Iron-Regulated Cyanobacterial Predominance and Siderophore Production in Oligotrophic Freshwater Lakes

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Biology

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IRON-REGULATED CYANOBACTERIAL PREDOMINANCE AND SIDEROPHORE PRODUCTION IN OLIGOTROPHIC FRESHWATER LAKES

(Thesis format: Integrated Article)

by

Ryan James Sorichetti

Graduate Program in Biology with Environment and Sustainability

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

The frequency and intensity of cyanobacterial blooms (cyanoblooms) is increasing globally. Contrary to existing phosphorus (P) and nitrogen (N) paradigms describing cyanobloom proliferation in eutrophic (nutrient-rich) freshwater lakes, many of the recent cyanobloom reports pertain to oligotrophic (nutrient-poor) freshwater lakes with no prior history of cyanobloom occurrence. There exists a critical research need to re-visit existing conceptual models, identify regulating factors currently unaccounted for and improve our ability to effectively detect and measure cyanobacterial toxins (cyanotoxins) in lakes. Iron (Fe) is required in nearly all pathways of cyanobacterial macronutrient use, though its direct role in regulating cyanobacterial biomass is not well understood. The hypotheses tested were: (1) cyanobacteria will predominate in lakes when concentrations of bioavailable Fe are low; and (2) cyanobacteria overcome this Fe limitation using the siderophore-based Fe acquisition strategy to scavenge Fe providing a competitive advantage over other phytoplankton. It was also hypothesized that (3) the rainbow trout gill cell-W1 cytotoxicity assay (RCA) will be effective in assessing the cytotoxicity of natural lake water samples. Among 25 oligotrophic lakes studied in the Algoma Highlands of central Ontario Canada, the proportion of cyanobacteria was highest at low Fe availability (< 1.0 × 10^{-19} mol L^{-1}). Within this range of low Fe, hydroxamate siderophore concentration was positively correlated to cyanobacterial density (r^2 = 0.77, p < 0.001). Dissolved organic matter (DOM) had an overriding control on the relationship between siderophore concentration and cyanobacterial density, with densities highest where DOM concentrations were low (< 5 mg L^{-1}) and with a degree of humification (HIX) < 5. These findings suggest labile DOM may be a source of Fe and/or siderophores, but refractory DOM may be a sink. The RCA successfully provided a measure of lake water toxicity that could not be reproduced using isolated cyanotoxin standards in the laboratory. These findings provide support for the critical role of Fe in regulating cyanobacterial biomass in lakes and represent the first record of siderophores in lakes in the Algoma Highlands of central Ontario. The RCA is sensitive to cyanotoxins in lakes and may be an important part of routine water quality biomonitoring programs.
Keywords
Cyanobacteria, phytoplankton, oligotrophic, freshwater lake, phosphorus, nitrogen, iron, siderophore, hydroxamate, catecholate, cyanotoxin, rainbow trout gill cell-W1 cytotoxicity assay.
Co-Authorship Statement

Chapter 2 was published in *Freshwater Biology* (reprint permission in Appendix C). Ryan J. Sorichetti (RJS) was the first author and the co-authors were Irena F. Creed (IFC) and Charles G. Trick (CGT). RJS conducted all fieldwork and laboratory sample analyses. Each of the authors contributed to the study design, synthesis of ideas and preparation of the final manuscript.

Chapter 3 was submitted as a manuscript for publication to the journal *Freshwater Biology* on June 1st, 2013. RJS was the first author and the co-authors were IFC and CGT. RJS conducted all fieldwork and laboratory sample analyses. Each of the authors contributed to the study design, synthesis of ideas and preparation of the submitted manuscript.

Chapter 4 was published in *Harmful Algae* (reprint permission in Appendix C). RJS was the first author and the co-authors were Jace T. McLaughlin (JTM), IFC and CGT. RJS conducted all fieldwork, conducted statistical analysis and contributed to the writing of the manuscript. JTM conducted all laboratory sample analyses. RJS, IFC and CGT contributed to the synthesis of ideas and preparation of the final manuscript.

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I dedicate this thesis to my wonderful loving family,

my parents, Jim and Donna

my brothers, Justin, Mark and Kevin

and my wife Dusa

This is for you.
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List of Abbreviations

°C: Degree Celsius
µm: Micrometer
µM: Micromolar
µg: Microgram
a.u.: Arbitrary units
ANOVA: Analysis of variance
Cells L⁻¹: Cells per liter
Chl-α: Chlorophyll α
CPCC: Canadian Phycological Culture Collection
Cₜ: Catecholate siderophore
Cyanobloom: Cyanobacterial bloom
cyanoHAB: Cyanobacterial harmful algal bloom
Cyanotoxin: Cyanobacterial toxin
DIN: Dissolved inorganic nitrogen
DMSO: Dimethylsulfoxide
DOC: Dissolved organic carbon
DOM: Dissolved organic matter
DON: Dissolved organic nitrogen
EDTA: Ethylenediamine tetra-acetic acid
EEM: Excitation – Emission Matrix
ELA: Erythrocyte lysis assay
Fe: Iron
Fe²⁺: Ferrous iron
Fe³⁺: Ferric iron
FBS: Fetal bovine serum
g: Relative centrifugal force
Ga: Billion years
HAB: Harmful algal bloom
HIX: Humification index
H₂: Hydroxamate siderophore
Hz: Hertz
L: Liter
LPH: Liter per hour
m: Meter
min: Minute
mL: Milliliter
mol L$^{-1}$: Moles per liter
mM: Millimolar
MWP: Micro well plate
N: Nitrogen (nutrient)
N$_2$: Molecular nitrogen (atmospheric dinitrogen)
NH$_4^+$: Ammonium
NiF: Nitrogenase
nm: Nanometer
nM: Nanomolar
NO$_3^-$: Nitrate
O$_2$: Molecular oxygen (atmospheric)
P: Phosphorus (nutrient)
PBS: Phosphate buffered saline
PARAFAC: Parallel factor
RFU: Raw fluorescence units
RTgill-W1: Rainbow trout gill-W1
S.E.: Standard Error
SHM: Stockholm Humic Model
sp./spp.: Species (singular/plural)
S/R: Signal ratio
SRP: Soluble reactive phosphorus
TDFe: Total dissolved iron
TN: Total nitrogen (nutrient)
TP: Total phosphorus (nutrient)
UV: Ultraviolet
v/v: Volume to volume ratio
W: Watts
X: Microscopy optical magnification factor
$Z_{\text{max}}$: Maximum depth
$Z_{\text{tc}}$: Thermocline boundary depth
Chapter 1

1 Introduction

1.1 Problem statement

The frequency and intensity of potentially harmful cyanobacterial blooms (cyanoblooms) is increasing within the Laurentian Great Lakes – St. Lawrence River Basin (Winter et al., 2011) and globally (Mur et al., 1999; Svrcek & Smith, 2004; Merel et al., 2010). Cyanoblooms are now reported in oligotrophic (nutrient-poor) lakes that have not had such reports in previous history (Carey et al., 2008, 2012a; Winter et al., 2011) and are occurring more intensively in eutrophic (nutrient-rich) lakes that have a history of cyanobloom presence (Paul, 2008). Existing conceptual models that attempt to describe the factors regulating cyanobacterial biomass are insufficient to explain the presence of cyanoblooms in lakes where the surface water chemistry should not have the capacity to support the cyanoblooms observed.

The Interagency, International Symposium on Cyanobacterial Harmful Algal Blooms (ISOC-HAB) working group has described the state of the science and identified critical research needs to address the emerging trends in cyanobloom occurrence in lakes (Hudnell & Dortch, 2008), including: (1) comprehensive data analysis on the physical, chemical and biological conditions controlling the variability in cyanobloom initiation and dynamics observed; and (2) improved and new analytical methods for the screening and detection of known and novel toxins. There exists urgency and interest, by both the scientific community and general public, to re-visit and revise existing conceptual models to generate a more comprehensive understanding of the physiological processes that contribute to cyanobacterial proliferation in oligotrophic lakes.

1.2 Cyanobacterial evolution through time and space

Morphological and isotopic evidence suggests that cyanobacteria are among the oldest organisms on Earth dating back to the early Archean time period, 2.5 – 3.5 billion years
Cyanobacteria existed in mats along the coastal floor in localized photic regions and have been identified as responsible for oxygenation of Earth’s atmosphere over evolutionary time through the production of oxygen oases in the Archean sea leading to the great oxidation event (Kasting, 1993). The Archean environment had a profound influence on the evolution of cyanobacterial anatomy and physiology that separates them from photosynthetic eukaryotic phytoplankton.

Cyanobacteria are photosynthetic chlorophyll-\(a\) (chl-\(a\)) containing prokaryotic phytoplankton. Compared to present day Earth, the Archean time period experienced higher doses of ultraviolet (UV) radiation (Cockell, 1998). Cyanobacteria evolved accessory pigments (phycobiliproteins), with a tolerance to high UV exposure, to capture light energy from a wide spectrum and pass this energy to chl-\(a\) and avoid photo-damage to the primary chl-\(a\) photosynthetic apparatus (Nisbett, 1985). These phycobiliproteins evolved into phycocyanin and phycoerythrin, which are used with chl-\(a\) in photosynthesis by cyanobacteria in the present day (Grossman et al., 1993).

Some genera of cyanobacteria are considered diazotrophs, having the ability to convert atmospheric nitrogen (\(N_2\)) into usable forms such as ammonium (\(\text{NH}_4^+\)) (Gallon, 1992). The Archean atmosphere and surface waters of the Archean sea were largely or entirely anoxic (Holland, 2006). Nitrogen fixation, through activity of the enzyme nitrogenase (NiF), is a strictly anaerobic process and was an important source of nitrogen (N) in the Archean sea (Berman-Frank et al., 2003). Cyanobacteria are the only diazotrophs that produce oxygen as a by-product of photosynthesis, restricting the functioning of NiF by introducing molecular oxygen into the surrounding environment. Cyanobacteria evolved differentiated cells known as heterocysts (Figure 1.1) where NiF is found, providing a micro-anaerobic environment to ensure NiF functioning and \(N_2\)-fixation capability in both aerobic and anaerobic environments (Berman-Frank et al., 2003).

Finally, sediments of the Archean sea were iron (Fe)-rich and due to the reducing conditions, there was an abundance of Fe in surface waters primarily in the ferrous (Fe\(^{2+}\)) form (Holland, 2006). Fe is required in NiF activity for \(N_2\)-fixation and so cyanobacteria were able to thrive in this Fe-rich environment. The appearance of small-scale banded Fe
Figure 1.1 Heterocyst cells, the site of N₂-fixation, on filaments of *Anabaena* spp. observed with the Fluid Imaging FlowCAM® in oligotrophic lake samples.
formations in sea sediments dating back to the end of the Archean time period suggests that as the great oxidation event proceeded, ferrous Fe in Archean sea surface waters was oxidized to the ferric (Fe$^{3+}$) form (Holland, 2006). The Fe-requiring biological processes that cyanobacteria depend on in the present day have persisted over geological and evolutionary time scales; the physical, chemical and biological conditions that influence Fe speciation and bioavailability to cyanobacteria have not. Less is known about evolutionary, adaptive or competitive strategies that allow cyanobacteria to overcome changes in supply of a nutrient it has depended on since its earliest existence.

### 1.3 The ecology of cyanobacteria in freshwater lakes

Cyanobacteria are ubiquitous in temperate, boreal and tropical freshwater lakes and are present in three main organizations: unicellular, colonial (multi-cellular) and filamentous (Mur et al., 1999). Cyanobacterial presence in lakes ranges from low biomass picocyanobacteria, to “transitionary” diverse communities containing large filamentous and colonial cyanobacteria, to high biomass “mono-specific” communities (Figure 1.2) (Paerl & Huisman, 2009). Currently, there is no consistent agreement on the definition of a cyanobacterial bloom (Carvalho et al., 20130). Usage of the term “bloom” has been associated with visible surface scums of phytoplankton, particularly cyanobacteria, for over 100 years (McGowan et al., 1999). The European Water Framework Directive proposes that any definition of a bloom should incorporate some metric of bloom intensity (e.g., magnitude or abundance) and an indication of how frequently they occur over a specified time period (European Commission, 2000). A recent review on the physical and chemical lake factors that regulate cyanobacterial biomass indicates that the term “bloom” can be defined as dominance by any one group of phytoplankton, constituting > 50% of total phytoplankton biomass during peak growing season regardless of whether surface accumulation occurs (Molot et al., 2013). When the bloom is comprised of cyanobacteria, it is then referred to as a cyanobloom.

