic prokaryotes that are ubiquitously distributed through most temperate and tropical aquatic environments (Adhikary 1996). Fossil evidence suggests that they were amongst the earliest of life forms and may well be responsible for the production of oxygen gas in the early history of Earth's atmosphere (Adhikary 1996, Carmichael 1994). In freshwater, cyanobacteria can range from 20-80% of the water column biomass as predicted by size-fractionated chlorophyll (Wilhelm et al.

2003, DeBruyn et al. 2004) and as such represent an important component of the whole ecosystem production.

Cyanobacteria have evolved specific mechanisms for proliferating under environmental stress. Under conditions of stress, normal biochemical pathways are often altered, as genes responsible for making or modifying different cellular products are up or down regulated. The result is an altered physiology that provides a competitive edge in the changing environment. For example, many cyanobacteria are capable of utilizing dinitrogen gas when levels of dissolved nitrate are low. This process, termed nitrogen fixation, exploits the iron-dependent enzyme nitrogenase to fix N_2 gas into cellular NH_4^+ (Bohme 1998). Not only does this process increase the availability of nitrogen to the organism itself, but it also acts as an entry point for nitrogen to enter the aquatic food chain (Rai 1996).

1.3 Iron

Iron is a micronutrient and the fourth most abundant element in the earth's crust (Briat 1992). In anoxic environments, iron is stable in both the ferric (Fe^{3+}) and ferrous (Fe^{2+}) valencies. Geologic records indicate that the Earth's early environment was anoxic (Carmichael 1994) and as such iron was readily available for incorporation into the enzymes of evolving microbes. However, in the presence of oxygen, at biological pH, iron reacts to form biologically unavailable iron hydroxides (Geider 1999). The evolution of oxygenic photosynthesis over millions of years (Carmichael 1994), and the subsequent accumulation of O_2 in the atmosphere and surface waters, constrained iron

from surface waters to precipitate and form the famous “red-beds” associated with many geological outcroppings (Laberge 1973). These chemical changes in iron availability coupled with the obligate cellular demand for iron sets up a unique problem for planktonic species (Wilhelm et al. 1996), leading to a strong natural selection for microbes that evolved methods to more efficiently scavenge iron from the environment (Briat 1992).

In many modern aquatic systems dissolved iron is present in only nano to picomolar amounts and its biological availability depends on a series of factors (Hutchins et al. 1993). Iron can be deposited by rain and aeolian flow into many aquatic environments as well as introduced *via* runoff and sediment disturbance by tributary merge flow or during a turnover event (Briat 1992). In the water column, iron is present in abiotic particles, in biological extracellular, and surface-associated, organic complexes formed in the water column (Hudson and Morel 1989). Typically, iron is deposited as Fe^{2+} (by rain or dust) and in 2- 200 milliseconds it is converted to Fe^{3+} , which, at physiological pH, rapidly reacts with oxygen to form iron hydroxides, which are insoluble and therefore unavailable to microorganisms (Morel and Herring 1993). The biogeochemical cycle of iron is further complicated by the tendency of iron to adsorb to particles in oxygen-rich waters and precipitate out of the water column (Hudson and Morel 1989).

1.4 Cyanobacteria and iron

In cyanobacteria iron is a major component in cytochromes, co-factors, and in iron-sulfur centers required for the completion of enzymes (Briat 1992, Butler, 1998, Geider and LaRoche 1994). Many iron-containing molecules are required for necessary biological pathways such as metabolic and catabolic pathways, nitrogen assimilation, electron transport, and chlorophyll production (Boyer et al. 1987).

During iron-replete conditions many cyanobacteria utilize non-specific transport systems to assimilate ferric iron. However, during conditions of iron stress many organisms induce a high affinity iron transport system (Wilhelm 1995). The ability of cyanobacteria to persist in low iron conditions is in great part due to these active iron-scavenging systems. During high affinity iron acquisition, many cyanobacteria produce low molecular weight Fe^{3+} binding compounds, called siderophores, which are excreted from the cell (Trick and Kerry 1992, Briat 1992). These siderophores bind tightly and specifically with ferric iron (Fe^{3+}) scavenged from the environment. Upon reencountering the cell, the ferrisiderophore compounds then bind with high affinity to protein receptors on the surface of the cell which mediate assimilation (Neilands 1995). Activation of these transport systems allows these cells to recover from stress. The growth kinetics of organisms known to produce siderophores as part of a high-affinity transport system under low nutrient conditions has been previously illustrated (Wilhelm 1995) (figure 1). This is demonstrated by a “recovery” of growth rate in strains that are able to activate these transporters after a lag in growth rate upon initial iron limitation. This lag time as well as the actual growth rate exhibited upon activation varies.

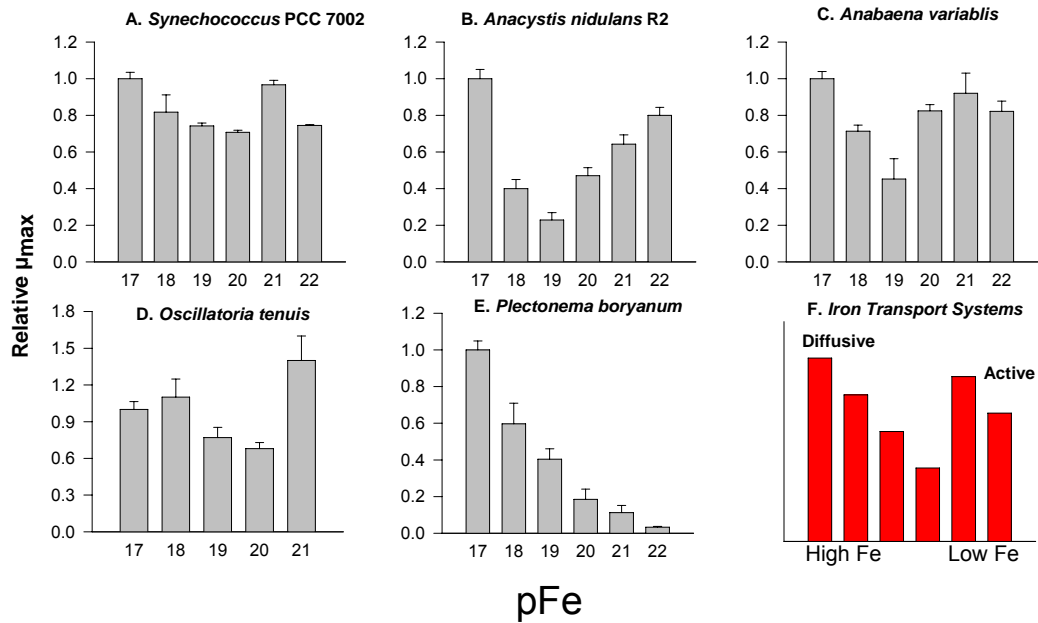


Figure 1: Active vs. Diffusive transport of iron

Graphs A-D Show growth rate of organisms known to activate a high-affinity transport system compared to graph E which illustrates growth for an organism that does not activate a high-affinity transport system. Graph F shows a generic trend in growth rate expected by activation of an active transport system. All growth rates are standardized to Fe-replete (pFe 17) conditions. (Wilhelm 1995).

Siderophore production has been documented in *Synechococcus* PCC 7002, *Anabaena variabilis* ATCC 29413, *Anacystis nidulans* R2, and *Oscillatoria tenuis*, as well as many other planktonic, cyanobacterial species (Trick and Kerry 1992, Wilhelm et al. 1996, Kerry et al. 1988, Brown and Trick 1992).

It is widely accepted that siderophore production is a response to iron limitation, but it has been difficult to accurately study the effects of trace metals in the past, due to the long process of developing trace metal clean techniques. Application of trace metal clean techniques (Bruland et al. 1979, Twiss 2000) has provided new opportunities to address questions concerning metal impacts on the productivity and physiology of aquatic organisms.

1.5 *Microcystis aeruginosa*

Microcystis aeruginosa is a cyanobacterium, belonging to the family Chroococcaceae, and is found in environments ranging from brackish water to freshwater (Codd 2000). It is coccoid and can be found as one cell or in loose colonial associations containing tens of thousands of cells (Reynolds et al. 1981). *Microcystis spp.* are non-nitrogen-fixing cyanobacteria with some strains capable of producing secondary metabolites called microcystins (Figure 2). With more than 60 different forms documented, microcystins are potent hepatotoxins that are a worldwide potential health concern (Lawton and Codd, 1991, Carmichael, 1994). Many cyanobacteria are thought to produce toxins in response to environmental stress (Lukac and Aegerter 1993, Lyck et al. 1996).

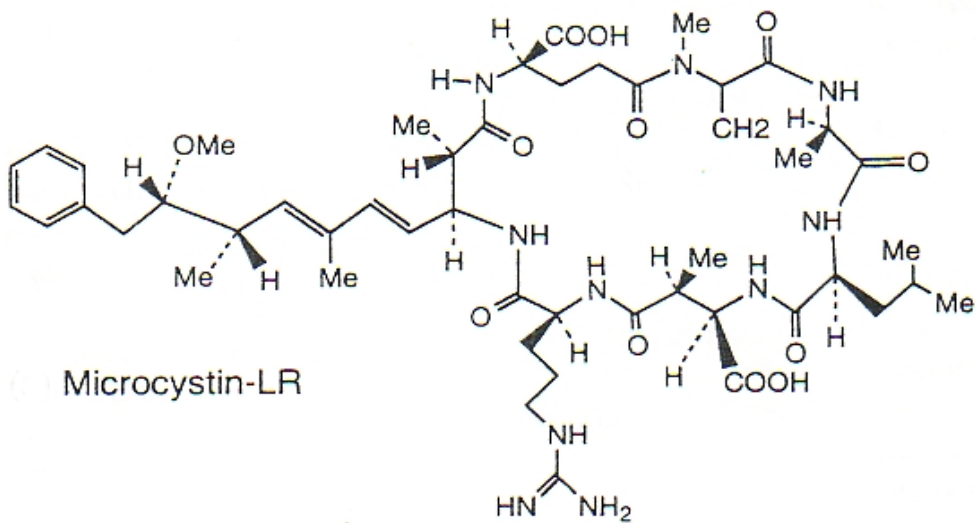


Figure 2: The structure of microcystin-LR
(Adapted from Kaebnick and Neilan 2001)

Since these toxins can affect livestock, fish, wildlife, pets and humans, it has become important to understand the environmental factors that influence their production (Frazier et al. 1998, Codd et al. 1999, Codd 2000, Chorus et al. 2000).