Cyanobacteria are strong competitors in the phytoplankton community assemblage providing the ability to become bloom-forming dominant phytoplankton. The relatively large size of cyanobacterial filaments and colonies provide predation defense against
Figure 1.2 Representation of cyanobacterial presence in freshwater lakes from [A] low biomass picocyanobacteria; [B] transitionary unicellular, colonial and filamentous forms; and [C] high biomass mono-specific communities.
zooplankton grazers (Whitton & Potts, 2000). High surface area to volume ratio of filaments and colonies enables higher transport efficiency of nutrients at lower concentrations of required metabolites (Whitton & Potts, 2000). *Anabaena, Aphanizomenon, Cylindrospermopsis, Nodularia* and *Lyngbya* are capable of N$_2$-fixation, providing the ability to fix atmospheric N$_2$ in N-limited conditions, an advantage over other eukaryotic phytoplankton (Paerl & Huisman, 2009). At low light intensities, cyanobacteria have higher growth rates relative to other phytoplankton and thus have the capacity to grow faster and increase density during blooms and turbid conditions (Van Liere *et al.*, 1979). *Anabaena, Aphanizomenon* and *Microcystis* produce gas vesicles providing the ability to control buoyancy in the vertical water column giving better access to light and at higher levels of biomass, often shade light access to other phytoplankton (Huisman *et al.*, 2005).

### 1.4 Human and ecosystem health implications of cyanobacteria and their toxins

Several genera of cyanobacteria have the capacity to produce toxic secondary metabolites known as cyanotoxins, classified as neurotoxins and hepatotoxins posing serious human, animal and ecosystem health consequences (Mur *et al.*, 1999). When a cyanobloom produces cyanotoxins, it is then referred to as a cyanobacterial harmful algal bloom (cyanoHAB). Table 1.1 presents cyanotoxins common to the Laurentian Great Lakes – St. Lawrence River Basin, the genera that produce the associated cyanotoxins, the human or animal organ affected by toxicity and the number of cyanotoxin variants identified.

The specific factors that contribute to or trigger cyanotoxin production, the mechanism of toxicity and the cumulative effects of cyanotoxins on human and ecosystem health are poorly understood (Watson, 2004). What is generally agreed upon within the research community, and forms the basis of research pertaining to this knowledge gap, is that there exists an inextricable link between cyanobacterial physiology and cyanotoxin production (Watson, 2003; Kaplan *et al.*, 2012). For example, nutrient limitation has been identified as a physiological control that triggers cyanotoxin production (Kaplan *et al.*, 2012), particularly Fe limitation (Alexova *et al.*, 2011; Kaplan *et al.*, 2012). Utkilen & Gjølme (1995) showed that microcystin-LR can serve as an intracellular Fe chelator with a high
Table 1.1 Cyanotoxins common to the Laurentian Great Lakes – St. Lawrence River Basin (from Sivonen & Börner, 2008; Watson et al., 2008).

<table>
<thead>
<tr>
<th>Cyanotoxin</th>
<th>Genera Produced</th>
<th>Affected Organ</th>
<th>Variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microcystins</td>
<td><em>Microcystis, Planktothrix, Anabaena, Oscillatoria, Nostoc</em> and <em>Anabaenopsis</em></td>
<td>Liver</td>
<td>&gt; 85</td>
</tr>
<tr>
<td>Nodularins</td>
<td><em>Nodularia</em></td>
<td>Liver</td>
<td>&gt; 6</td>
</tr>
<tr>
<td>Cylindrospermopsins</td>
<td><em>Cylindrospermopsis, Umezakia, Aphanizomenon, Raphidiopsis</em> and <em>Anabaena</em></td>
<td>Liver, Kidneys, Spleen, Thymus and Heart</td>
<td>4</td>
</tr>
<tr>
<td>Anatoxin-a</td>
<td><em>Anabaena, Aphanizomenon, Cylindrospermum, Oscillatoria, Planktothrix</em> and <em>Phormidium</em></td>
<td>Brain</td>
<td>4</td>
</tr>
<tr>
<td>Anatoxin-a(S)</td>
<td><em>Anabaena</em></td>
<td>Brain</td>
<td>1</td>
</tr>
<tr>
<td>Saxitoxins</td>
<td><em>Anabaena, Aphanizomenon, Cylindrospermopsis, Lyngbya</em> and <em>Planktothrix</em></td>
<td>Brain</td>
<td>26</td>
</tr>
<tr>
<td>Aplysiatoxins</td>
<td><em>Lyngbya, Oscillatoria</em> and <em>Schizothrix</em></td>
<td>Skin</td>
<td>80</td>
</tr>
<tr>
<td>Lyngbyatoxins</td>
<td><em>Lyngbya</em></td>
<td>Skin</td>
<td>&gt; 1</td>
</tr>
<tr>
<td>β-methyl amino alanine</td>
<td>Potential in all genera</td>
<td>Brain</td>
<td>1</td>
</tr>
</tbody>
</table>
affinity for Fe as a means to store it within the cell during Fe-limited conditions. Cyanobacterial growth stage has also been identified as a physiological control on cyanotoxin production. Alexova et al. (2011) and Kaplan et al (2012) found that cyanotoxin production rate is highest when cells are in an exponential stage of growth and attribute this to the fact that all cellular processes, including photosynthesis, are occurring at a relatively high rate during this growth stage. Quantifying the effects of complex mixtures of cyanotoxins, as often found in lakes experiencing cyanoHABs, has proven difficult, although cell-based bioassays offer intriguing opportunities to address the limitations of traditional cyanotoxin analytical methods (Fent, 2001; Graham et al., 2008; Lee et al., 2009). For example, the rainbow trout gill cell-W1 cytotoxicity assay (RCA) has been used to assess the cytotoxicity of phytoplankton secondary metabolites on cellular metabolism in marine environments (Ling & Trick, 2010; Dorantes-Aranda et al., 2011) with promise for adaptive applicability in freshwater environments.

1.5 Macronutrient controls on cyanobacterial biomass

Early studies indicate that total phosphorus (TP) concentration is the best predictor of cyanobacterial biomass in freshwater lakes (Trimbee & Prepas, 1987). Under this scheme, cyanobacterial biomass is expected to increase with increased TP supply to lakes. Total nitrogen (TN) concentration has also been identified as an important nutrient regulating cyanobacterial biomass in lakes (Downing et al., 2001) with emphasis on the availability of organic or inorganic N forms (Berman, 2001). Other studies indicate the importance of the nitrogen (N) to phosphorus (P) (N:P) ratio with low ratios (< 16:1) favouring cyanobacterial biomass due to the capacity for some species to fix N\(_2\) (Smith, 1983).

At present day, disagreement still exists in the identification of important macronutrient concentrations and ratios controlling cyanobacterial biomass in lakes (Schindler, 2012). This disparity suggests that there may be a critically important underlying factor that is not traditionally considered when investigating the biogeochemical nutrient controls on cyanobacterial biomass. The study of inland freshwater lakes, limnology, is over 100 years old and throughout this time, the role of trace metals in regulating cyanobacterial
biomass has rarely been discussed (Rueter & Petersen, 1987). However, those few studies that have considered the importance of trace metals have typically focused on Fe (Morton & Lee, 1974; Murphy et al., 1976).

1.6 The requirement of iron in cyanobacterial physiology

In aerobic lake surface waters, Fe commonly exists in the oxidized ferric form, which must be reduced to the ferrous form at the cyanobacterial cell surface prior to assimilation via the enzyme ferric reductase (FeR) (Figure 1.3) (Kranzler et al., 2011). In oligotrophic lakes, Fe often exists as a limiting trace metal with low bioavailability to phytoplankton (Davison, 1993). This may be due to the tendency for Fe to bind to dissolved organic matter (DOM), forming a DOM-Fe complex that is not readily assimilated (Baken et al., 2011).

The low bioavailability of Fe to cyanobacteria in oligotrophic lakes is problematic because Fe is required in nearly all pathways of macronutrient assimilation (Figure 1.3). For N assimilation, ammonium can be directly assimilated into the cyanobacterial cell but nitrate must first be reduced to ammonium at the cell surface prior to assimilation. Nitrate reduction is made possible with the enzyme nitrate reductase (NtR), which requires Fe for functioning (Lin & Stewart, 1998). The assimilation of N₂ is made possible with the enzyme NiF which also requires Fe for functioning (Murphy et al., 1976). The assimilation of phosphate is dependent on redox conditions and in aerobic surface waters; Fe has the potential to bind to phosphate forming the FePO₄ complex that is not readily assimilated by cyanobacteria and so will precipitate out of surface waters in lakes (Moore & Reddy, 1994). It is evident that Fe plays a critical role in cyanobacterial macronutrient use efficiency.

1.7 Organic iron-binding ligands (siderophores)

One possible mechanism in which cyanobacteria may have evolved to overcome Fe limitation in freshwater lakes is through the use of siderophores. Siderophores are low molecular weight organic Fe-binding ligands produced by bacteria and fungi during Fe-limited conditions as a competitive Fe-scavenging strategy (Neilands, 1995).
Figure 1.3 The critical role of Fe in macronutrient assimilation by cyanobacteria. Black circled “Fe” represents enzymatic processes in which Fe is required for nutrient assimilation: NtR, NiF and FeR.
Cyanobacteria are the only group of phytoplankton that possesses an Fe-siderophore uptake system and so have a competitive advantage for Fe scavenging over eukaryotic phytoplankton in Fe-limited conditions (Wilhelm & Trick, 1994). Two main types of functional siderophores exist that cyanobacteria can use: (1) hydroxamate siderophores are water-soluble and have relatively weak Fe-binding capacity; and (2) catecholate siderophores are fat-soluble and have relatively strong Fe-binding capacity (Neilands, 1995). The mechanisms of Fe binding by the two siderophore types differ as described by Neilands (1995) and are presented in Figure 1.4. Hydroxamate siderophores are produced intracellularly and are transported to the external environment via specialized membrane-bound protein channels where they bind soluble ferric Fe. Upon contact of the Fe-siderophore complex at the cell surface, ferric Fe is reduced to ferrous Fe (via FeR) and assimilated. Catecholate siderophores are cell membrane-bound and Fe binding occurs at the cell surface where ferric Fe is reduced to ferrous Fe (via FeR) and assimilated.

The current state of knowledge concerning cyanobacterial siderophore use to overcome Fe limitation has primarily been derived from laboratory studies (Kerry et al., 1988; Wilhelm & Trick, 1994; Wilhelm et al., 1996; Wilhelm et al., 1998). These principles have been investigated almost exclusively in marine environments (Barbeau et al., 2003; Eldridge et al., 2004) with very few field studies conducted in freshwater environments (e.g., Murphy et al., 1976). Investigating the presence and potential utilization of siderophores in oligotrophic freshwater lakes may provide helpful insights as to why cyanoblooms can develop in lakes with an insufficient chemical capacity to support cyanoblooms based on traditional conceptual models.

1.8 Changes in the seasonality and distribution of cyanobacteria

Cyanoblooms are traditionally associated with eutrophic, macronutrient replete, freshwater systems often a result of direct anthropogenic activity including forest harvesting, agricultural fertilization and urban expansion resulting in increased nutrient supply to lakes (Downing et al., 2001). Studies projecting the benefit of global climate change to cyanoblooms in lakes typically focus on eutrophic systems where increased growth rates, dominance, persistence and geographical distribution are expected with
Figure 1.4 Mechanism of Fe binding for hydroxamate and catecholate siderophores. Hydroxamate siderophores are released into the external environment while catecholate siderophores are cell membrane-bound.
rising temperatures, enhanced vertical stratification and changes in seasonal and interannual weather patterns (Wiedner et al., 2007; Johnk et al., 2008; Paepl & Huisman, 2009). Very few studies consider how global climate change will influence cyanobacterial proliferation in oligotrophic, nutrient depleted freshwater systems (e.g., Carey et al., 2012b). However, recent trends in cyanobloom dynamics indicate that our fundamental understanding of important factors contributing to cyanobacterial biomass is incomplete. Blooms are now reported in oligotrophic systems (Carey et al., 2008) and it is becoming clear that cyanoblooms are no longer a strict eutrophication (nutrient enrichment) problem. Reports of cyanoblooms have increased over the past several decades in the Laurentian Great Lakes – St. Lawrence River basin in unmanaged oligotrophic lakes with no immediate point source of nutrients (Winter et al., 2011; Carey et al., 2012a). It remains unclear whether increasing reports correspond to truly new appearances of cyanoblooms in lakes or whether this is due to increased public awareness, expansion of cottage living in more remote locations or recent encouragement by regional ministries to report bloom events. Nonetheless, the underlying importance remains that when regional ministries do respond to reported blooms for sampling purposes, blooms are found in oligotrophic lakes where cyanoblooms would not otherwise be expected based on current conceptual models.

1.9 Thesis questions, hypotheses and objectives

In direct response to the ISOC-HAB identified critical research needs, the primary research questions addressed in this thesis include:

1. What are the biogeochemical nutrient conditions that result in elevated cyanobacterial biomass in oligotrophic freshwater lakes?

2. Do cyanobacteria in oligotrophic freshwater lakes employ a competitive Fe scavenging strategy through the utilization of organic Fe-binding ligands (siderophores)?

3. Can an existing cell-based bioassay be adapted and utilized to evaluate the cytotoxic effects of phytoplankton secondary metabolites using analytical
standards, complex mixtures extracted from laboratory cultures and in naturally occurring oligotrophic freshwater lake samples?

To address these research questions, the following research hypotheses were tested:

1. Cyanobacteria will predominate in the phytoplankton community when concentrations of bioavailable ferric Fe are restricted; specifically, where ferric Fe is not readily accessible to eukaryotic phytoplankton (ferric Fe concentrations < \(1.0 \times 10^{-19} \text{ mol L}^{-1}\)).