1.6 Cyanobacterial toxin production and microcystin

Cyanobacterial toxins, including microcystin, are secondary metabolites (Codd 1995). Production of most cyanobacterial toxins is thought to be triggered by a specific environmental condition or set of conditions (Oh 1999). Although there remains no conclusive evidence regarding the environmental conditions that induce toxin production,

several studies have characterized the response of cyanobacterial toxin production to environmental stressors (Lyck et al. 1996, Lukac and Aegerter 1993).

Many conditions, or sets of conditions, have been studied to observe the effect on toxin production. There is evidence to suggest that microcystin is produced in response to decreases in iron availability and as such, it may be tied to changes in cellular physiology associated with growth recovery under iron-limiting conditions (Lukac and Aegerter 1993, Utkilen and Gjølme 1995, Lyck 1996). Some studies suggest that microcystin may act as a siderophore due to its similarity in structure to known siderophores and its affinity for other metals (Utkilen and Gjølme 1995, Codd 1995) although the compound itself has recently been shown to have no affinity for iron (Wilhelm and Witter, results unpublished).

It has been difficult to compare studies of microcystin content. The shortcomings of such studies involve the difficulty in normalizing toxin into an easily reproducible and comparable form due to the variation between parameters, such as dry weight or total protein, used to normalize toxin data (Kaebernick and Neilan 2001, Lyck et al. 1996). This, coupled with the fact that microcystin can exist in over 60 different chemical forms, has made accurate toxin analysis difficult and has led to extensive variation in results (Kaebernick and Neilan 2001).

1.7 Lake Erie

The largest system of potable fresh surface water in the world is the Laurentian Great Lakes Basin (Wilhelm 2003). Lake Erie is the smallest by volume and shallowest

of the Great Lakes. It warms quickly in the summer months and on occasion, will freeze over in the winter. This lake is directly surrounded by 17 metropolitan areas and has approximately 11.6 million people in its drainage basin (Wilhelm et al. 2003). Morphometrically, Lake Erie can be divided into three functional basins: the central basin, the western basin and the eastern basin (figure 3 in meters). Each of these has unique characteristics.

The western basin has an average depth of 24 feet with a maximum depth of 62 feet. The central basin is characterized by a fairly uniform depth between 62-80 feet and is much larger than either the eastern or western basins. The eastern basin is the deepest with a maximum depth of 210 feet and an average of 82 feet. Field studies were performed in the Western basin of Lake Erie during the MELEE (Microbial Ecology of the Lake Erie Ecosystem) cruise aboard the Canadian Coast Guard Ship ‘*Limnos*’ during July 2000.

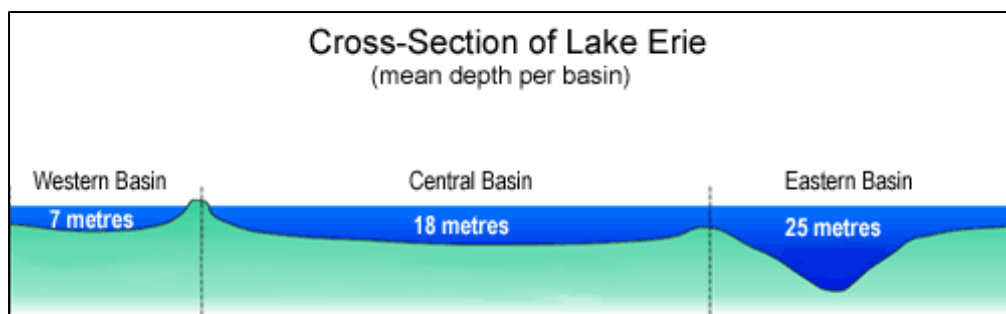


Figure 3: Cross-section of Lake Erie
Image taken with permission from the Delta Institute
(<http://erieforum.org/lakeerie.php>)

1.8 Eutrophication and Lake Erie

Eutrophication is the process of “aging” whereby nutrient content and sediment loading is increased. Naturally, this process occurs over a geologic time scale (millions of years) but human influence can dramatically speed up the process so that changes may be seen in as little as a decade. In Lake Erie, the speed and scope of eutrophication has been greatly magnified by anthropogenic influences.

Eutrophic and hypereutrophic freshwater bodies tend to favor a food web structure dominated by cyanobacteria (Haider et al. 2003, Codd 2000, Carmichael 1994). Large, bio-fouling blooms of cyanobacteria can occur very quickly, clogging filters, impairing water usage and leaving water with an unpleasant odor and taste. Such blooms may also contain harmful cyanobacterial toxins (Ouellette and Wilhelm 2003). As eutrophication of a waterbody progresses, the period of cyanobacterial dominance intensifies, and the problems associated with blooms are magnified (Haider et al. 2003, Codd 2000). Blooms of *Microcystis aeruginosa* are extremely common in eutrophic water bodies around the globe (Oh, 2000). *M. aeruginosa* is present in Lake Erie and has proliferated extensively since 1995. In 2003 a large bloom in the Western Basin of the Lake produced toxin concentrations that were 14-20 times the World Health Organizations limit ($1 \mu\text{g L}^{-1}$) (Rinta-Kanto and Wilhelm, unpublished). Australia, England and Japan have all reported chronic blooms of microcystin producing strains of *Microcystis* for many years, and this problem will be magnified worldwide as eutrophication is amplified by anthropogenic activities (Haider et al. 2003).

This thesis addresses the following hypothesis:

Hypothesis: Under conditions of iron limitation, *Microcystis aeruginosa* undergoes physiological changes that allow them to proliferate.

To be able to reject this hypothesis (or its corresponding null) I have addressed the following questions.

- 1) How do changes in total iron availability affect growth of *M. aeruginosa*?
- 2) How does the sequestration of iron by organic iron-binding compounds affect iron availability to cells?
- 3) How does iron availability affect toxin production by *M. aeruginosa*?

2 Methods and Materials

2.1 Organisms and media preparation

Microcystis aeruginosa LE3 was the gift of Dr. Wayne Carmichael (Wright State University). It was isolated from Lake Erie 1995 and is known to produce the toxin microcystin in fairly high quantities (Brittain et al. 2000). *Microcystis aeruginosa* UTEX 2385 was purchased from the University of Texas Culture Collection for Algae. This isolate is also known to produce the toxin microcystin (Rinehart et al. 1994, Ouellette and Wilhelm 2003). All studies were performed using UTEX 2385 unless otherwise indicated.

Both cell types were maintained in BG-11 media (Rippka et al. 1979) with pH maintained at 7.0. For studies concerning nutrient Fe, the medium was modified according to Kerry et al. (1988) by removing the Fe-chelator citric acid. Trace metal impurities (including iron) were removed from water and nutrient stocks by passing them through a cation exchange (chelex-100) resin column (Price et al. 1989). All nutrient stocks were filter-sterilized through 0.2 μm nominal pore-size polycarbonate syringe filter. To prevent iron contamination that can be introduced from steam pipes in autoclaves, media were sterilized by Tyndalisation using Keller's microwave sterilization method (Keller et al. 1988). The media were microwave heated to almost boiling and then left to cool under a trace metal clean laminar-flow hood. This was repeated three consecutive times for sterilization. Cultures were maintained under continuous illumination with full spectrum fluorescent lights (*ca* 80 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) at a constant temperature of 25°C.

2.2 Iron concentration

The chemical speciation of iron in the BG-11 medium has been previously characterized (Wilhelm 1994) using the Mineql+ software package (Westall et al. 1976, Schecher and McAvoy 1992). The concentrations of total and free ferric iron (Fe^{3+}) used are shown in Table 1. The concentration of free ferric iron in solution is calculated similarly to pH, denoted by the $-\log [\text{Fe}^{3+}]$ and expressed as pFe. For these experiments, varying iron concentrations were generated by an addition of specific amounts from characterized iron stock into iron-free BG-11 media. Iron stock solutions were generated by dissolving FeCl_3 into 0.75% HCl (generated with Chelex-100 treated water). A primary stock solution consisting of 8.4×10^{-3} M FeCl_3 and a secondary stock solution consisting of 6.2×10^{-6} M FeCl_3 were prepared to provide for the range of media needed.

2.3 Preparing cultures for experiments at iron limiting concentrations

Cells were physiologically equilibrated in the appropriate medium during a preacclimation step prior to each experiment (Wilhelm 1995). Aliquots of iron-replete stock cultures were transferred into fresh medium containing the desired iron concentration. The new culture was grown to mid-log phase before it was transferred into fresh media a second time. This process is repeated once more, for a total of three transfers. This allows the iron in the media and in intracellular iron storage to equilibrate to the desired iron concentration (Kerry et al. 1988).

Table 1: Concentration of iron at different pFe levels
 Concentration of available iron for each pFe designation and the iron added to obtain each concentration (Kerry et al. 1988).

Designation	Iron added (M)	Available Iron (M)
pFe 17	4.2×10^{-5}	10^{-17}
pFe 18	4.7×10^{-6}	10^{-18}
pFe 19	4.1×10^{-7}	10^{-19}
pFe 20	3.1×10^{-8}	10^{-20}
pFe 21	5.1×10^{-9}	10^{-21}

2.4 Chelators

For general growth experiments, iron free BG-11 medium, as prepared above, was equilibrated to pFe 18 for 24 hours. Then each chelator was added from stock solutions made with chelex-100 treated water. When the final concentration of chelator was achieved, the new medium solution was left for 24 hours to equilibrate before inoculation. Each culture was inoculated from pFe 18 [10^{-18} M] cultures in log growth as determined by preliminary growth rate determinations (data not shown). Of the chelators Rhodotruclic Acid (RA) is unique in that it complexes iron at a 3:2 ratio, whereas the other chelators in this study bind iron at a 1:1 ratio (Mioni et al. 2003). Therefore the RA

addition experiments were performed at 1.5-fold higher concentrations than the other three chelators in order to have comparable iron binding. In addition to laboratory experiments, there were field studies performed to further review the effect of DFB, and EDTA on *M. aeruginosa*. Table 2 describes the four chelators used through these studies. Each chelator is characterized by, type, origin and iron-binding ratio.

2.5 Estimation of cyanobacterial biomass

Measuring the biomass of *Microcystis spp.* has been found to be a challenging task (Ouellette and Wilhelm 2003). Direct cell counts of cyanobacterial cells can be done with an epifluorescence microscope, since the phytopigments the cells contain allow them to autofluorescence under UV illumination (Vanliere 1989). However, determining cyanobacterial biomass through microscopy can be tedious, and samples from field studies must be preserved and brought back to the laboratory for analysis.

A common method to estimate biomass is by chlorophyll *a* extraction. This has often been used as the most convenient way of determining biomass. We estimated total chlorophyll *a* using the non-acidification extraction protocol of Welschmeyer (1994) and a Turner Designs, TD-700 fluorometer standardized with known chlorophyll standards and equipped with optical filters for extracted chlorophyll (Turner Designs). Chlorophyll *a* extraction requires collection of cells onto a filter, adding 90% acetone and incubation of the samples for approximately 24 hours (Welschmeyer 1994).

Table 2: A list of chelators, their physical distinction and iron-binding ratios

Ligand	Desferrioxamine B	Ferrichrome	Rhodotruclic acid	Dipyridyl
<u>Abbreviation</u>	DFB	FC	RA	DPP
<u>Origin</u>	Fungal Siderophore	Fungal Siderophore	Bacterial Siderophore	Synthetic
<u>Type</u>	Linear tetrapeptide	Cyclic Hexapeptide	Cyclic dipeptide	Cyclic Amine
<u>Iron binding ratio</u>	1:1	1:1	3:2	1:1
<u>Relative stability constants</u>	21.65	21.6	19.39	16.3
$\log K_{\text{Fe}^{3+}\text{L}}$				
<u>Reference</u>	Granger and Price 1999, Orr 1998, Hutchins et al. 1999, Mioni et al. 2003.	Orr 1998, Hider 1984, Mioni et al. 2003.	Orr, 1998, Atkin et al. 1970, Mioni et al. 2003.	Liu and Hider 2001,

2.6 Growth rate

Growth rate was determined from estimates of cellular biomass based on *in vivo* chlorophyll *a* fluorescence over time with an appropriately equipped Turner Designs TD-700 fluorometer. This is an easy and quick method to determine changes in cyanobacterial biomass in a culture without filtering, preserving or using any solvents (Wilhelm and Poorvin 2001) The process measures *in vivo* fluorescence by excitation of chlorophyll *a* molecules using a standard, *in vivo* chlorophyll fluorescence filter set and a chlorophyll *a* solid standard. The standard allows for normalization of data between time-points within an experiment and between separate experiments.

Growth rate studies were performed with triplicate treatments. Fluorescence was measured every 12-24 hours for each individual culture. The growth rate was determined as follows: for each replicate, the growth rate (d^{-1}) was calculated from a first order regression of the natural logarithm of fluorescence over time. The mean value (of the individual replicate) and standard deviation was taken from these regressions and recorded as doublings per day. Statistical analysis of this data was by standard two-tailed paired t-tests. Results were considered significant when $p < 0.05$.

2.7 Direct counts of *M. aeruginosa* cells

All field and laboratory samples were preserved in 2.5% glutaraldehyde (final concentration) and stored at 4°C prior to enumeration. Cultures were diluted with sterile (0.2- μ m filtered) Milli-Q water as needed, based on the density of cells of the preserved sample and the number of replicates to be obtained from one preserved sample.

Typically, 1 - 1.5 mL of sample was preserved and, due to cell density, 300 μ l of the sample was diluted with 500 μ l of water. The diluted sample (\sim 800 μ l) was vacuum filtered onto a 0.22- μ m nominal pore-size black polycarbonate filter (HTBP) backed with a nitrocellulose 0.45 μ m pore size filter (HAWP). Each sample was filtered with less than 15 kPa on a vacuum manifold (Hoeffler Scientific) to prevent breakage of cells. After filtration each HTBP filter was transferred to a clean glass and prepared for enumeration. One drop of type FF low fluorescence immersion oil (Cargille Laboratories, Inc.) was placed directly on top of the filter and a glass cover slip was placed on top. Slides were either enumerated immediately or frozen at -20°C until they could be observed. A Leica DMRXA epifluorescence microscope equipped with wide blue filter set with an ocular grid was used. For each sample 200 cells or 20 fields of view were counted. A calibration factor was determined using a grid size calibrated with a stage micrometer. All enumerations were performed under oil immersion at 1000x total magnification.

2.8 Iron uptake by laboratory cultures

Radioactive iron was added as 0.1 Ci of ^{55}Fe (as $^{55}\text{FeCl}_3$ in 1% HCl (5.592×10^{-5} M) per bottle plus 0.47 nM stable iron for a total iron concentration equal to pFe18. All samples containing radioactive Fe were filtered onto a 0.45 μ m pore size nitrocellulose filter (HAWP) and rinsed with a Titanium-Citrate-EDTA solution to remove superficially associated iron (Hudson and Morel 1989). Filters were placed into 7 mL scintillation vials. Scintillation fluid (5 mL of EcoScint) was added into vials and samples were left overnight to dissolve the filter. Then samples were placed in a Beckmann Coulter, LS

381 liquid scintillation counter equilibrated with a Fe^{55} -specific quench curve. Sample readings were recorded as disintegrations per minute(DPM). These values were then converted to fg iron per sample or per cell

2.9 CHN analyses and for laboratory iron uptake experiments

Parallel cultures were prepared at the time of, and maintained at the same temperature and light conditions as the cultures prepared for radioactive iron quota studies. Cultures used for CHN analysis did not have radioactive iron but needed to be compared to cultures used in uptake experiments in order to determine if iron affects carbon and nitrogen uptake and vice versa.

Carbon-Nitrogen-Hydrogen analysis samples were cultured to early exponential phase based on previous growth rate studies (final data in section 3.1). Then 10 mL of each sample was filtered with less than 15 kPa on a vacuum manifold (Hoeffler Scientific) onto a precombusted, 25 mm Glass Microfiber Filter (GFF Millipore). The filter was then dried atop an aluminum boat in a glass Petri dish at 60°C for 24 hours. Each filter was then folded, packaged in an individual aluminum foil pouch and sent to Bowling Green State University. Samples were processed in Dr. McKay's laboratory using a Perkins Elmer 2400 combustion furnace and analyzer according to the Perkins Elmer manual. The basis of this procedure is to use a combustion method to convert the sample elements into CO_2 , NH_2 and H_2 . The gases are detected with a thermal conductivity detector. The analyzer reported values, in this case, of absolute amount of carbon, hydrogen and nitrogen in μg

A standard ANOVA model was performed to determine the significant difference of values obtained for carbon and nitrogen during iron uptake experiments in the laboratory. To assure that the assumption of equality of variance was correct, the Lavine's test of homogeneity of variance was performed to confirm the quality of the ANOVA test. Differences were considered significant when $p < 0.05$. For iron uptake at one time point, the lack of replication and extreme variances between some values within one treatment indicated that a non-parametric test should be used, not an ANOVA model. A Kruskal-Wallis One-Way Analysis of Variance was performed to determine significant differences between these values.

2.10 X-Ray fluorescent trace metal quantification

Benjamin Twining and Steven Baines of the Marine Sciences Research Center at the State University of New York, processed several of our samples using X-ray fluorescence to characterize cellular trace metal content. The process works by scanning an object with X-rays and measuring the resulting spectra. This utilizes the fact that each metal reflect different energies as a specific "wavelength fingerprint". Each metal has a standard energy and a very sophisticated computer can process these for strength and clarity and calculates the quantity of each metal. Three cells were used per iron concentration for original determinations. Those values were averaged together and results are reported as mean fg/cell \pm 1 SD. However, data were normalized to estimated cellular Carbon by estimating cell volume. The Titanium III wash was not utilized for these studies due to several reasons. The Ti (III) rinse is formulated for use with unstable

Fe⁵⁵ or Fe⁵⁹, not for stable Fe. Preliminary numbers indicated that the Ti (III) rinse showed no significant difference from those samples analyzed without the solution

2.11 Toxin analysis

Toxin analysis was performed by Dr. Mike McKay at Bowling Green State University. Quantitative detection of microcystin was done using an EnviroLogix QuantiPlate Microcystin Kit utilizing a competitive Enzyme-Linked ImmunoSorbent Assay (ELISA). This ELISA test is designed for the quantitative detection of microcystin in surface water samples with a range from 0.16 to 2.5 ppb. The toxin in the sample competes with enzyme-labeled microcystin for antibody binding sites located on the inside surface of the test wells (EnviroLogix catalog Number EP 022). This test does not screen for different variants of microcystin but for the quantity of total microcystin in each sample. Samples were run in triplicate at two different dilutions (1.0 and 0.1) to assure that they were within the detection range. The sample kit has a built-in negative control factor to determine if the concentration in the sample is above or below range of detection. Other reports have shown that much more in-depth analysis is required to adequately determine toxin content (Orr 1998).