2. Cyanobacterial density will be highest in lakes with low total dissolved Fe (TDFe) due to the siderophore Fe scavenging system that gives cyanobacteria a competitive advantage over other phytoplankton. However, the presence of DOM and its composition will influence the bioavailability of Fe to cyanobacteria. Lakes with refractory DOM will have low cyanobacterial density due to strong Fe complexation while lakes with labile DOM will allow cyanobacteria to scavenge Fe from DOM complexes with siderophores and overcome Fe limitation.

3. The application of analytical standards of phytoplankton secondary metabolites to the RCA will result in concentration dependent decreases in the viability of tested cells; the application of complex mixtures of phytoplankton secondary metabolites extracted from cultures of toxic and/or noxious compound producing phytoplankton will result in a concentration dependent decrease in the viability of tested cells; and that the RCA will be effective in assessing cytotoxicity in natural lake water samples collected as part of a routine water quality sampling or biomonitoring strategy.

To test the research hypotheses, the following objectives were completed using 25 oligotrophic freshwater lakes in the Algoma Highlands of central Ontario, Canada during periods of peak phytoplankton biomass:

1. Determine if P or N limits cyanobacterial biomass.
2. Determine the role Fe may play in alleviating P or N limitation on cyanobacterial biomass.
3. Investigate the presence of hydroxamate and catecholate siderophores in lakes.
(4) Determine whether the presence of siderophores alleviates Fe limitation on cyanobacterial biomass.

(5) Investigate role DOM may play in regulating the bioavailability of Fe and siderophores to cyanobacteria.

(6) Investigate the effectiveness and adaptability of the RCA by applying it to analytical standards, complex mixtures (extracted from laboratory culture) of phytoplankton secondary metabolites and natural lake water samples.
1.10 References


Chapter 2

2 Evidence for iron-regulated cyanobacterial predominance in oligotrophic lakes

This chapter was published in *Freshwater Biology* (Sorichetti *et al*., 2013).

2.1 Introduction

The ecosystem services provided by freshwater lakes are threatened by photosynthetic cyanobacteria (Keeler *et al*., 2012), which are widely distributed in temperate and boreal freshwater ecosystems (Fogg *et al*., 1973). Cyanobacteria in lakes range from low biomass picocyanobacteria, to “transitionary” diverse communities containing large filaments and colonies, to high biomass “mono-specific” communities (cyanoblooms) (Paerl & Huisman, 2009). The term “bloom” is often defined as dominance by cyanobacteria, constituting >50% of phytoplankton biomass, regardless of whether surface accumulation is visible (Downing *et al*., 2001). At higher levels of biomass, cyanobacteria have the capacity to produce toxic secondary metabolites known as cyanotoxins, often classified as neurotoxins and hepatotoxins, that may pose serious human, animal and ecosystem health concerns (Mur *et al*., 1999). Under these situations, the term cyanobacterial harmful algal bloom (cyanoHAB) is used.

In oligotrophic lakes, the low nutrient supplies have traditionally led to dominance by picocyanobacteria (Callieri & Stockner, 2000). However, even in oligotrophic lakes the dominance by picocyanobacteria can be usurped by larger, filamentous or colonial cyanobacteria with small changes in light and competition for nutrient flux (Callieri & Stockner, 2000). These transitional cyanobacteria represent the low biomass cyanobacteria that may be stimulated by other environmental factors to form cyanoHABs. Recent trends in cyanobloom dynamics indicate that our understanding of important factors contributing to cyanobacterial growth is insufficient. Cyanoblooms are now being reported in oligotrophic, macronutrient poor freshwater systems including *Gloeotrichia echinulata* blooms in northeastern USA (Carey *et al*., 2008) and *Anabaena spp.*, *Aphanizomenon spp.*, *Microcystis spp.*, *Gloeotrichia spp.* and *Oscillatoria spp.*
blooms in Ontario Canada (Winter et al., 2011). It is becoming clear that cyanoblooms are no longer strictly associated with eutrophication.

Most models dealing with cyanobacteria-eukaryotic phytoplankton competition or dominance are based on macronutrient use efficiency and the consequences of adding more phosphorus (P) and nitrogen (N) into a lake ecosystem. Reports of emerging cyanobloom occurrence have increased over the past several decades and are thought to be due to increased P supply as a result of land use and land cover change (Foley et al., 2005) and/or global N enrichment (Bergström & Jansson, 2006). However, these explanations provide little insight into cyanobloom occurrence in unmanaged oligotrophic lakes with no increase in point or non-point sources of nutrients that would lead to increased nutrient loading (Carey et al., 2008). In fact, stoichiometric studies of controls on cyanobacterial biomass have often failed to account for micronutrients such as iron (Fe), which has been shown to contribute significantly to cyanobacterial growth in laboratory studies (Kerry et al., 1988; Wilhelm, 1995) and in lakes (Vrede & Tranvik, 2006; Downs et al., 2008; Molot et al., 2010). Bioavailable Fe in aerobic oligotrophic surface waters exists in its ferric form (Fe\(^{3+}\)), which is reduced to its ferrous form (Fe\(^{2+}\)) at the cell surface prior to assimilation (Kranzler et al., 2011). Cyanobacteria require Fe for nitrogenase activity in N\(_2\)-fixation (Murphy et al., 1976) and for nitrate reductase activity in N-assimilation (Kranzler et al., 2011).

The purpose of this study was to determine if the availability of Fe can be related to the presence of cyanobacteria in oligotrophic lakes in a region with increased unexplained occurrences of cyanoblooms (Winter et al., 2011). For a set of oligotrophic lakes with similar physical characteristics but different macro- and micronutrient conditions, we set out to determine if P or N limits cyanobacterial biomass and what role ferric Fe may play in alleviating P or N limitation on cyanobacterial biomass. Our hypothesis was that cyanobacteria will predominate in the community when concentrations of ferric Fe are restricted; specifically, where ferric Fe is not readily accessible to eukaryotic phytoplankton (ferric Fe concentrations <1.0 \times 10^{-19} \text{ mol L}^{-1}). A better understanding of P, N and Fe limitation on cyanobacterial biomass will provide insight into the risk of cyanoblooms in lakes with a relatively low macronutrient supply.
2.2 Methods

2.2.1 Study sites

For this study, 25 oligotrophic lakes from the Algoma Highlands in the Laurentian Great Lakes-St. Lawrence River Basin were selected based on minimal direct anthropogenic influences and public concern about the potential of cyanoblooms in the lakes (Fig. 2.1). The lakes are relatively shallow, thermally stratified during the warm summer months and are dimictic with major mixing events occurring during the spring snowmelt and fall storms. These two major periods of hydrologic connectivity between land and lake represent important episodes of terrestrial nutrient input to lakes (Creed & Band, 1998).

2.2.2 Fieldwork

Lakes were monitored throughout the growth season, with major sampling performed during the peak phytoplankton biomass period (September-October) in 2009, 2010 and 2011. This peak biomass period was identified based on a 2009 survey of these lakes from June to November (Fig. 2.2), which also matched well with reports by Winter et al. (2011) that cyanoblooms are occurring later in the growing season (October-November) compared to previous decades in this region of Ontario. At the lake, temperature, pH, dissolved O\textsubscript{2} and conductivity were measured at 1.0 m depth below the lake surface and maximum lake depth (Z\textsubscript{max}) and thermocline boundary depth (Z\textsubscript{tc}) were measured using a YSI 600 QS multi-parameter sonde with a YSI 650 MDS display (YSI Incorporated, Yellow Springs, OH).

Surface water samples integrated to 1.0 m depth were collected in 500 mL pre-rinsed polyethylene bottles near the centre of the lake, outside a phytoplankton bloom if present, and stored in the dark on ice in a cooler until return to the field laboratory. Median Z\textsubscript{max} among the lakes is 5.3 m and median Z\textsubscript{tc} is consistently 1.5 m among years (25\textsuperscript{th} percentile = 0.81 m, 75\textsuperscript{th} percentile = 2.4 m) so integrated water sampling to 1.0 m was chosen to standardize sampling among all lakes at a depth close enough to the thermocline boundary, and not directly through it, to capture phytoplankton populations and nutrients that would diffuse out of the hypolimnion. When visible blooms were present, the highest biomass was in areas where the phytoplankton material had accumulated due to winds. To avoid this
Figure 2.1 Location of the surveyed oligotrophic lakes in the Algoma Highlands of central Ontario, Canada.
Figure 2.2 Oligotrophic lake biomass (chl-α, +/- S.E.) time series collected in 2009 to identify peak seasonal biomass. September and October biomass was significantly higher than early season (June) based on Friedman Repeated Measures ANOVA on Ranks with Tukey Test (critical α = 0.05). Lettering denotes significant differences in chl-α among sample periods. A decline in biomass was observed in November but was excluded from statistical analyses since not all lakes were accessible for sampling due to ice cover. Two lakes were excluded from the time series since early season sampling was not conducted, thus N = 23.
bias, samples were made outside of, but adjacent to, the area of the visible phytoplankton bloom. Within the area of highest bloom density, some cells would be actively growing, some in stationary phase and some would be senescent. An additional pool of re-mineralized nutrients from biomass turnover would be expected in the centre of the bloom that would not be present on the bloom fringe where cells rely on ambient lake nutrients, which we measured and used for our correlative analyses. All lake water samples were processed within 12 hours of collection and analyzed immediately upon return to the laboratory.

2.2.3 Phytoplankton

A 500 mL sub-sample of lake water was filtered onto Whatman GF/F filters and analyzed for chlorophyll-\(a\) (chl-\(a\)) according to EPA Method 445.0 (Arar & Collins, 1997). Chl-\(a\) was extracted from filters using an acetone:ultra-pure water solvent (90:10 v/v) in 20 mL scintillation vials and stored in the dark at -20 °C for 20 hours. Samples were brought to room temperature in the dark and measured using a Turner 10-AU field fluorometer with a 680 nm emissions filter (Turner Designs, Sunnyvale, CA).

A 3.5 mL sub-sample of unfiltered lake water was preserved with 1% buffered formaldehyde (v/v) in sterile Wheaton 5 mL cryule vials and phytoplankton community composition was assessed using the BD FACSCalibur flow cytometer (BD Biosciences, Sparks, MD) according to Marie et al. (1999). Water samples were vortexed to break apart colonies in best efforts to count single cells, but at low speeds and in pulses to avoid breaking cells. The vortexed water samples were then introduced to the flow cytometer where the fluorescent properties of cyanobacteria (orange fluorescence from phycobiliproteins) and eukaryotes (red fluorescence from chlorophyll) were detected for each cell in the sample. Cells were identified as cyanobacteria or eukaryote and total cell density was recorded. The proportion of cyanobacteria was enumerated by dividing the cell count for cyanobacteria by the total cell count (cyanobacteria + eukaryote) and multiplied by 100 to obtain a percentage of cyanobacteria in each sample. A 10 mL subsample of unfiltered lake water was used for taxonomic identification of phytoplankton genera with the Fluid Imaging FlowCAM (Fluid Imaging, Yarmouth, ME).
Flow cytometry using fluorescence was selected over traditional microscopy methods for phytoplankton community composition for several reasons: (1) samples preserve well and remain usable for this analysis for long periods of time while in the field; (2) flow cytometry is very sensitive to both small and large cells, which is important in oligotrophic lakes where picocyanobacteria biomass may be significant and undifferentiated from debris in unfiltered samples; and (3) assurance that small picocyanobacteria abundance is not underestimated relative to larger cyanobacterial cells.

2.2.4 Nutrients

A 90 mL sub-sample of unfiltered lake water was preserved with 10% H₂SO₄ (v/v) in screw-top borosilicate tubes. TP concentration was assessed by autoclaving for 30 minutes at 121 °C in sulphuric acid-persulphate medium to convert all P to orthophosphate and presented to the Technicon AutoAnalyzer (AAII) System (SEAL Analytical, Mequon, WI) where the colorimetric reaction was measured on the inline spectrophotometer at 883 nm.

A 300 mL sub-sample of lake water was filtered through a 0.45 µm Pall Life Sciences polysulphonate membrane disc filter and analyzed for nitrate and ammonium (colorimetry, analytical range is 10 to 1,000 µg L⁻¹ as N) and metals (inductively coupled plasma spectrometry with a detection limit of 0.83 µg L⁻¹) according to Ontario Ministry of the Environment and Energy Standards Development Branch (1996). TN (unfiltered) and dissolved organic carbon (DOC, 0.45 µm filtered) were assessed using a Shimadzu TOC-VCPH with TNM-1 and ASI-V auto-sampler with detection limits of 100 µg L⁻¹ as N and 4.0 µg L⁻¹ as DOC (Shimadzu, Kyoto, Japan). DON concentration was calculated by difference of TN – DIN [NO₃⁻ + NH₄⁺].