2.12 Field study experimental design

Field studies were performed in Lake Erie from July 4 to July 14 2000 on the Canadian Coast Guard Ship, *Limnos*. All trace-metal clean iron uptake experiments were performed in a portable particle free room onboard the CGGS *Limnos*. The facility was

an antechamber with a metal free interior. The walls, floor and ceiling were coated in an epoxy-coated laminate. The work area was a HEPA-filtered laminar flow bench made of polyethylene. The vacuum pump utilized nitrogen gas and no metal or glass materials were used (Twiss et al. 2000). All materials were triple acid washed prior to installation in the facility.

2.12.1 Size fractionated iron uptake by the natural community

Water for this experiment was collected at 3 meters using an all Teflon pumping system designed to move water directly from the lake's surface to a class 100 clean facility within the ship. Immediately after collection, this water was passed through a Nyltex 210- μm filter to remove most of the grazers. This pre-filtered water was then transferred into acid washed polyethylene carboys and dispensed in 1200 mL aliquots into 1.2 L acid washed polycarbonate culture bottles. Bottles were filled completely as to leave less space for oxygen. All treatments were performed in triplicate. Table 3 illustrates each experimental treatment. Where nutrient addition is indicated, 10 μM NO_3 , 1 μM PO_4 and 10 μM Si were added. Where iron addition is indicated, iron was added as 0.1 Ci (5.592×10^{-5} M) of ^{55}Fe (as $^{55}\text{FeCl}_3$ in 1% HCl) per bottle plus 0.47 nM stable iron for a total iron concentration equal to pFe18. Ultra-filtered water was used to prepare all nutrient and chelator solutions and to rinse the final filters. Ultra-filtrate water was produced by pressure filtering lake water before passing it through the Millipore M12 Tangential Flow Filtration System (Wilhelm and Poorvin 2001).

Table 3: Experimental treatments added during iron uptake
 Experiments performed in triplicate on Lake Erie in July 2000.

Culture #	Nutrients	EDTA	DFB	Fe ⁵⁵
1-3	∅	∅	∅	+
4-6	∅	∅	∅	∅
7-9	+	50nM	∅	+
10-12	+	100nM	∅	+
13-15	+	200nM	∅	+
16-18	+	500nM	∅	+
19-21	+	∅	50nM	+
22-24	+	∅	100nM	+
25-27	+	∅	200nM	+
28-30	+	∅	500nM	+

All bottles were incubated in an onboard incubator covered with fine mesh screen to simulate *in situ* light conditions (ca 37% of surface ambient levels). *In situ* temperature was maintained by a constant flow of lake water pumped directly into the incubator. After 24 hours the samples were removed from incubation and placed in a portable particle-free facility onboard. Samples of water after pre-filtration, and from final ultrafiltrate were preserved in 2.5% glutaraldehyde. These samples were then enumerated to determine the efficiency of the ultra-filtrate procedure (data not shown).

From each bottle, a 2mL sample was taken at time zero as a baseline measurement. Parallel dilutions were used to determine iron uptake by different size fractions of the natural plankton community. Each culture sample was filtered, in the particle-free room, as follows: 250 mL were filtered through a 20.0 μm nominal pore size filter, 100 mL through a 2.0 μm filter and 50 mL through a 0.2 μm filter (Millipore). Filtration force of < 20 kPa was applied to reduce cell breakage due to sheering. After filtration of culture aliquots, 3 mL of the Ti(III) solution was applied to each filter and let sit for 3 minutes with vacuum turned off. After three minutes, vacuum was reapplied to remove all of the Ti (III) solution and 3 mL of ultra-filtered lake water was applied as a rinse. That rinse was allowed to sit for three minutes without vacuum before vacuum was reapplied to remove the final rinse water. After completing the final rinse, the filters were placed in 7 mL polycarbonate scintillation vials and radioassayed by liquid scintillation counter (courtesy Dr. Michael Twiss, Ryerson Polytechnic University).

Statistical significance for this set of data was determined using paired, two-tailed t-tests. Statistical results are usually considered significant if $p < 0.05$. However all of

the significant results for this data set are $p < 0.02$. Due to the low p-value, it is probable that there are very few type 1 errors, which are invariably introduced when there are a high number of comparisons.

2.12.2 Effect of DFB on growth of LE3

This experiment was performed to see how the growth of *M. aeruginosa*, LE3 would be affected by addition of DFB lake water. Table 4 explains treatments performed in triplicate into 1L of ultrafiltered lake water. After addition of nutrients and DFB, the new solution was allowed to equilibrate for 12 hours before addition of 50 mL of LE3 stock culture in mid log growth at pFe 18. Upon inoculation, after gently swirling to obtain a homogeneous distribution of cells, 50 mL was removed, filtered and frozen for chlorophyll *a* extraction and a baseline measurement. The process was repeated at 24 hours and again at 48 hours.

An ANOVA model was used to determine significant differences between the chlorophyll *a* values obtained during studies of the effect of DFB in waters of Lake Erie. This analysis was performed as described above and homogeneity of variance was confirmed with a Lavine's test. Differences were considered significant when $p < 0.05$.

Table 4: Experimental treatments for studies of the effect of DFB on *M. aeruginosa* cultured in ultrafiltered lake water

Concentration of DFB added (nM)	Nutrients Added?
0	No
10	yes
25	yes
50	yes
100	yes

3 Results

3.1 Growth rates

The growth rate of *Microcystis aeruginosa* under conditions of increasing iron limitation is shown in figure 4. A decrease in total iron did not result in a linear decrease in growth rate. There is not a significant difference in growth rate of cultures maintained at pFe 17 and pFe 18. (iron-replete). Cultures grown at pFe 19, 20 and 21 are significantly lower than cultures maintained at either pFe 17 ($p < 0.0047$) or pFe 18 ($p < 0.0028$). The growth rate of pFe 19 cultures is significantly lower than cultures a pFe 21 ($p = 0.014$), where the “recovery” of growth rate is obvious, but not significantly different from pFe 20 cultures ($p = 0.054$). There is no significant difference between cultures grown at pFe 20 and pFe 21.

3.2 Influence of chelators on growth rate

Addition of DFB at 5 nM ($p = 0.007$) and 25 nM ($p = 0.0029$) results in growth rates significantly higher than pFe 17, but not significantly higher than pFe 18 (figure 5). The 500 nM DFB treatment resulted in growth rates significantly lower than the growth rate at pFe 17 ($p = 0.023$) and pFe 18 ($p = 0.01$). All DFB treatments in this study resulted in growth rates significantly higher than growth exhibited by cultures grown at pFe 19 ($p < 0.009$) and pFe 20 ($p < 0.02$). The addition of 500 nM DFB resulted in growth rates not dissimilar to growth rates shown for cultures grown in iron-limited conditions (pFe 19, 20 and 21). The addition of 500 nM DFB resulted in growth rates significantly lower ($p < 0.037$) than all other DFB treatments in these batch culture experiments.

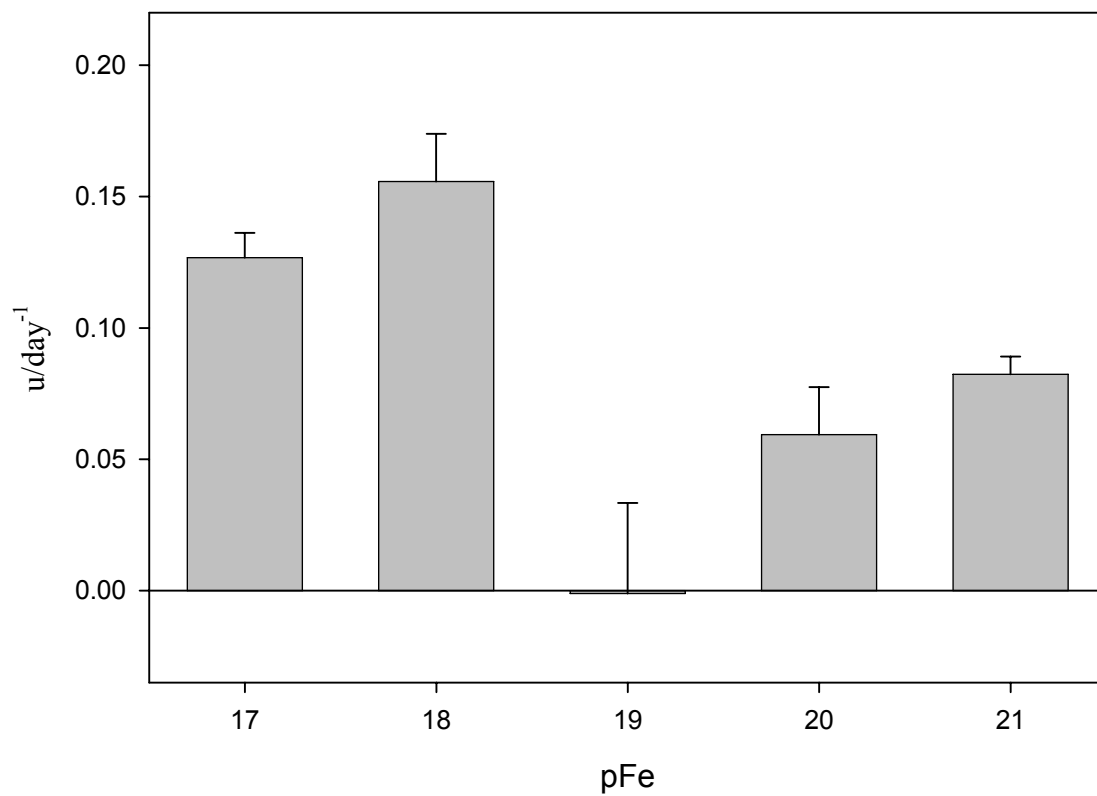


Figure 4: Average growth rate of *M. aeruginosa* at varying iron concentrations
Determined by in vivo fluorometric analysis. n= 3.

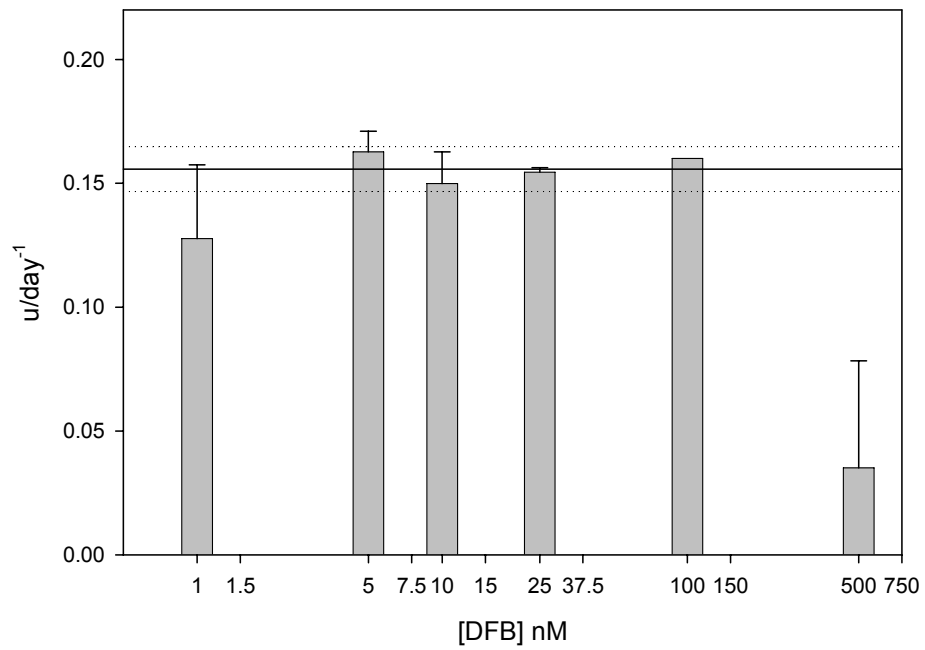
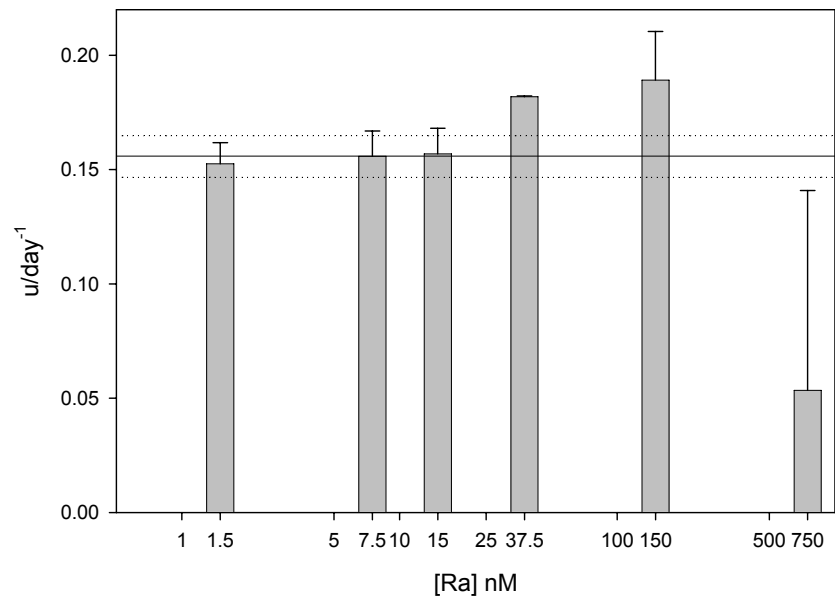


Figure 5: The growth of *M. aeruginosa* affected by the addition of chelators Rhodotrucic acid (Ra), Desferrioxamine-B (DFB), Dipyrityl (DPP), and Ferrichrome (FC). (n=3). Upper specification line is equal to growth rate observed at pFe 18 with standard error. Continued on next page.

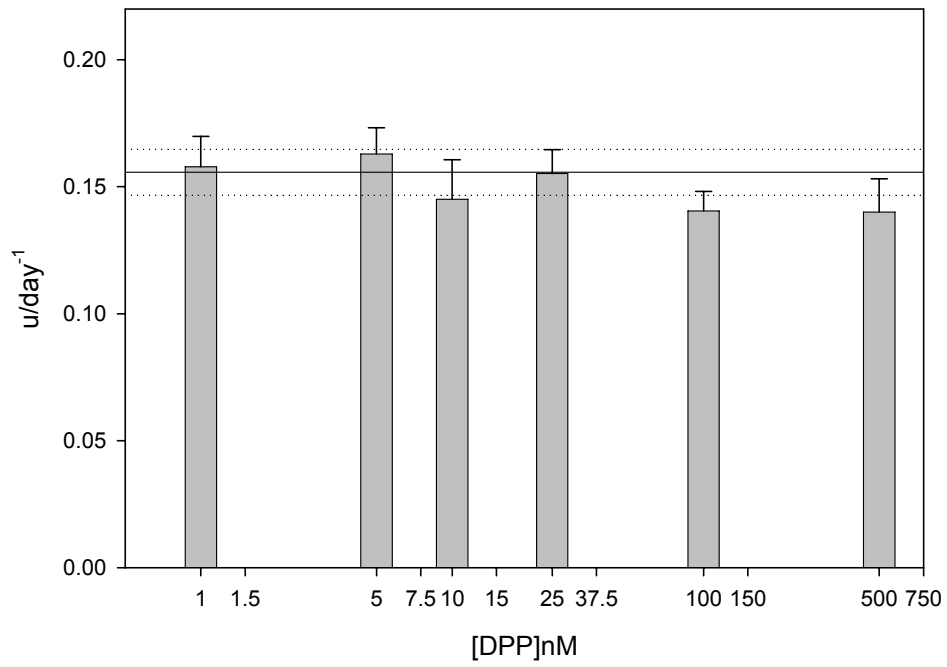
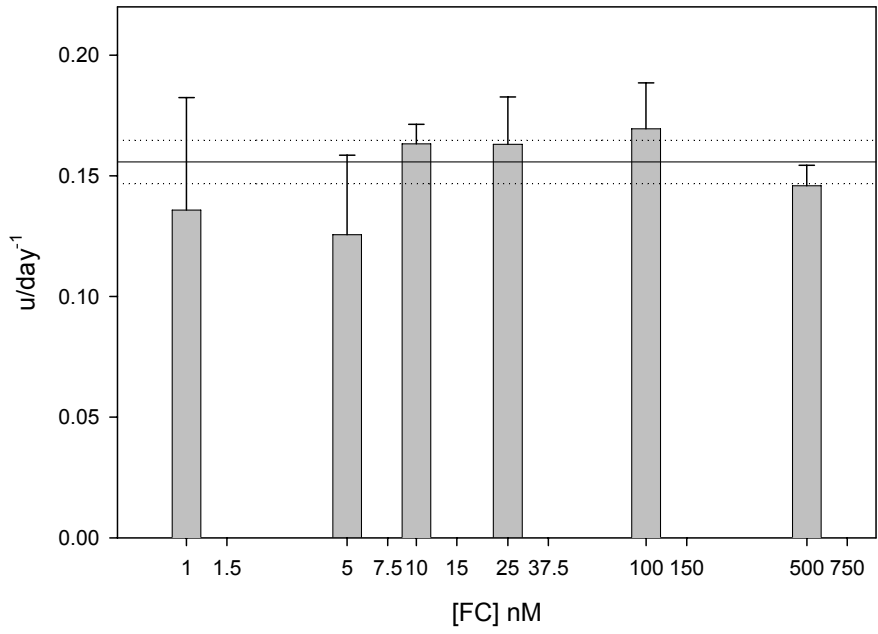


Figure 5 (continued)

Studies observing the effects of Ferrichrome showed no significant difference between treatments. Addition of FC resulted in growth rates not dissimilar from iron-replete conditions at pFe 18. All treatments of FC addition resulted in growth rates significantly higher ($p < 0.028$) than pFe 17 cultures with the exception of the 500nM treatment, but not dissimilar from pFe 18 cultures. All treatments exhibited growth rates significantly higher ($p < 0.0057$) than those seen at the induction of iron limitation at pFe 19. With the exception of the 500 nM addition, FC treatments had significantly higher growth rates compared to cultures in iron limited conditions at pFe 20 (< 0.0029) or pFe 21 ($p < 0.001$).