Ferric Fe concentration in lakes was modelled in Visual MINTEQ (v.3.0). Concentrations of the following nutrients and lake parameters were incorporated into the model: DOC, TP, nitrate, ammonium, sulphate, total dissolved Fe (TDFe), calcium, magnesium, chloride, pH and surface water temperature. The Stockholm Humic Model (SHM) of humic organic matter and metal complexation in Visual MINTEQ was used (Gustafsson, 2001). The SHM model was run with an assumed ratio of fulvic:humic acids of 50% for
lakes in the Algoma Highlands of central Ontario (Thurman & Malcolm, 1981). The model output provided molar concentrations for all nutrients, metals and complexes upon which the modelled ferric Fe concentration was recorded for each lake.

2.2.5 Statistical analyses

A Friedman Repeated Measures ANOVA on Ranks with Tukey Test (critical $\alpha = 0.05$) was used to identify the peak biomass period in 2009 by investigating differences in biomass (chl-$a$) throughout the growing season (June to November). A Kruskal-Wallis ANOVA on Ranks with Dunn's Test (critical $\alpha = 0.05$) was used to investigate differences in total and dissolved nutrients, chl-$a$, phytoplankton densities (cyanobacteria, eukaryote and total) and pH during peak biomass between each of three years (2009, 2010 and 2011). Pearson correlations were used to investigate auto-correlation among lake nutrients (critical $\alpha = 0.05$). Exponential regression was used to investigate the relationship between thermocline boundary depth and cyanobacterial proportion.

A baseline threshold of cyanobacterial proportion (5% cyanobacteria by cell density) that appeared to be common to all lakes was observed and selected so the specific factors leading to the growth of cyanobacteria above this threshold could be investigated. Lakes in all years combined were grouped into below-baseline (<5% cyanobacteria) and above-baseline (>5% cyanobacteria). A Mann-Whitney $U$ Rank Sum Test (critical $\alpha = 0.05$) was used to investigate differences in total and dissolved nutrients, chl-$a$, phytoplankton densities (cyanobacteria, eukaryote and total), pH and surface water temperature between below-baseline and above-baseline lakes. All statistical measures were performed in SigmaPlot (v.11.0). Regression tree analysis was performed in R (v.2.15.3) using the ‘rpart’ package to investigate the chemical determinants of chl-$a$, total phytoplankton density, eukaryote density and cyanobacterial proportion in lakes. Chemical parameters incorporated into the regression tree model included TP, TN, TN:TP, nitrate, ammonium, DIN, DIN:TP, DON, TDFe, modelled ferric Fe, sulphate, DOC, calcium, magnesium, chloride, pH and surface water temperature.
2.3 Results

2.3.1 Community structure during peak phytoplankton biomass

Lake phytoplankton community characteristics including total phytoplankton biomass (estimated by chl-a), total phytoplankton density and the density of cyanobacteria and eukaryotes that comprised total phytoplankton density in each of three years (2009, 2010 and 2011) and for all years combined are presented in Table 2.1.

For all years combined, chl-a ranged from 0.5 to 54.7 µg L\(^{-1}\), eukaryote density ranged from \(3.9 \times 10^7\) to \(1.0 \times 10^9\) cells L\(^{-1}\), cyanobacterial density ranged from \(4.8 \times 10^5\) to \(4.6 \times 10^7\) cells L\(^{-1}\) and total phytoplankton density ranged from \(4.6 \times 10^7\) to \(1.0 \times 10^9\) cells L\(^{-1}\) during peak phytoplankton biomass. Cyanobacterial density was found to be significantly higher in 2009 \((1.1 \times 10^7\) cells L\(^{-1}\)) vs. 2011 \((2.1 \times 10^6\) cells L\(^{-1}\)) \((p < 0.05)\) and total phytoplankton density was found to be significantly higher in 2010 \((2.2 \times 10^8\) cells L\(^{-1}\)) vs. 2011 \((1.6 \times 10^8\) cells L\(^{-1}\)) \((p < 0.05)\). No significant differences were found in chl-a or eukaryote density among years.

Among all three years, cyanobacteria comprised a range of \(<1\) to 36% of the total phytoplankton density with the remaining density comprised of chlorophyll-containing eukaryotic phytoplankton including chlorophytes, chrysophytes and diatoms. Lakes with the lowest total phytoplankton density had the highest cyanobacterial proportion, whereas lakes with relatively high total phytoplankton density had the lowest cyanobacterial proportion within phytoplankton communities (Fig. 2.3 A). Lakes with the lowest chl-a had the highest proportion of cyanobacteria, whereas lakes with the highest chl-a had the lowest proportion of cyanobacteria (Fig. 2.3 B). These trends were consistent throughout each of the three years of sampling during peak biomass. Cyanobacterial genera observed in above-baseline lakes included *Microcystis spp.*, *Anabaena spp.* and *Aphanizomenon spp.*; no one individual genus was exclusively present in any lake at the time of sampling. Picocyanobacteria were more prominent in the below-baseline lakes with an absence of the colonial and filamentous species.
Table 2.1 Lake phytoplankton community characteristics: median, minimum, maximum and range for chl-α, eukaryotes, cyanobacteria and total phytoplankton density in each of three years (2009, 2010 and 2011) and in all years combined. Superscript letters in bold indicate significant differences among the three years based on Kruskal-Wallis ANOVA on Ranks with Dunn's Test (critical α = 0.05).

<table>
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<tr>
<th>Year</th>
<th>Statistic</th>
<th>Chl-α (μg L⁻¹)</th>
<th>Eukaryotes (cells L⁻¹)</th>
<th>Cyanobacteria (cells L⁻¹)</th>
<th>Total Density (cells L⁻¹)</th>
</tr>
</thead>
<tbody>
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<td>1.1 × 10⁷ᵇ</td>
<td>1.9 × 10⁸ᵃᵇ</td>
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<tr>
<td></td>
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<td>3.9 × 10⁷</td>
<td>1.8 × 10⁶</td>
<td>4.6 × 10⁷</td>
</tr>
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<td>3.9 × 10⁷</td>
<td>5.2 × 10⁸</td>
</tr>
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<td>4.7 × 10⁸</td>
<td>3.7 × 10⁷</td>
<td>4.7 × 10⁸</td>
</tr>
<tr>
<td>2010</td>
<td>Med.</td>
<td>2.7</td>
<td>2.2 × 10⁶</td>
<td>5.1 × 10⁶ᵃᵇ</td>
<td>2.2 × 10⁶ᵇ</td>
</tr>
<tr>
<td></td>
<td>Min.</td>
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<td>5.8 × 10⁷</td>
<td>8.2 × 10⁵</td>
<td>7.0 × 10⁷</td>
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<td>2.9 × 10⁷</td>
<td>1.0 × 10⁹</td>
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<td>Range</td>
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<td>9.4 × 10⁸</td>
<td>2.8 × 10⁷</td>
<td>9.3 × 10⁸</td>
</tr>
<tr>
<td>2011</td>
<td>Med.</td>
<td>3.5</td>
<td>1.6 × 10⁸</td>
<td>2.1 × 10⁶ᵃᵃ</td>
<td>1.6 × 10⁶ᵃᵃ</td>
</tr>
<tr>
<td></td>
<td>Min.</td>
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<td>5.1 × 10⁷</td>
<td>4.8 × 10⁵</td>
<td>5.7 × 10⁷</td>
</tr>
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<td>4.6 × 10⁷</td>
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<td>4.5 × 10⁸</td>
<td>4.6 × 10⁷</td>
<td>4.4 × 10⁸</td>
</tr>
<tr>
<td>All Years</td>
<td>Med.</td>
<td>3.2</td>
<td>2.0 × 10⁸</td>
<td>5.9 × 10⁶</td>
<td>2.0 × 10⁸</td>
</tr>
<tr>
<td></td>
<td>Min.</td>
<td>0.5</td>
<td>3.9 × 10⁷</td>
<td>4.8 × 10⁵</td>
<td>4.6 × 10⁷</td>
</tr>
<tr>
<td></td>
<td>Max.</td>
<td>54.7</td>
<td>1.0 × 10⁹</td>
<td>4.6 × 10⁷</td>
<td>1.0 × 10⁹</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>54.2</td>
<td>9.6 × 10⁸</td>
<td>4.6 × 10⁷</td>
<td>9.5 × 10⁸</td>
</tr>
</tbody>
</table>
Figure 2.3 Relationship between cyanobacterial proportion and A) total phytoplankton density and B) chl-α in the oligotrophic lakes during the peak biomass in 2009 to 2011.
2.3.2 Nutrient ranges, ratios and measures of limitation

The median thermocline boundary depth ($Z_{tc}$) was 1.5 m and the median maximum depth ($Z_{max}$) was 5.3 m among all lakes. $Z_{tc}$ was found to exhibit a significant exponential decay trend when correlated to lakes with above baseline cyanobacteria ($r^2 = 0.81$, $p < 0.05$) while no significant relationship was found in the below baseline lakes (Fig. 2.4).

According to the Pearson correlation matrix (Table 2.2), modelled ferric Fe was not auto-correlated with any other lake nutrients. Lake chemical characteristics in each of three years (2009, 2010 and 2011) and for all years combined are presented in Table 2.3. For all years combined, TP in the lakes ranged from 2.2 to 59.7 µg L$^{-1}$. Soluble reactive P (SRP) was not detected in any lake samples as concentrations were below method detection limit (<0.2 µg L$^{-1}$) at the time of sampling. For all years combined, TP determined total phytoplankton biomass (chl-$a$) in the lakes ($r^2 = 0.54$, $p < 0.001$). TP was found to be significantly higher in 2009 (13.9 µg L$^{-1}$) vs. 2011 (8.9 µg L$^{-1}$) ($p < 0.05$). Lakes with the lowest TP had the highest cyanobacterial proportion whereas lakes with the highest TP had the lowest cyanobacterial proportion (Fig. 2.5). For all years combined, TN in the lakes ranged from ~200 to 2400 µg L$^{-1}$; no significant differences were found in TN among years and no significant relationship was found between TN and cyanobacterial proportion. The majority of TN was organic N (95%), with nitrate being the primary form of inorganic N (5%). Nitrate was significantly higher in 2009 (13.3 µg L$^{-1}$) vs. 2010 (1.7 µg L$^{-1}$) ($p < 0.05$), while ammonium was significantly different among all three years in 2009 (29.0 µg L$^{-1}$) vs. 2010 (3.0 µg L$^{-1}$) vs. 2011 (8.0 µg L$^{-1}$) (all $p < 0.05$). DIN was significantly higher in 2009 (46.7 µg L$^{-1}$) vs. 2010 (7.7 µg L$^{-1}$) vs. 2011 (13.9 µg L$^{-1}$) ($p < 0.05$) and vs. 2011 (13.9 µg L$^{-1}$) ($p < 0.05$). No significant difference was found in DON among years.

For all years combined, the molar ratio of TN:TP in the lakes ranged from 19:1 to 406:1. TN:TP was significantly higher in 2011 (102:1 molar) vs. 2009 (64:1 molar) ($p < 0.05$). The highest cyanobacterial proportion occurred at a TN:TP molar ratio of 66:1 (36% cyanobacteria), with high variability in cyanobacterial proportion at TN:TP molar ratios greater than 66:1 (Fig. 2.6 A). For all years combined, the molar ratio of DIN:TP in the lakes ranged from 0.3:1 to 193:1. DIN:TP was significantly higher in 2009 (6.1:1 molar)
Figure 2.4 Relationship between cyanobacterial proportion and thermocline boundary depth (m) in the oligotrophic lakes. Lakes with cyanobacterial communities greater than 5% exhibited an exponential decay trend with increasing thermocline boundary depth.
Table 2.2 Pearson correlation matrix for lake nutrients (critical $\alpha = 0.05$). Modelled ferric Fe was the only nutrient not auto-correlated with any other nutrient in the lakes. Data are presented as Pearson R (sample N). Positive Pearson R indicates a positive linear relationship between nutrients and a negative Pearson R indicates a negative linear relationship. Pearson R values in bold indicate a statistically significant relationship between lake nutrients ($p < 0.05$) and italicized Pearson R indicates no significant relationship.

<table>
<thead>
<tr>
<th></th>
<th>TP</th>
<th>TN</th>
<th>TN:TP</th>
<th>NO$_3^-$</th>
<th>NH$_4^+$</th>
<th>DON</th>
<th>DIN</th>
<th>DIN:TP</th>
<th>TDFe</th>
<th>Fe$^{3+}$</th>
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</thead>
<tbody>
<tr>
<td>DOC</td>
<td>0.19 (75)</td>
<td>0.22 (75)</td>
<td>-0.20 (74)</td>
<td>-0.32 (74)</td>
<td>0.23 (74)</td>
<td>0.28 (74)</td>
<td>-0.25 (74)</td>
<td>-0.33 (74)</td>
<td>0.67 (75)</td>
<td>-0.15 (75)</td>
</tr>
<tr>
<td>TP</td>
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<td>0.15 (74)</td>
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<td>-0.17 (74)</td>
<td>-0.34 (74)</td>
<td>0.24 (75)</td>
<td>0.03 (75)</td>
<td></td>
</tr>
<tr>
<td>TN</td>
<td>0.49 (74)</td>
<td>-0.06 (74)</td>
<td>0.19 (74)</td>
<td>0.97 (74)</td>
<td>-0.02 (74)</td>
<td>-0.07 (74)</td>
<td>0.06 (75)</td>
<td>0.07 (75)</td>
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<td></td>
</tr>
<tr>
<td>TN:TP</td>
<td>0.26 (74)</td>
<td>-0.09 (74)</td>
<td>0.42 (74)</td>
<td>0.22 (74)</td>
<td>0.52 (74)</td>
<td>-0.24 (74)</td>
<td>-0.02 (74)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>NO$_3^-$</td>
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<td>0.98 (74)</td>
<td>0.85 (74)</td>
<td>-0.24 (74)</td>
<td>-0.06 (74)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH$_4^+$</td>
<td>0.06 (74)</td>
<td>0.50 (74)</td>
<td>0.19 (74)</td>
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<td>0.07 (74)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DON</td>
<td>-0.27 (74)</td>
<td>-0.28 (74)</td>
<td>0.10 (74)</td>
<td>0.08 (74)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>DIN</td>
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<td>-0.07 (74)</td>
<td></td>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>DIN:TP</td>
<td>-0.22 (74)</td>
<td>-0.06 (74)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TDFe</td>
<td>0.06 (75)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.3 Lake chemical characteristics – median, minimum, maximum and range in each of three years (2009, 2010 and 2011) and in all years combined. Superscript letters in bold indicate significant differences among the three years based on Kruskal-Wallis ANOVA on Ranks with Dunn's Test (critical α = 0.05).

<table>
<thead>
<tr>
<th>Year</th>
<th>Statistic</th>
<th>pH</th>
<th>DOC (µg L⁻¹)</th>
<th>TDFe (µg L⁻¹)</th>
<th>TP (µg L⁻¹)</th>
<th>TN (µg L⁻¹)</th>
<th>NO₃⁻ (µg L⁻¹)</th>
<th>NH₄⁺ (µg L⁻¹)</th>
<th>DIN (µg L⁻¹)</th>
<th>DON (µg L⁻¹)</th>
<th>TN:TP</th>
<th>DIN:TP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Med.</td>
<td></td>
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<td>15.7</td>
<td>13.9b</td>
<td>421.7</td>
<td>13.3b</td>
<td>29.0c</td>
<td>46.7b</td>
<td>297.9</td>
<td>63.8a</td>
<td>6.1b</td>
</tr>
<tr>
<td>2009</td>
<td>Min.</td>
<td>5.4</td>
<td>2624.2</td>
<td>3.3</td>
<td>8.0</td>
<td>216.8</td>
<td>10.0</td>
<td>7.7</td>
<td>20.7</td>
<td>173.8</td>
<td>22.8</td>
<td>1.3</td>
</tr>
<tr>
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<td>Max.</td>
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<td>18578.0</td>
<td>267.3</td>
<td>59.7</td>
<td>2415.5</td>
<td>230.1</td>
<td>61.7</td>
<td>248.3</td>
<td>2371.4</td>
<td>375.8</td>
<td>65.8</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>3.7</td>
<td>15953.8</td>
<td>264.0</td>
<td>51.7</td>
<td>2198.7</td>
<td>220.1</td>
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<td>227.6</td>
<td>2197.6</td>
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</tr>
<tr>
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<td>Med.</td>
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<td>12.0</td>
<td>10.4ab</td>
<td>338.1</td>
<td>1.7a</td>
<td>3.0a</td>
<td>7.7a</td>
<td>303.4</td>
<td>72.3ab</td>
<td>1.9a</td>
</tr>
<tr>
<td></td>
<td>Min.</td>
<td>5.8</td>
<td>651.6</td>
<td>2.9</td>
<td>3.6</td>
<td>211.8</td>
<td>1.7</td>
<td>3.0</td>
<td>4.7</td>
<td>142.9</td>
<td>19.4</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
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<td>39.4</td>
<td>579.4</td>
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<td>571.5</td>
<td>178.2</td>
<td>96.4</td>
</tr>
<tr>
<td></td>
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<td>11594.6</td>
<td>133.9</td>
<td>35.8</td>
<td>367.6</td>
<td>178.2</td>
<td>5.0</td>
<td>178.1</td>
<td>428.6</td>
<td>158.8</td>
<td>96.1</td>
</tr>
<tr>
<td>2011</td>
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<td>3076.1a</td>
<td>10.0</td>
<td>8.9a</td>
<td>364.8</td>
<td>4.0ab</td>
<td>8.0b</td>
<td>13.9a</td>
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<td>230.1</td>
<td>1.7</td>
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<td>19.6</td>
<td>20.0</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>Max.</td>
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<td>19588.4</td>
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<td>37.4</td>
<td>547.0</td>
<td>311.9</td>
<td>64.0</td>
<td>375.8</td>
<td>529.7</td>
<td>406.4</td>
<td>193.2</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>1.9</td>
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<td>143.9</td>
<td>35.2</td>
<td>316.9</td>
<td>310.2</td>
<td>61.0</td>
<td>371.1</td>
<td>510.1</td>
<td>386.4</td>
<td>192.9</td>
</tr>
<tr>
<td>All Years</td>
<td>Med.</td>
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<td>4666.6</td>
<td>10.2</td>
<td>11.4</td>
<td>369.0</td>
<td>10.8</td>
<td>8.0</td>
<td>21.0</td>
<td>306.9</td>
<td>71.6</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>Min.</td>
<td>5.4</td>
<td>449.8</td>
<td>2.9</td>
<td>2.2</td>
<td>211.8</td>
<td>1.7</td>
<td>3.0</td>
<td>4.7</td>
<td>19.6</td>
<td>19.4</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>Max.</td>
<td>9.1</td>
<td>19588.4</td>
<td>267.3</td>
<td>59.7</td>
<td>2415.5</td>
<td>311.9</td>
<td>64.0</td>
<td>375.8</td>
<td>2371.4</td>
<td>406.4</td>
<td>193.2</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>3.7</td>
<td>19138.6</td>
<td>264.4</td>
<td>57.5</td>
<td>2203.7</td>
<td>310.2</td>
<td>61.0</td>
<td>371.1</td>
<td>2351.8</td>
<td>387.0</td>
<td>192.9</td>
</tr>
</tbody>
</table>
Figure 2.5 Relationship between cyanobacterial proportion and TP in the oligotrophic lakes during peak biomass.
Figure 2.6 A) Relationship between cyanobacterial proportion and TN:TP (molar ratio) in the oligotrophic lakes during peak biomass. Dashed vertical line represents the threshold for N-limitation (TN:TP < 16:1) and P-limitation (TN:TP > 16:1) according to the Redfield nutrient limitation principles on biomass (Redfield et al., 1963). B) Relationship between cyanobacterial proportion and DIN:TP (molar ratio) in the oligotrophic lakes during the peak biomass. Dashed vertical lines represent the threshold for N-limitation (DIN:TP < 2) and P-limitation (DIN:TP > 5) and lakes that fall between these lines represent co-limited lakes for DIN and TP according to the Ptacnik et al. (2010) nutrient limitation principles. X-axes are log_{10} scaled.
vs. 2010 (1.1:1 molar) \( (p < 0.05) \). The highest cyanobacterial proportion among all lake samples was observed at a DIN:TP molar ratio of 8:1 (36% cyanobacteria) (Fig. 2.6 B).

Additionally, pH was significantly different among all three years in 2009 (7.9) \( \text{vs.} \) 2010 (6.9) \( \text{vs.} \) 2011 (7.4) \( (p < 0.05) \). DOC was significantly higher in 2009 (5610 µg L\(^{-1}\)) \( \text{vs.} \) 2011 (3076 µg L\(^{-1}\)) \( (p < 0.05) \). No significant difference was found in TDFe among years.

2.3.3 Nutrients and phytoplankton in lakes above and below baseline

For all years combined, modelled ferric Fe in the lakes ranged from \( 1.0 \times 10^{-25} \) to \( 1.0 \times 10^{-13} \) mol L\(^{-1}\). The highest cyanobacterial proportion occurred at a modelled ferric Fe concentration of \( 1.0 \times 10^{-21} \) mol L\(^{-1}\) (36% cyanobacteria). All lakes with above-baseline cyanobacteria corresponded to modelled ferric Fe concentrations < \( 1.0 \times 10^{-19} \) mol L\(^{-1}\) (Fig. 2.7).

Chemical and phytoplankton characteristics in lakes with above- and below-baseline cyanobacteria are presented in Table 2.4. Lakes with above-baseline cyanobacteria had significantly higher cyanobacterial density \( (1.6 \times 10^7 \text{ vs.} 3.3 \times 10^6 \text{ cells L}^{-1}) \), lower chl-a \( (1.7 \text{ vs.} 4.8 \mu \text{g L}^{-1}) \), lower eukaryote density \( (9.5 \times 10^7 \text{ vs.} 2.5 \times 10^8 \text{ cells L}^{-1}) \) and lower total phytoplankton density \( (1.1 \times 10^8 \text{ vs.} 2.2 \times 10^8 \text{ cells L}^{-1}) \) compared to lakes with below-baseline cyanobacteria (all \( p < 0.05 \)).

Lakes with above-baseline cyanobacteria had significantly higher surface temperature \( (17.4 \text{ vs.} 16.3 \degree \text{C}) \), nitrate \( (18.2 \text{ vs.} 4.9 \mu \text{g L}^{-1}) \), TN:TP \( (104.7:1 \text{ vs.} 63.1:1 \text{ molar}) \), DIN \( (49.0 \text{ vs.} 15.5 \mu \text{g L}^{-1}) \), and DIN:TP \( (10.2:1 \text{ vs.} 2.3:1 \text{ molar}) \). Other factors such as TDFe \( (6.2 \text{ vs.} 30.2 \mu \text{g L}^{-1}) \), TP \( (6.5 \text{ vs.} 14.1 \mu \text{g L}^{-1}) \), DON \( (245.5 \text{ vs.} 354.8 \mu \text{g L}^{-1}) \) and DOC \( (3981 \text{ vs.} 5370 \mu \text{g L}^{-1}) \) were significantly lower compared to lakes with below-baseline cyanobacteria (all \( p < 0.05 \)). No significant difference was found in TN between lakes with above- and below-baseline cyanobacteria.
Figure 2.7 Relationship between cyanobacterial proportion and modelled ferric Fe in the oligotrophic lakes during peak biomass. The highest cyanobacterial proportion was observed at [modelled ferric Fe] < $1.0 \times 10^{-19}$ mol L$^{-1}$. The dashed vertical line represents a threshold in ferric Fe where cyanobacteria have shown to be competitive for Fe when limiting in laboratory experiments (Kerry et al., 1988).
Table 2.4 Lake chemical (above) and phytoplankton (below) characteristics: median, minimum, maximum and range in lakes with less than 5% baseline cyanobacteria vs. lakes with greater than 5% baseline cyanobacteria. Superscript letters in bold indicate significant differences among between above and below baseline lakes based on Mann-Whitney U Rank Sum Test (critical α = 0.05).

<table>
<thead>
<tr>
<th>Cyanobacteria</th>
<th>Statistic</th>
<th>Temp. (°C)</th>
<th>DOC (μg L⁻¹)</th>
<th>TDFe (μg L⁻¹)</th>
<th>TP (μg L⁻¹)</th>
<th>TN (μg L⁻¹)</th>
<th>NO₃⁻ (μg L⁻¹)</th>
<th>DIN (μg L⁻¹)</th>
<th>DON (μg L⁻¹)</th>
<th>TN:TP (molar)</th>
<th>DIN:TP (molar)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Med.</td>
<td>17.4b</td>
<td>3,981.1a</td>
<td>6.2a</td>
<td>6.5a</td>
<td>331.1</td>
<td>18.2b</td>
<td>49.0b</td>
<td>245.5b</td>
<td>104.7b</td>
</tr>
<tr>
<td>&gt;5%</td>
<td>Min.</td>
<td>13.7</td>
<td>449.8</td>
<td>2.9</td>
<td>2.2</td>
<td>211.8</td>
<td>1.7</td>
<td>4.7</td>
<td>19.6</td>
<td>23.1</td>
<td>1.6</td>
</tr>
<tr>
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<td>267.3</td>
<td>59.7</td>
<td>1534.6</td>
<td>311.9</td>
<td>375.8</td>
<td>1513.6</td>
<td>406.4</td>
<td>193.2</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>6.5</td>
<td>18128.2</td>
<td>264.4</td>
<td>57.5</td>
<td>1322.8</td>
<td>310.2</td>
<td>371.1</td>
<td>1494.0</td>
<td>383.3</td>
<td>191.6</td>
</tr>
<tr>
<td>&lt;5%</td>
<td>Med.</td>
<td>16.3a</td>
<td>5370.3b</td>
<td>30.2b</td>
<td>14.1b</td>
<td>371.5</td>
<td>4.9b</td>
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<td>63.1a</td>
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<td>2415.5</td>
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<td>Range</td>
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<td>2198.7</td>
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<td>95.3</td>
<td>2197.6</td>
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</table>