Observation of the effects of RA addition (figure 5) revealed that growth rate of cultures at 37.5 nM RA is significantly higher ($p = 0.024$) than the 1.5 nM treatment and the treatment and 150 nM RA results in growth rates significantly higher ($p = 0.036$) than cultures maintained at the 750 nM RA addition. All treatments were not dissimilar to growth rates seen in iron-replete (pFe 18) cultures. However, all treatments, with the exception of the 750 nM addition, resulted in growth rates significantly higher than pFe 17 ($p < 0.028$), pFe 19 ($p < 0.006$), pFe 20 ($p < 0.003$) and pFe 21 ($p < 0.0007$).

The study of the effects of DPP on *M. aeruginosa* show that there is no significant difference in growth as affected by changing concentrations of DPP from 1 nM to 500 nM. The addition of DPP at 1 nM, 5 nM and 25 nM resulted in growth rates significantly different from the growth rate at pFe 17 ($p < 0.025$). All concentrations of DPP added resulted in growth rates significantly higher than those seen at iron-limited conditions, as

indicated by pFe 19 ($p < 0.003$), pFe 20 ($p < 0.004$), and pFe 21 ($p < 0.007$) but not dissimilar to growth rates seen at iron-replete conditions as indicated by pFe 18.

3.3 Iron uptake by laboratory cultures

The average uptake of iron by *M. aeruginosa*, at pFe 17-pFe 21, during logarithmic growth, is illustrated by figure 6. This graph shows average iron uptake in fg/mL. Because this measure is small, and there was a lack of replication, statistical analysis of these values did not indicate any significant difference in uptake between each pFe treatment. The only statistical differences were due to variation between duplicate samples. The iron uptake illustrated in this figure mirrors the standard trend in growth rate determined for *M. aeruginosa* at varying iron concentrations (figure 4). The uptake at pFe 17 is not significantly lower than the uptake at pFe 18. Iron uptake falls dramatically at pFe 19 and then shows a recovery at pFe 20. It is interesting to notice that while iron uptake is the lowest for the pFe 19 treatment, the total carbon and nitrogen determined in this study (table 5), are significantly higher at this pFe as compared to other treatments (Anova, $p < 0.001$). Nitrogen values are significantly different at every pFe treatment level ($p < 0.001$, 0.001, 0.003). These variations in carbon and nitrogen are highly significant.. Effectively, the Anova model used to determine statistical significance explains 96.3% of the variation in carbon values and 99.3% of the variation in nitrogen values.

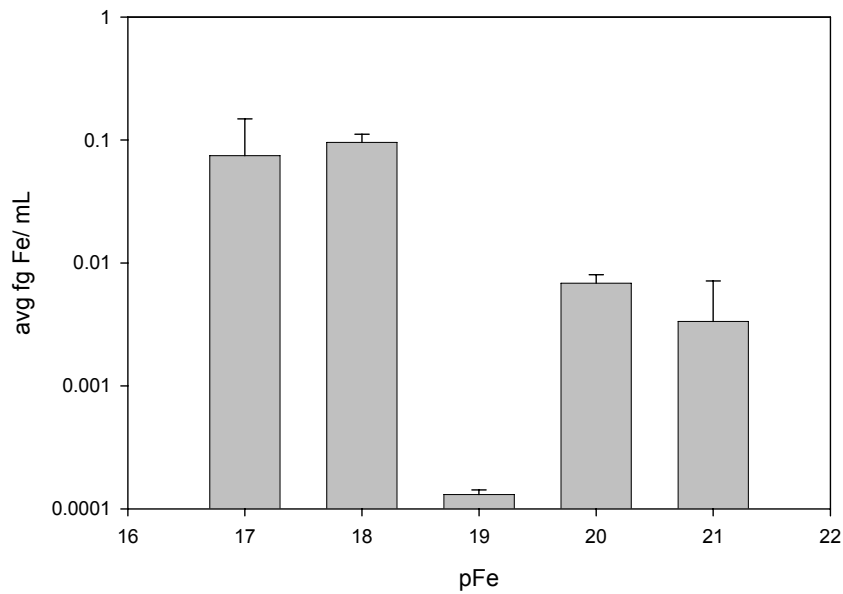


Figure 6: Average iron uptake (fg Fe/mL) in laboratory cultures

Table 5: Carbon and nitrogen content at varying iron concentrations
Asterisks indicate significant difference ($p < 0.05$) using Bonferroni post hoc test.

Iron Concentration	Average $\mu\text{g C/ mL}$	Average $\mu\text{g N/ mL}$
pFe 17	7.00	4.34* ($p < 0.001$)
pFe 19	13.30* ($p < 0.001$)	5.73* ($p < 0.001$)
pFe 21	8.90	2.72* ($p = 0.003$)

3.4 Effects of DFB on LE3

To demonstrate the effects of the fungal siderophore DFB on the growth of *M. aeruginosa*, LE3 under ambient conditions, ultrafiltered lake water was amended with increasing concentrations of DFB. Total chlorophyll was monitored over time (figure 7). The chlorophyll *a* of the control (DFB = 0) is significantly higher ($p < 0.001$) than the treatments with DFB added but here appears to be no significant difference between treatments with DFB addition ($p = 0.08$) (figure 7). This work indicates that a reduction in bioavailable iron due to DFB 10 nM treatment (the smallest concentration used) decreased *Microcystis* growth. Statistical analysis by linear regression indicated that, with proper replication, a linear relationship between increasing chlorophyll *a* concentration and increasing chelator concentration would likely be seen but the statistical model of this data was not conclusive (figure 7).

3.5 Lake Erie iron uptake by size class

When iron uptake was compared in different size fractions of the natural community, we observed that 60-70% of total iron uptake occurs in the 0.2-2.0 μm size fraction (pico plankton), 20% in the 2.0-20 μm size fraction and the $>20 \mu\text{m}$ size fraction is responsible for less than 10% of the total iron uptake (Figure 8). The effect of EDTA and DFB additions on Fe uptake was also determined. Iron uptake rates for the DFB treatments are significantly lower than the control at the 0.02 - 2 μm size fraction ($p = 0.0047$), the 2-20 μm size fraction ($p = 0.003$) and the $> 20 \mu\text{m}$ size fraction ($p = 0.028$). DFB, at 50 nM, is significantly different from the treatments of 100 nM ($p = 0.01$), 200 nM ($p = 0.001$) and

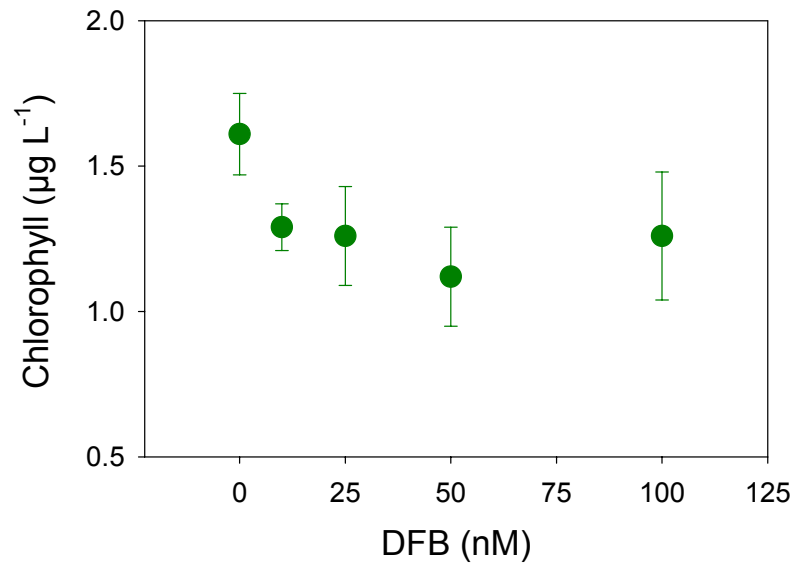


Figure 7: The response of *M. aeruginosa*, LE3 to addition of DFB
Measured by chlorophyll *a* extraction. n=2

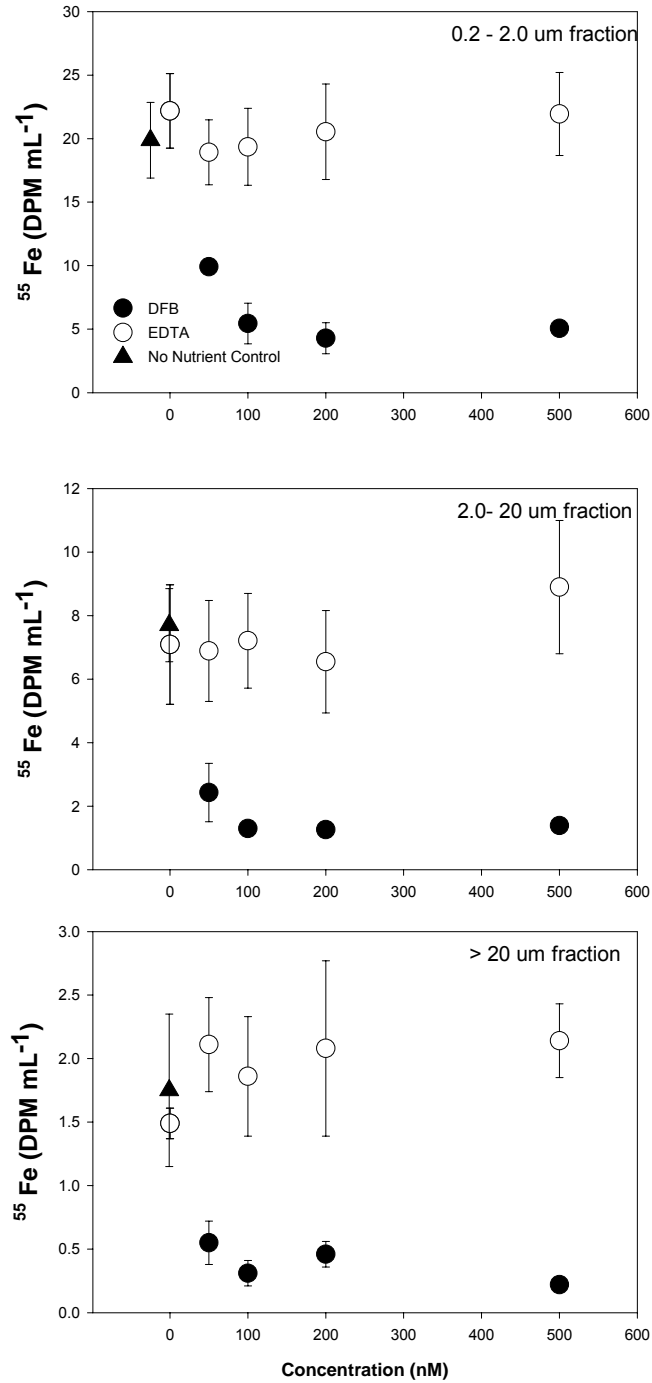


Figure 8: Size fractionated iron uptake by the natural community of Lake Erie
 Experiments were carried out over 48 hours, n=3.500nM (p= 0.001).