<table>
<thead>
<tr>
<th>Cyanobacteria</th>
<th>Statistic</th>
<th>Chl-α (μg L⁻¹)</th>
<th>Cyanobacteria (%)</th>
<th>Cyanobacteria (cells L⁻¹)</th>
<th>Eukaryotes (cells L⁻¹)</th>
<th>Total Density (cells L⁻¹)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Med.</td>
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<td>14.5b</td>
<td>1.6 × 10⁶b</td>
<td>9.5 × 10⁷a</td>
</tr>
<tr>
<td>&gt;5%</td>
<td>Min.</td>
<td>0.6</td>
<td>5.2</td>
<td>5.9 × 10⁶</td>
<td>3.9 × 10⁷</td>
<td>4.6 × 10⁷</td>
</tr>
<tr>
<td>N = 28</td>
<td>Max.</td>
<td>54.7</td>
<td>36.0</td>
<td>4.6 × 10⁷</td>
<td>3.4 × 10⁸</td>
<td>3.8 × 10⁸</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>54.1</td>
<td>30.8</td>
<td>4.0 × 10⁷</td>
<td>3.0 × 10⁸</td>
<td>3.3 × 10⁸</td>
</tr>
<tr>
<td>&lt;5%</td>
<td>Med.</td>
<td>4.8b</td>
<td>1.4a</td>
<td>3.3 × 10⁵a</td>
<td>2.5 × 10⁸b</td>
<td>2.2 × 10⁹b</td>
</tr>
<tr>
<td>N = 47</td>
<td>Min.</td>
<td>0.5</td>
<td>0.2</td>
<td>4.8 × 10⁵</td>
<td>6.4 × 10⁷</td>
<td>6.7 × 10⁷</td>
</tr>
<tr>
<td></td>
<td>Max.</td>
<td>20.9</td>
<td>4.9</td>
<td>1.2 × 10⁷</td>
<td>1.0 × 10⁹</td>
<td>1.0 × 10⁹</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>20.4</td>
<td>4.7</td>
<td>1.2 × 10⁷</td>
<td>9.4 × 10⁸</td>
<td>9.3 × 10⁸</td>
</tr>
</tbody>
</table>
2.3.4 Combinatorial nutrient determinants of phytoplankton community structure in lakes

Regression tree analysis of the lake chemical and biological data indicated that there were complex combinatorial determinants that separated the eukaryotic phytoplankton from the cyanobacteria. TP, with additional influence of DON and DOC, was the prime environmental factor that determined chl-\(a\) in these oligotrophic lakes (Fig. 2.8 A). In contrast, the ratio of TP to nitrogen (both TN and DIN) determined the phytoplankton density (Fig. 2.8 B). Differentiating between the eukaryotic and prokaryotic phototrophic plankton required two independent sets of environmental parameters. The eukaryotic phytoplankton density was positively correlated to the ratio of TN to TP (Fig. 2.8 C). In contrast, distinctly independent parameters of low modelled ferric Fe and available nitrogen correlated most strongly with the cyanobacterial proportion (Fig. 2.8 D).

2.4 Discussion

The primary objective of this study was to determine if P or N limits cyanobacterial growth and what role ferric Fe may have in alleviating P or N limitation on cyanobacterial biomass. We found that the influence of P and N limitation are minimal and that cyanobacteria are most competitive in low Fe environments.

2.4.1 Community structure during peak phytoplankton biomass

With the highest proportion of cyanobacteria found to be 36% among three years of sampling, none of the oligotrophic lakes were considered to be experiencing cyanoblooms during peak biomass period, outside of visible biomass accumulation due to wind. The nutrient controlling factors on cyanobacterial growth presented in this study are thus focused on those that result in shifts from picocyanobacteria to more transitionary cyanobacterial communities. We investigated whether patterns existed among lakes with respect to total phytoplankton density and cyanobacterial proportion in oligotrophic lakes. The observed trend of highest cyanobacterial proportion occurred when total phytoplankton density and chl-\(a\) was lowest in lakes and is supported by Watson et al. (1997) who found that in temperate oligotrophic lakes, phytoplankton biomass and species diversity is low and cyanobacteria can dominate the
Figure 2.8 Regression trees depicting the chemical determinants of A) chl-$\alpha$, B) total phytoplankton density, C) eukaryotic phytoplankton and D) cyanobacterial proportion in the oligotrophic lakes during peak biomass.
picophytoplankton community, but with nutrient enrichment, taxonomic diversity increases. Picocyanobacteria dominated the oligotrophic lakes when overall phytoplankton biomass was low but in higher biomass lakes with higher nutrient levels, the community was comprised of transitionary cyanobacteria (*Microcystis* spp., *Anabaena* spp. and *Aphanizomenon* spp.) with more taxonomic diversity. None of the lakes achieved high biomass mono-specific communities, which would constitute a cyanobloom.

### 2.4.2 Nutrient ranges, ratios and inferences of limitation

All lakes with cyanobacterial proportions above the baseline (>5% cyanobacteria) were significantly and negatively correlated to increasing $Z_{tc}$, suggesting that cyanobacteria in these surface water communities may use buoyancy control to exploit diffusing hypolimnetic nutrient sources, although no vertical depth samples were available to confirm this. The negative trend observed may indicate that with a deeper $Z_{tc}$ it is more difficult to migrate vertically and obtain hypolimnetic nutrients to satisfy metabolic demand. Genera observed in the above-baseline lakes, including *Microcystis* spp. (Mur et al., 1999) and *Anabaena* spp. (Kinsman et al., 1991), have been shown to utilize the buoyancy control strategy; this may enable them to obtain internally loaded nutrients from the hypolimnion, which are an important source of P and Fe in other shallow Canadian freshwater lakes (Petticrew & Arocena, 2001).

The observation of highest cyanobacterial proportion at lowest TP in lakes may be attributed to cyanobacteria having the ability of luxury P uptake, high internal cellular storage capacity of P relative to the other phytoplankton (Mur *et al*., 1999) and high P transport affinity at low P concentrations (Molot & Brown, 1986). When P concentration is low, cyanobacteria may out-compete other phytoplankton groups with lower P transport affinity and an inferior internal cellular storage capacity resulting in a higher proportion of cyanobacteria within the phytoplankton community.

According to Redfield ratio stoichiometry (Redfield *et al*., 1963), phytoplankton biomass in the lakes may have been P-limited at the time of sampling with TN:TP >16:1 as a molar ratio. Other studies have found that DIN:TP predicts phytoplankton biomass better
than TN and TP concentrations or TN:TP because DIN:TP provides a more representative indication of bioavailable inorganic N where N limitation may occur at DIN:TP <2:1 and P limitation may occur at DIN:TP >5:1 as molar ratios (Ptacnik et al., 2010). Since about 95% of TN in the lakes was DON, which is a fraction of N in the total N pool that is not immediately bioavailable to phytoplankton, and SRP was undetectable, investigation using DIN:TP was warranted. The highest cyanobacterial proportion (36%) across a gradient of DIN:TP in the lakes was at DIN:TP = 8:1 as a molar ratio, a potentially P-limited condition. By DIN:TP limitation definitions, biomass may have been P-limited in 47% of lakes, DIN-limited in 26% of lakes and co-limited by DIN and TP in 27% of lakes at the time of sampling.

Both metrics presented here (TN:TP and DIN:TP) suggest different nutrient limitation conditions on biomass in the same lakes. Schindler (2012) discussed common errors in the interpretation of ecosystem processes and indicators, one of which is the incorrect assumption that ratios of total, suspended or dissolved nutrients are reliable indicators of nutrient limitation. Although we agree that DIN:TP may be a more reliable indicator of nutrient limitation of biomass in the lakes than TN:TP, we also agree with Schindler (2012) that additional factors must be considered when evaluating nutrient limitation of biomass in lakes. We argue that Fe plays an important role in nutrient limitation since Fe is directly involved in phytoplankton macronutrient P and N use efficiency.

2.4.3 Nutrients and phytoplankton in lakes above and below baseline

We hypothesized that cyanobacterial growth would be highest in a range of modelled ferric Fe not readily accessible to eukaryotic phytoplankton (<1.0 × 10⁻¹⁹ mol L⁻¹). Naturally occurring concentrations of ferric Fe are expected to range from 1.0×10⁻¹⁷ to 1.0 × 10⁻²¹ mol L⁻¹ in neutral aquatic systems (Schneider & Schwyn, 1987). We found that while some of the lakes fell within this expected range of ferric Fe, the majority of lakes had far lower concentrations of modelled ferric Fe that gave rise to the highest cyanobacterial proportions, <1.0 × 10⁻¹⁹ mol L⁻¹. The ability of cyanobacteria to thrive and increase relative to the other phytoplankton in these lakes with low modelled ferric Fe could be due to the siderophore-based Fe acquisition strategy used by some
cyanobacteria providing a competitive advantage during Fe stress (Neilands, 1995).

Studies of siderophores in oligotrophic lakes within the Laurentian Great Lakes-St. Lawrence River Basin are lacking, with only one record documenting the presence and utilization of hydroxamate-type siderophores by *Anabaena flos-aquae* in the Bay of Quinte, a eutrophic bay on the northern shore of Lake Ontario (Murphy *et al.*, 1976).

Kerry *et al.* (1988) found that siderophore production by cyanobacteria (*Anacystis nidulans*) was initiated at a ferric Fe concentration of 1.0 × 10^{-19} mol L^{-1}, which matches well with the modelled ferric Fe concentrations in the oligotrophic lakes with the highest cyanobacterial proportions. This suggests that cyanobacteria may be utilizing siderophores as a mechanism to obtain ferric Fe, providing cyanobacteria with a competitive advantage in this range of ferric Fe over other phytoplankton that do not have the capacity to produce siderophores. Oligotrophic lakes with ferric Fe concentrations >1.0 × 10^{-20} mol L^{-1} may provide sufficient ferric Fe to promote and sustain growth of all phytoplankton, thus eliminating any competitive advantage by cyanobacteria for Fe acquisition. Cyanobacterial genera known to have the capacity to produce and utilize siderophores and that were observed in the oligotrophic lakes included *Anabaena spp.* (Wilhelm & Trick, 1994) and *Microcystis spp.* (Murphy *et al.*, 1976). Although consensus pertaining to the minimum Fe requirement to promote and sustain cyanobacterial growth is lacking, the decline in cyanobacterial proportion observed at modelled ferric Fe concentrations <1.0 × 10^{-22} mol L^{-1} may represent the minimum ferric Fe concentration required to support cyanobacterial growth in these oligotrophic lakes.

Ferric Fe bioavailability for N-assimilation and N2-fixation may be an important regulator of cyanoblooms in oligotrophic lakes. We suspect that the limited amount of modelled ferric Fe in lakes above the baseline may be accessible exclusively to cyanobacteria via the siderophore-based Fe acquisition strategy. By scavenging and thus dictating the bioavailability of ferric Fe to other phytoplankton in surface waters, cyanobacteria have the capacity to be competitive for and assimilate DIN or fix N2 when nitrate supplies are low, while increasing in proportion relative to eukaryotic phytoplankton.
2.4.4 Combinatorial nutrient determinants of phytoplankton community structure in lakes

The regression tree evaluation clearly documents that the factor primarily correlating to the rise of the transitionary colonial or filamentous cyanobacteria is the ability to thrive in low Fe environments, regardless of the macronutrient composition. The dichotomy between the factors regulating the eukaryote cells and the cyanobacteria shows almost no overlap. As observed in all above-baseline oligotrophic lakes where TDFe was low and DIN was high, cyanobacteria may have used siderophores to scavenge Fe required for N assimilation, restricting Fe access, and thus N access, to eukaryote phytoplankton. This leads us to conclude that there is very little direct competition for nutrients, but rather each group exists in a very specific ecological homeostasis.

A possible, but presently untested, scenario is that the initial state, physiological health potential, of the community (eukaryotic phytoplankton vs. transitionary cyanobacteria) at the time of addition of internally or externally derived phosphorus would lead one population to out-grow the other, leading to predominance (bloom) of that one ecotype. Given the rise in cyanobacteria in oligotrophic lakes, future research is needed on landscape controls on the mobilization of Fe to lakes and/or lake controls on the fate of Fe to complement recent research on changing P (Eimers et al., 2009) and N (Kothawala et al., 2011) concentrations in oligotrophic lakes.
2.5 References


assessments and valuations of ecosystem services. Proceedings of the National Academy of Sciences of the United States of America, 109, 18619-18624.


Chapter 3

3 Regulation of cyanobacterial biomass in oligotrophic lakes with competing iron, siderophore and refractory DOM supply

This chapter has been re-submitted as a manuscript for publication to the journal *Freshwater Biology*.

3.1 Introduction

Lakes in Ontario Canada and in other parts of northeastern North America have undergone some dramatic changes over the past two decades (Carey, Weathers & Cottingham, 2008; Winter *et al.*, 2011). These changes pertain to an increased frequency and duration of cyanobacterial harmful algal blooms (cyanoHABs) both within and among lakes. Original perceptions were that cyanoHABs occur strictly in high macronutrient eutrophic lakes (Schindler, 2006). However it is now recognized that cyanoHABs occur in low macronutrient oligotrophic lakes as well, such as those on the Precambrian Shield within the Laurentian Great Lakes-St. Lawrence River Basin (Carey *et al.*, 2008; Winter *et al.*, 2011).

Public concern for the emergence of cyanoHABs in oligotrophic lakes is rising. This concern is not trivial. Cyanobacteria are often bloom forming, having the capacity to grow to high levels of biomass (Paerl & Huisman, 2009). Several genera of cyanobacteria have the capacity to produce toxic secondary metabolites known as cyanotoxins, often classified as neurotoxins and hepatotoxins posing serious human, animal and ecosystem health concerns (Mur, Skulberg & Utkilen, 1999).

Studies pertaining to macronutrient controls on cyanobacterial growth are well documented, particularly on the importance of nitrogen (N) (Berman, 2001), phosphorus (P) (Downing, Watson & McCauley, 2001) and the ratio of N/P (Smith, 1983). The importance of macronutrient concentrations or their ratios in predicting phytoplankton biomass remains highly debated (Schindler, 2012). Despite these debates, macronutrient explanations of controls on cyanobacterial biomass are insufficient to explain cyanoHAB
occurrence in oligotrophic lakes where macronutrient supplies for phytoplankton metabolic demand are often not met.

Studies that investigate controls on cyanobacterial biomass often fail to account for micronutrients such as iron (Fe), which has been shown to contribute significantly to cyanobacterial growth in laboratory studies (Kerry, Laudenbach & Trick, 1988; Wilhelm, 1995). Cyanobacteria require Fe in nitrogenase (NiR) activity for N$_2$ fixation (Murphy, Lean & Nalewajko, 1976) and in nitrate reductase (NtR) activity for nitrate assimilation (Lin & Stewart, 1998). Morel et al. (1991) showed that N$_2$-fixing cyanobacteria require Fe at concentrations up to 20 times higher than a eukaryotic phytoplankton dividing once per day. Wilhelm (1995) showed that even non-N$_2$-fixing cyanobacteria require Fe at concentrations higher than that for eukaryotic phytoplankton (Wilhelm, 1995). Molot et al. (2010) provide both laboratory and field evidence that cyanobacterial growth is restricted by limited access to bioavailable Fe.

Fe in aerobic surface waters commonly exists in the ferric form (Fe$^{3+}$), which must be reduced to the ferrous form (Fe$^{2+}$) at the cell surface prior to phytoplankton assimilation via the enzyme ferric reductase (FeR) (Kranzler et al., 2011). Siderophores are low molecular weight organic Fe-binding ligands produced by bacteria and fungi during Fe-limited conditions as an Fe-scavenging strategy (Neilands, 1995). Hydroxamate siderophores are water-soluble and have relatively weak Fe-binding capacity, whereas catecholate siderophores are fat-soluble and have relatively strong Fe-binding capacity (Neilands, 1995). The mechanisms of Fe-binding in the two siderophore types differ as described by Neilands (1995). Hydroxamate siderophores are produced within the cell, are transported to the external environment via specialized membrane-bound protein channels where they bind soluble ferric Fe, and ferric Fe is reduced to ferrous Fe (via FeR) upon contact of the Fe-siderophore complex at the cell surface and then assimilated. Catecholate siderophores are cell membrane-bound and Fe-binding occurs at the cell surface where ferric Fe is reduced to ferrous Fe (via FeR) and assimilated.

Cyanobacteria are the only phytoplankton group possessing a Fe-siderophore uptake system and so have a competitive advantage for Fe scavenging over eukaryote
phytoplankton in Fe-limited conditions (Wilhelm & Trick, 1994). Fe is essential in regulating the efficiency of macronutrient use by cyanobacteria playing a critical role in N and P uptake. The bioavailable N pool is dependent on Fe supply required for N assimilation (NtR) and N₂ fixation (NiR). The bioavailable P pool is dependent on the potential for Fe to bind to phosphate and precipitate from aerobic surface waters (Moore & Reddy, 1994). Hydroxamate and catecholate siderophores may be important sources of Fe to cyanobacteria, in turn regulating important macronutrient uptake.

In oligotrophic lakes, Fe often exists as a limiting trace metal with low bioavailability to pelagic phytoplankton (Davison, 1993). This may be due to the tendency for Fe to bind to dissolved organic matter (DOM). DOM with refractory properties has relatively high Fe-binding capacity due to its humic acid composition, comprised mainly of phenolic and carboxylic acid groups with high affinity for metal ions (Baken et al., 2011). DOM with labile properties has relatively low Fe-binding capacity due to its high protein content and low humic acid composition (Baken et al., 2011). In addition to binding Fe, DOM may also bind siderophores through mixed-ligand complexation with bound Fe (Chen & Wang, 2008). Therefore, DOM and its chemical composition may be an important determinant of bioavailable Fe and siderophores in natural waters. Insight into the use of siderophores by cyanobacteria in oligotrophic lakes and the potential for DOM to bind Fe and siderophores in lakes is needed.

The purpose of this study is to determine if the presence of siderophores results in elevated cyanobacterial biomass in oligotrophic lakes and if so, under what macro- and micronutrient conditions. Our hypothesis was that cyanobacterial density would be highest in lakes with low total dissolved Fe (TDFe) due to the siderophore Fe scavenging system that gives cyanobacteria a competitive advantage for Fe acquisition over other phytoplankton. However, the presence of DOM and its composition will influence the bioavailability of Fe to cyanobacteria. Lakes that have DOM with refractory properties will have low cyanobacterial density due to strong Fe complexation, whereas lakes that have DOM with labile properties will allow cyanobacteria to Fe from DOM complexes and overcome Fe limitation. For a set of oligotrophic lakes with similar physical characteristics but different macro- and micronutrient conditions, we set out to determine
if Fe limits cyanobacterial growth and if the presence of Fe-binding siderophores alleviates Fe limitation.

3.2 Methods

3.2.1 Study sites

For this study, 25 oligotrophic lakes were selected based on public concern for the potential of cyanoHABs in the lakes (Table 3.1). The lakes were characteristically shallow, thermally stratified during the warm summer months, and dimictic with major mixing events occurring during the spring snowmelt and autumn storms. These two major periods of hydrologic connectivity between land and lake represent important episodes of terrestrial nutrient input to lakes (Creed & Beall, 2009; Mengistu, Quick & Creed, 2013).

3.2.2 Lake water chemistry sample collection

Lakes were sampled once during peak phytoplankton biomass in 2011, which has been identified as the period from late September to late October based on the previous years growing season biomass data on the same lakes (Sorichetti, Creed & Trick, 2013). While on the lake, temperature and pH were measured at 1 m depth below the lake surface using a YSI 600 QS multi-parameter sonde with a YSI 650 MDS display (YSI Incorporated, Yellow Springs, OH, USA). Secchi depth was measured and lake surface water samples integrated to 1 m depth were collected in 500 mL pre-rinsed polyethylene bottles near the center of the lake, outside of a phytoplankton bloom if present, and stored in the dark on ice in a cooler until returning to the field laboratory. Best efforts were made to sample outside of a phytoplankton bloom and not directly in the highest density to avoid sampling of senescent cells and capture actively growing biomass.
Table 3.1 Coordinates (lat/long) and physical characteristics (max depth, thermocline depth and surface water temperature) of lakes in the Algoma Highlands of central Ontario.

<table>
<thead>
<tr>
<th>Lake</th>
<th>Latitude (N)</th>
<th>Longitude (W)</th>
<th>Max Depth (m)</th>
<th>Thermocline Depth (m)</th>
<th>Surface Temp. (ºC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negick</td>
<td>47°12'20.35&quot;</td>
<td>84°29'22.81&quot;</td>
<td>5.3</td>
<td>3.3</td>
<td>14.0</td>
</tr>
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<td>Caysee</td>
<td>47°10'58.05&quot;</td>
<td>84°39'22.84&quot;</td>
<td>1.3</td>
<td>1.1</td>
<td>14.8</td>
</tr>
<tr>
<td>Huff</td>
<td>47°10'15.40&quot;</td>
<td>84°33'56.25&quot;</td>
<td>4.0</td>
<td>1.0</td>
<td>16.0</td>
</tr>
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<td>Tay</td>
<td>47°10'01.60&quot;</td>
<td>84°17'13.23&quot;</td>
<td>0.8</td>
<td>0.4</td>
<td>13.0</td>
</tr>
<tr>
<td>Airport</td>
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<td>84°17'34.80&quot;</td>
<td>7.7</td>
<td>2.2</td>
<td>13.6</td>
</tr>
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<td>Mystery</td>
<td>47°07'24.18&quot;</td>
<td>84°16'25.46&quot;</td>
<td>0.6</td>
<td>0.4</td>
<td>13.0</td>
</tr>
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<td>Upper Griffin</td>
<td>47°05'09.96&quot;</td>
<td>84°24'05.27&quot;</td>
<td>8.1</td>
<td>2.5</td>
<td>16.5</td>
</tr>
<tr>
<td>Lower Griffin</td>
<td>47°04'53.95&quot;</td>
<td>84°25'11.11&quot;</td>
<td>7.8</td>
<td>2.2</td>
<td>16.3</td>
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<td>Big Turkey</td>
<td>47°02'51.59&quot;</td>
<td>84°25'10.39&quot;</td>
<td>42.7</td>
<td>3.7</td>
<td>15.7</td>
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<td>Little Turkey</td>
<td>47°02'31.15&quot;</td>
<td>84°24'28.20&quot;</td>
<td>7.3</td>
<td>2.4</td>
<td>15.8</td>
</tr>
<tr>
<td>Upper Tilley</td>
<td>47°00'56.46&quot;</td>
<td>84°23'15.65&quot;</td>
<td>6.1</td>
<td>1.4</td>
<td>16.6</td>
</tr>
<tr>
<td>Lower Tilley</td>
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<td>84°23'13.91&quot;</td>
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<td>0.5</td>
<td>15.5</td>
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<td>Carp</td>
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<td>84°33'38.93&quot;</td>
<td>1.5</td>
<td>0.3</td>
<td>15.2</td>
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<td>Sill</td>
<td>46°46'20.22&quot;</td>
<td>84°15'14.01&quot;</td>
<td>7.3</td>
<td>2.6</td>
<td>16.2</td>
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<td>46°26'19.92&quot;</td>
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<td>7.4</td>
<td>2.0</td>
<td>17.2</td>
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<td>83°49'37.43&quot;</td>
<td>3.1</td>
<td>1.1</td>
<td>15.5</td>
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<td>Desbarats</td>
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<td>83°55'55.66&quot;</td>
<td>9.1</td>
<td>2.2</td>
<td>16.8</td>
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<td>Ottertail</td>
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<td>83°45'01.56&quot;</td>
<td>3.0</td>
<td>0.7</td>
<td>14.8</td>
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<td>83°53'30.37&quot;</td>
<td>6.2</td>
<td>1.5</td>
<td>15.1</td>
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<tr>
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<td>83°16'47.00&quot;</td>
<td>3.1</td>
<td>1.1</td>
<td>16.4</td>
</tr>
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<td>Twin</td>
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<td>83°55'43.38&quot;</td>
<td>3.5</td>
<td>1.6</td>
<td>16.1</td>
</tr>
<tr>
<td>Dean</td>
<td>46°13'39.17&quot;</td>
<td>83°10'43.87&quot;</td>
<td>14.9</td>
<td>n.a.</td>
<td>16.8</td>
</tr>
</tbody>
</table>
3.2.3 Lake siderophore sample collection

Siderophore water sample collection and filtration methodology was adapted from Macrellis et al. (2001). Large volume water filtration was conducted lakeside at all 25 lakes immediately upon returning to shore within 20 min of water sample collection. A total of 40 L of bulk lake water was collected into a 60 L cleaned and pre-rinsed polyethylene bin. Water filtration was conducted in a stepwise method using a Wayne RUP160 1/6-horsepower 3000 GPH oil-less utility submersible water pump. Filtration was conducting at the low speed setting using the following stepwise methodology to ensure filtration efficiency:

1) Bulk lake water was pumped into a second cleaned and pre-rinsed polyethylene bin through a sponge pre-filter to exclude large particulate matter to obtain primary filtered water.

2) An in-line canister filter chamber was installed with a 60 μm filter cartridge and the submersible water pump was placed into the bin containing the primary filtered water and filtered into a cleaned and pre-rinsed polyethylene bin to obtain secondary filtered water.

3) A 20 μm filter cartridge was installed and the submersible water pump was placed into the bin containing secondary filtered water and filtered into a cleaned and pre-rinsed polyethylene bin to obtain tertiary filtered water.

A 1 μm filter cartridge was installed and the submersible water pump was placed into the bin containing tertiary filtered water and filtered into a cleaned and pre-rinsed polyethylene bin to obtain final filtered water. A total of 40 L of final filtered water for all 25 lakes was transported back to the field laboratory for further processing.

3.2.4 Laboratory Work

All lake water samples were processed within 12 h of sample collection and analyzed immediately upon returning to the laboratory.
3.2.5 Phytoplankton

A 500 mL sub-sample of lake water was filtered through 0.7 µm Whatman GF/F filters (GE Healthcare Life Sciences, Baie d’Urfe, QC, Canada) and analyzed for chlorophyll-α (chl-α) using a Turner 10-AU field fluorometer (Turner Designs, Sunnyvale, CA, USA) according to EPA Method 445.0 (Arar & Collins, 1997). A 3.5 mL sub-sample of unfiltered lake water was preserved with 1% buffered formaldehyde (v/v) in sterile 5 mL cryule vials (Wheaton, Millville, NJ, USA) and phytoplankton community composition was assessed using a BD FACSCalibur flow cytometer (BD Biosciences, Sparks, MD, USA) according to Marie et al. (1999). Water samples were vortexed to break apart colonies in best efforts to count single cells. A 10 mL subsample of unfiltered lake water was used for taxonomic identification of phytoplankton genera with a Fluid Imaging FlowCAM (Fluid Imaging, Yarmouth, ME, USA).