500 nM ($p < 0.02$). There is not significant difference ($p > 0.05$) between 100 nM, 200 nM and 500 nM additions for this size class. In the 2.0-20 μm size class, there is no significant difference between concentrations of DFB added or between concentrations of EDTA added. However in all size classes, there is a significant drop in iron uptake by culture exposed to DFB at every concentration (p-values in table 6). For all three size classes, there was no significant difference between treatments at varying concentrations of EDTA. For the 2-20 μm and the 20 μm size classes, there was no significant difference between treatments at varying concentrations of DFB.

3.6 Trace metals by X-ray fluorescence imagery

X-ray fluorescence data, normalized to estimates of cellular C (figure 9), reveals that, as ambient iron decreases from pFe 17 to pFe 19, there is an increase in Mn and Zn but not in Cu. At pFe 21, Mn drops slightly lower, but is still higher than that observed at pFe 17. The pFe 21 values for Zn were lost due to processing complications. This data illustrates that decreasing iron levels do have an effect on cellular levels of Mn and Zn. Figure 10 is an X-ray image of cellular trace metals. The image shows the light pattern for each metal inside a cell, except for an iron particle that was outside the cell. This particle probably affected the calculated total iron value, but relative metal amounts were able to be determined.

Table 6: DFB and EDTA direct concentration statistical comparison
 p-values to validate significant differences between iron uptake in the presence of EDTA and DFB at specific concentrations. The sequence of t-tests performed for triplicate cultures at each EDTA concentration is represented here by the highest p-value in that sequence.

[DFB]	0.2 – 2 μm	2-20 μm	>20 μm
50 nM	p = 0.0083	p = 0.018	p = 0.019
100nM	p = 0.003	p = 0.004	p = 0.01
200nM	p = 0.002	p = 0.004	p = 0.015
500nM	p = 0.002	p = 0.005	p = 0.009

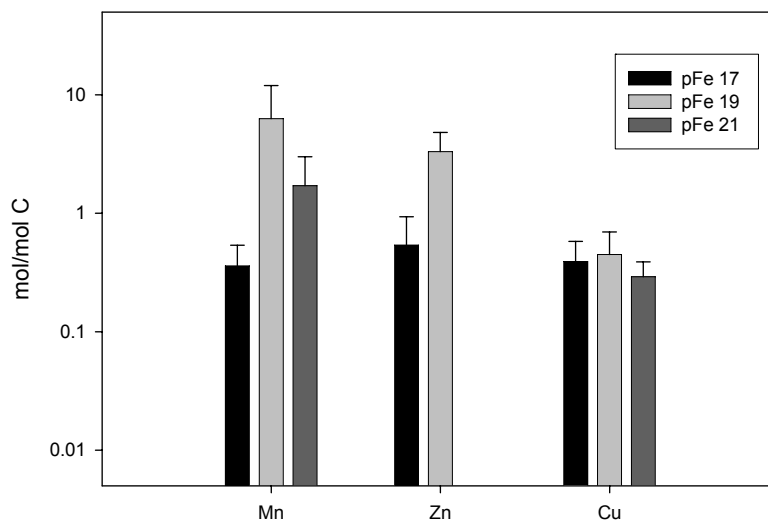
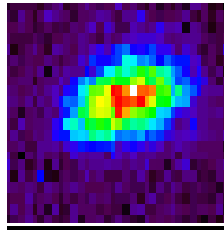
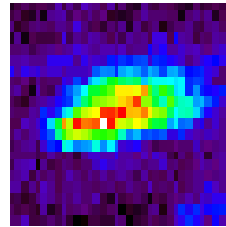


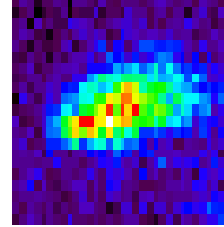
Figure 9: Intracellular trace metal concentration of *M. aeruginosa*
 Cells cultured at varying iron concentrations. (n=3)



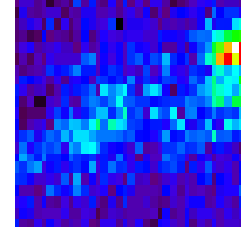
K



Zn



Ca



Fe

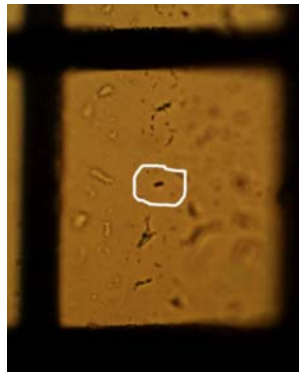


Figure 10: XRF Imagery of pFe 19 *M.aeruginosa* cell

3.7 Toxin production

Production of microcystin was measured using the ELISA detection kit (EnviroLogix). Due to calibration errors, these tests did not render a suitable number of acceptable values from which to draw an accurate conclusion. Only one data point was within calibration range for pFe 17 and 18 while two values were valid for pFe 18 and 21. There was no usable data for cultures maintained at pFe 20. Figure 11 shows the placement of these six data points along the standard curve for the ELISA test for microcystin. A basic trend of the effect of iron concentration on toxin production is indicated. Toxin production appears to be highest for cultures maintained at pFe 19. There is little difference in toxin production seen in cultures at pFe 17, 18 and 21. No statistical analysis was performed on this data to determine if there are any significant differences.

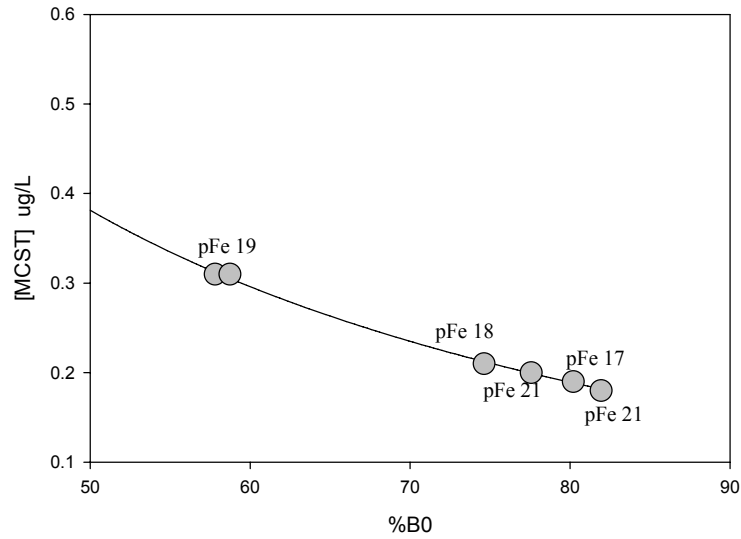


Figure 11: Results for ELISA test for microcystin

Microcystin concentration ($\mu\text{g}/\text{L}$) along a standard calibration curve at four iron concentrations. %BO = (OD of sample or calibrator/ OD of negative control) x 100.

4 Discussion

In this thesis I have focused on confirming that *M. aeruginosa* can proliferate at reduced iron concentrations. I have reviewed the effects of total iron availability and sequestration of iron by organic chelators on the growth of *M. aeruginosa* in laboratory cultures and on the growth of the natural planktonic community in an area where *M. aeruginosa* blooms have been commonly documented. I have also tried to resolve the relationship between iron availability and microcystin production by *M. aeruginosa*.

4.1 Growth rate of *M. aeruginosa* at decreasing iron concentrations

Experiments to determine the growth rate of *M. aeruginosa* at varying iron concentrations provide evidence that *M. aeruginosa* utilizes an inducible high affinity transport system during times of iron limitation. If *M. aeruginosa* did not activate a high affinity transport system, one would expect the growth rate to be directly proportional to the iron concentration (Kerry et al. 1988, Wilhelm and Trick 1994, Wilhelm 1995). However, I did not observe such a trend throughout the laboratory set of experiments. Growth studies clearly show that growth rate decreases with initial iron limitation followed by a recovery in growth rate as iron decreases further. This trend is well documented for organisms that are known to activate a high-affinity transport system and the concomitant production of siderophores and surface-associated receptor proteins during conditions of iron limitation (Wilhelm 1995, Wilhelm et al. 1998). Studies on a *M. aeruginosa* strain isolated from Lake Kasumigaura agree with the idea of siderophore production (Imai et al 1999). Authors of these studies have demonstrated that *M. aeruginosa* produce compounds consistent with hydroxamate-type siderophores. Unlike

our observations in this study, *M. aeruginosa* isolates from Lake Kasumigaura were not effective in enabling substantial recovery of growth rate. However, the lowest iron concentration utilized in their study was 3.1 order of magnitude higher than pFe 17 and 6.1 order of magnitude higher than pFe 20 where the recovery of growth rate is seen in this studies and confirmed in the literature.

In this study cyanobacterial biomass was estimated by direct fluometric analysis. However, during these studies direct cell counts, *via* epifluorescence microscopy were also made. This portion of the experiment was somewhat erratic and did not yield any valuable results. Biases were due to various complications such as separation and dilution of the thick, aggregated cultures, problems with initial immersion oil, and rapid quenching of autofluorescence. Effectively, recent studies of the effects of iron limitation on the growth of toxin-producing cyanobacteria have shown that molecular techniques, such as qPCR, may be easier and more efficient than direct culture methods (Ouellette and Wilhelm 2003).

4.2 Growth rate of *M. aeruginosa* as affected by addition of organic chelators

The results of this study provide some additional evidence that *M. aeruginosa* uses a high affinity transport system during periods of iron limitation. Within these experiments, it appears that these chelators help to maintain a fairly steady level of bioavailable iron. RA is the only dihydroxamate, bacterial siderophore tested and the only chelator that binds in a 3:2 ratio and it resulted in the highest growth rates produced in this study. The majority of treatments in this study resulted in growth rates higher than those seen at pFe 17 but not dissimilar compared with the pFe 18 treatment.

Iron sequestration was only beginning to be observed at 500 nM and 750 nM with RA and DFB addition, and those treatments were not dissimilar from pFe 21 cultures. However, all other growth rates in this study are comparable to, or higher than the growth rates seen for iron-replete cultures because the chelators maintained a level of ambient available iron similar to pFe 17 or pFe 18. In future studies of this kind, chelators should be observed at steady increments up to and above 500 nM to tease apart the effects these chelators have on iron availability for this organism. Growth rate is not a good indication of the physiologic state of cyanobacteria in regard to iron stress. Iron uptake is a more reliable parameter by which to measure physiological response to iron by cyanobacteria. (Wilhelm 1995)

4.3 Iron uptake by laboratory cultures

Iron uptake rates, by cultures of *M. aeruginosa* in pure culture follow the same U-shaped trend observed for growth rate over the experimental range of pFe 17-pFe 21. The minimal uptake rate was observed at pFe 19, while uptake rates in Fe-replete (pFe 18) and Fe-depleted (pFe 20 and 21) treatments were comparable. This provides further evidence to support the idea that *M. aeruginosa* does activate some mechanism to increase its ability to take up iron during iron limitation. Effectively, no recovery in iron uptake rate for the most iron-depleted treatments would be expected if *M. aeruginosa* were not activating an active transport system.

The carbon and nitrogen content of the cells in the pFe 19 treatment, is significantly higher compared to the other treatments ($p < 0.001$). This indicates that, at pFe 19, when growth rate and iron uptake are lowest, cells are decreasingly biologically

active and are not utilizing cellular stores of nutrients such as carbon and nitrogen. (Wilhelm 1995) Another possibility is that cells may be able to alter metabolic pathways which effect carbon and nitrogen content. Many studies have shown that iron availability affects the cellular carbon and nitrogen content in other photosynthetic organisms (Rueter and Ades 1987, Milligan and Harrison 2000)

4.4 Iron uptake in the natural community of Lake Erie by size class

One outstanding question in metal's chemistry is the effect of DFB on iron uptake by natural communities. Several groups have argued that DFB renders iron unavailable to the planktonic community (Wells 1999, Hutchins et al. 1999, Mioni et al. 2003, Eldridge et al. 2004) while others have suggested plankton can access iron in this form (Maldonado and Price 1999). As such, evaluation of this technique to manipulate iron availability in Lake Erie is of significant importance for future research. These results indicate that DFB, at increasing concentrations, inhibits iron uptake by the natural community. EDTA has not effect on the iron uptake by the natural community. This study was utilized in Mioni 2003 to validate the use of DFB to sequester iron in a natural community.

The results demonstrate that there is clearly a trend whereby DFB addition acts to effectively draw down the available iron. In comparison, our control chelate (EDTA) allows iron to remain bioavailable, which confirms what was observed by Lukac and Aegerter (1993). The results confirm that DFB can be used to reduce iron availability to a natural freshwater community. While there is still some background uptake of the

radiotracer, it is at a reduced rate and is most likely not at sufficient rates to satisfy community growth (although more research is needed to confirm this)

Studies published in 2003 indicate the station at which this experiment was carried out, is phosphate limited during summer months (Wilhelm et al. 2003). This finding makes the control value for these uptake experiments extremely important. The control value, with no nutrients added, gives an indication that our results were not a response to the nutrients added. The lack of significant statistical differences between EDTA and DFB treatments, and between concentrations of chelators added, may be due to the high variations in standard error reported for EDTA treatments.

4.5 Effects of DFB on LE3

Chlorophyll *a* extraction techniques were used to further measure the effect of DFB on *M. aeruginosa* LE3. This experiment, coupled with studies of iron uptake by the natural community of Lake Erie in the presence of organic chelators, shows that DFB, at as little as 1nM, affects the iron uptake and productivity of *M. aeruginosa*. Growth rate studies in the laboratory indicated that DFB does not directly lower the growth rate of *M. aeruginosa* in batch culture, grown in iron-replete medium, until it is added in concentrations >100 nM. However as most aquatic systems do not maintain an ambient iron level comparable to pFe 18, it is expected that the effect of DFB addition should be more pronounced in a natural community than in an iron-replete laboratory culture.

4.6 Toxin Production

As of the time of these studies (pre-2001) most studies focused on determining a specific condition responsible for toxin production (Oh, 1999). One study suggested that microcystin production is not controlled through direct metabolic pathways, but instead by the rate of cell division (Orr, 1998). At least 30 papers were published in 2003 to attempt to validate or repudiate this hypothesis and it seems that this is a new focus of toxin research. The point of contention among researchers now is whether it is specific environmental conditions or general cell division rate that is the direct cause of toxin production (Lyck, 2003, Orr, 1999). Our attempt to determine the role of iron in this process is but a small piece of the puzzle. Each environmental condition or set of conditions and their effect on toxin production and now, on general cell division rate, brings us closer to predicting conditions that may be conducive to harmful toxic blooms of cyanobacteria in freshwater.

Due to extreme calibration and dilution problems, the data presented by this experiment can only provide an indication of how iron may affect toxin production by *M. aeruginosa*. It may provide a general starting point and ways to avoid the same errors in future studies of this kind. We can only suggest that at pFe 19, when iron uptake and growth rate are lowest, toxin production appears to increase. This indicates that there may be a relationship between reduced ambient iron and increased toxin production in *M. aeruginosa*. Since this data is not normalized to cell number, it is difficult to say if this is a direct result of decreasing iron concentrations or an artifact of reduced growth rate brought about by iron limitation. A focus for current toxin research is on molecular

techniques, such a quantitative qPCR, to measure the expression of the genes involved in the production of cyanotoxins (Ouellette and Wilhelm 2003).

4.7 Cellular metal content by X-Ray fluorescence

The cellular content of Mn, Zn and Cu as shown in these studies indicates that when cells activate a high-affinity transport system during iron limitation it may stimulate the uptake of other metals. Using this technique, X-Ray fluorescence, we looked at cellular trace metal levels. While only preliminary, the results do suggest that growth, under conditions of iron limitation, may lead to an increase in the uptake of non-specific metals by *Microcystis* spp.

In previous studies, Wilhelm and Trick (1994) postulated that the part of the iron transport system that implied specificity to metals was the presence of a catecholate-type siderophore working in conjunction with a hydroxamate-type siderophore. This two-step chelator system would benefit from both the specificity of the catecholate for iron while exploiting the high assimilation rate (with lower specificity) of the hydroxamate transport system. In the current study the transport system appears to facilitate an increase in other extra-cellular metals. There have been previous studies that indicate that *M. aeruginosa* produces a hydroxamate- type siderophore (Imai et al. 1999) and this data could support that claim.

4.8 Conclusion and future studies

It is determined that the cyanobacterium, *M. aeruginosa*, exhibits growth rate and iron uptake that support the hypothesis that a high affinity transport system is induces to

help alleviate iron-limitation, whether that limitation is brought about by direct manipulation of bioavailable iron or by the addition of organic chelators. Since many organisms that utilize a high affinity transport system produce siderophores as part of that system. It has been suggested that *M. aeruginosa* also produces a siderophore although no isolation of such siderophore has been made (Imai et al, 1999). It remains unknown if microcystin production is induced by iron limitation and what the biological role of microcystin is. Studies to determine the factors affecting growth and proliferation of *M. aeruginosa* and production of microcystin are essential to water quality and public health worldwide. The threat of blooms of increasing magnitude and toxicity in Lake Erie makes studies like this one important. Satellite pictures have confirmed massive cyanobacterial blooms in the western basin for several years (LandSat5 courtesy of OhioView and Dr. R.M.L. McKay). Field studies have indicating that the dominant species in those blooms may *M. aeruginosa* (Brittan et al. 2000). Identification of the conditions that induce toxin production is extremely important to reduce the public health impact of toxic blooms. Studies have indicated that toxin production increases as iron availability decreases and that iron effects the concentration of toxin produced (Utkilen and Gjolme 1995, Lyck et al. 1996). One of these studies (Utkilen and Gjolme 1995) have also shown that limitation by phosphorous does not increase toxicity of *M. aeruginosa*. The growth rate in the presence of iron limitation, as shown in this body of work, indicate a mechanism that allows for recovery of growth rate at decreasing ambient iron. This mechanism may prove to have an effect on the production of microcystin by this organism.

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