3.2.6 Nutrients

A 90 mL sub-sample of unfiltered lake water was preserved with 10% H₂SO₄ (v/v) in screw-top borosilicate tubes. Total phosphorus (TP) concentration was assessed by autoclaving for 30 min in sulfuric acid-persulphate media to convert all P to orthophosphate at 121 ºC and presented to a Technicon AutoAnalyzer (AAII) System with a method detection limit (MDL) of 0.02 µM (SEAL Analytical, Mequon, WI, USA).

Total N was assessed using a Shimadzu TOC-VCPH with TNM-1 and ASI-V auto-sampler (MDL = 100 µg L⁻¹) (Shimadzu, Kyoto, Japan). A 300 mL sub-sample of lake water was filtered through 0.45 µm Pall Life Sciences (Mississauga, ON, Canada) polysulfonate membrane disc filters and analyzed for nitrate and ammonium (colorimetry, MDL = 3.5 and 5.9 µg L⁻¹ respectively) and TDFe (inductively coupled plasma spectrometry, MDL = 0.83 µg L⁻¹) according to Ontario Ministry of the Environment and Energy Standards Development Branch (1996).

3.2.7 DOM

Dissolved organic matter (0.45 µm filtered) was assessed using a Shimadzu TOC-VCPH with TNM-1 and ASI-V auto-sampler (MDL = 4 µg L⁻¹) (Shimadzu, Kyoto, Japan). A
100 mL sub-sample of 0.45 µm filtered lake water was stored in an amber polyethylene bottle in the refrigerator at 4 °C for excitation-emission matrix (EEM) and parallel factor (PARAFAC) statistical analysis. EEMs were run according to Cory & McKnight (2005) on a Cary Eclipse spectrofluorometer (Agilent Technologies, Santa Clara, CA, USA) with a 75 Hz xenon lamp as the excitation source. Scans were run on ratio (S/R) mode with 1.5 mL of filtered lake water at room temperature in a 1.0 cm quartz cuvette within 10 days of sample collection. EEM scans were run with an excitation wavelength range of 240 nm to 450 nm at 2 nm intervals and with an emissions wavelength range of 300 nm to 600 nm at 2 nm intervals. Blank EEM and Raman interference scans were run using ultra-pure Milli-Q water. Absorbance scans for the correction of inner filter effect, the quenching of excitation light and subsequent emitted radiation, were run from 200 nm to 800 nm on a Cary 300 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). PARAFAC analysis on EEMs was conducted in MATLAB R2009B (MathWorks, Natick, MA, USA) according to Cory & McKnight (2005) to evaluate the degree of DOM humification (HIX) in the study lakes.

3.2.8 Siderophores

Final filtered water for siderophore analysis was processed using column chromatography to isolate siderophores. All 40 L of final filtered water from each lake was passed through a column at a maximum rate of 1.2 L min⁻¹ to ensure maximum adsorption of siderophores to 200 mL of XAD-16 (amberlite) resin (Macrellis et al., 2001). Once all final filtered water was passed through the column, 200 mL of ultra-pure Milli-Q water was passed through the column to thoroughly rinse the XAD-16 resin loaded with siderophore sample. Finally, 500 mL of methanol was passed through the column to elute the isolated siderophore sample, retained in a polyethylene bottle and stored in the dark and in a freezer until further processing and analysis.

Methanol eluted samples were then concentrated by rotary evaporation at 30 °C to a final volume of 20 mL (Macrellis et al., 2001). The Czaky test was used to quantitatively determine the concentration of hydroxamate siderophores in the concentrated methanol eluent (MDL = 0.02 µM), using hydroxylamine hydrochloride as standards (Gillam, Lewis & Andersen, 1981). The Arnow test was used to quantitatively determine the
concentration of catecholate siderophores in the concentrated methanol eluent (MDL = 0.02 µM), using 2,3-dihydroxybenzoic acid as standards (Arnow, 1937).

3.2.9 Statistical analysis

A baseline threshold of cyanobacterial density ($5 \times 10^6$ cells L$^{-1}$) that appeared to be common to all lakes was observed and selected so the specific factors leading to the growth of cyanobacteria above this threshold could be investigated. Linear regression (critical $\alpha = 0.05$) was used to investigate relationships between: TP and chl-$\alpha$ concentrations; DOM and the concentration of siderophores; HIX and the concentration of siderophores; the concentration of hydroxamate siderophores and cyanobacterial density; and the concentration of nitrate and cyanobacterial density. Pearson correlation analysis (critical $\alpha = 0.05$) was used to investigate auto-correlation among lake nutrient concentrations, siderophore concentrations and cyanobacterial density. Statistical measures were performed in SigmaPlot (v.11.0, SYSTAT Software, Chicago, IL, USA).

Regression tree analysis was performed in R (v.2.15.3, Lucent Technologies, Murray Hill, NJ, USA) using the ‘rpart’ package to investigate the chemical determinants of chl-$\alpha$ and cyanobacterial density. Chemical parameters incorporated into the regression tree model included TP, TN, TN/TP, nitrate, ammonium, TDFe, sulfate, DOC, calcium, magnesium, chloride, pH and surface water temperature.

3.3 Results

3.3.1 Phytoplankton community structure during peak biomass

Lake phytoplankton community characteristics including chl-$\alpha$ concentration, total phytoplankton density and the density of cyanobacteria and eukaryotes that comprised total density are presented in Table 3.2.

Among the study lakes, total phytoplankton biomass (estimated by the concentration of chl-$\alpha$) ranged from 1.1 to 20.9 µg L$^{-1}$ and total phytoplankton density ranged from $5.7 \times 10^7$ to $5.0 \times 10^8$ cells L$^{-1}$ during peak biomass. Cyanobacterial density ranged from $4.8 \times 10^5$ to $4.6 \times 10^7$ cells L$^{-1}$ and eukaryote density ranged from $5.1 \times 10^7$ to $5.0 \times 10^8$ cells L$^{-1}$. We observed a separation point in cyanobacterial density and considered $>5.0 \times 10^6$
Table 3.2 Lake phytoplankton community characteristics – median, range, 25th and 75th percentile for measured chl-\(\alpha\), eukaryotes, cyanobacterial and total phytoplankton density in lakes in the Algoma Highlands of central Ontario.

<table>
<thead>
<tr>
<th></th>
<th>N = 25</th>
<th>Chl-(\alpha) ((\mu)g L(^{-1}))</th>
<th>Eukaryotes (cells L(^{-1}))</th>
<th>Cyanobacteria (cells L(^{-1}))</th>
<th>Total Density (cells L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>3.5</td>
<td>1.6 \times 10^8</td>
<td>2.1 \times 10^6</td>
<td>1.6 \times 10^8</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>19.8</td>
<td>4.5 \times 10^8</td>
<td>4.6 \times 10^7</td>
<td>4.5 \times 10^8</td>
<td></td>
</tr>
<tr>
<td>25%</td>
<td>1.8</td>
<td>1.1 \times 10^8</td>
<td>1.0 \times 10^6</td>
<td>1.1 \times 10^8</td>
<td></td>
</tr>
<tr>
<td>75%</td>
<td>6.3</td>
<td>2.2 \times 10^8</td>
<td>7.1 \times 10^6</td>
<td>2.2 \times 10^8</td>
<td></td>
</tr>
</tbody>
</table>
cells L$^{-1}$ as lakes having high cyanobacteria and $<5.0 \times 10^6$ cells L$^{-1}$ as lakes having low cyanobacteria.

### 3.3.2 Nutrient ranges during peak biomass

Lake chemical characteristics are presented in Table 3.3. TP in the study lakes ranged from 2.2 to 37.4 µg L$^{-1}$; soluble reactive P (SRP) was not detected in any lake samples as concentrations were below MDL at the time of sampling. TN ranged from 230 to 547 µg L$^{-1}$; the majority of TN was organic N (95%), with nitrate being the primary form of inorganic N (5%). Nitrate ranged from 1.8 to 312 µg L$^{-1}$ and ammonium ranged from 3.0 to 64.0 µg L$^{-1}$. The inorganic N sample bottle for Mystery Lake was lost in transit, thus $N = 24$ for nitrate and ammonium. TDFe ranged from 0.4 to 151 µg L$^{-1}$. The pH in lakes ranged from 6.1 to 8.0 during peak biomass.

### 3.3.3 Phytoplankton and nutrients during peak biomass

TP was positively correlated total phytoplankton biomass, as estimated by chl-$a$ ($r^2 = 0.83$, $P < 0.001$) (Fig. 3.1). Cyanobacteria had highest density when total phytoplankton biomass was relatively low, and lowest density when total phytoplankton biomass was relatively high (Fig. 3.2a). In contrast, eukaryotes had highest density when total phytoplankton biomass was relatively high and lowest density when total phytoplankton biomass was relatively low (Fig. 3.2b). Nutrient conditions that occurred when cyanobacterial density was highest included low concentrations of TP (Fig. 3.3a), variable (low and high) nitrate (Fig. 3.3b) and low TDFe (Fig. 3.3c). Regression tree multivariate analysis of lake nutrient concentrations, siderophore concentrations and cyanobacterial density indicated non-overlapping chemical determinants of cyanobacterial density (TDFe) and total phytoplankton biomass, using chl-$a$ as a proxy (TP) (Fig. 3.4a and 3.4b).

Pearson correlation analysis for lake nutrient concentrations, siderophore concentrations and cyanobacterial density in lakes with TDFe concentration $<3.2$ µg L$^{-1}$ showed that cyanobacterial density was positively (Pearson $R = 0.88$, $P < 0.05$; Table 3.4) and linearly ($r^2 = 0.77$, $P = 0.01$) correlated to the concentration of hydroxamate siderophores.
Table 3.3 Lake chemical characteristics – median, range, 25\textsuperscript{th} and 75\textsuperscript{th} percentile for measured chemical characteristics of lakes in the Algoma Highlands of central Ontario.

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>DOM (µg L\textsuperscript{-1})</th>
<th>TP (µg L\textsuperscript{-1})</th>
<th>TN (µg L\textsuperscript{-1})</th>
<th>NO\textsubscript{3}\textsuperscript{-} (µg L\textsuperscript{-1})</th>
<th>NH\textsubscript{4}\textsuperscript{+} (µg L\textsuperscript{-1})</th>
<th>TDFe (µg L\textsuperscript{-1})</th>
<th>Hydroxamate (µg L\textsuperscript{-1})</th>
<th>Catecholate (µg L\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>7.4</td>
<td>3075.6</td>
<td>8.9</td>
<td>364.6</td>
<td>4.0</td>
<td>8.0</td>
<td>7.7</td>
<td>111.3</td>
<td>4109.3</td>
</tr>
<tr>
<td>Range</td>
<td>1.9</td>
<td>19117.9</td>
<td>35.2</td>
<td>316.8</td>
<td>310.3</td>
<td>61.1</td>
<td>151.1</td>
<td>825.7</td>
<td>6172.8</td>
</tr>
<tr>
<td>25%</td>
<td>7.3</td>
<td>2318.7</td>
<td>5.4</td>
<td>305.3</td>
<td>1.8</td>
<td>3.0</td>
<td>3.2</td>
<td>0.0</td>
<td>3782.2</td>
</tr>
<tr>
<td>75%</td>
<td>7.5</td>
<td>4666.8</td>
<td>15.0</td>
<td>426.0</td>
<td>43.5</td>
<td>12.0</td>
<td>35.8</td>
<td>327</td>
<td>5279.0</td>
</tr>
</tbody>
</table>
Figure 3.1 Total phosphorus versus phytoplankton biomass, using chl-α as a proxy, during peak biomass in lakes in the Algoma Highlands of central Ontario. Vertical lines represent trophic status definitions based on TP for ultra-oligotrophic (TP = 0 to 8 μg L⁻¹), oligotrophic (TP = 8 to 26.7 μg L⁻¹) and mesotrophic (TP = 26.7 to 84.4 μg L⁻¹) lakes according to Wetzel (2001). By removing the mesotrophic study lake, the significant linear relationship between TP and phytoplankton biomass holds true ($r^2 = 0.65$, $P < 0.001$).
Figure 3.2 Chlorophyll-α concentration versus (a) cyanobacterial and (b) eukaryote density during peak biomass in lakes in the Algoma Highlands of central Ontario.
Figure 3.3 (a) Total phosphorus (b) nitrate and (c) total dissolved iron concentration versus cyanobacterial density during peak biomass in lakes in the Algoma Highlands of central Ontario. Three study lakes (solid circle) deviated from the hypothesized trend having high cyanobacterial density and high nitrate concentration. Cyanobacterial communities in these lakes were compared to three study lakes (dashed circle) with high cyanobacterial density and low nitrate concentration. Black-filled data points are lakes with high cyanobacterial density (>5.0 × 10^6 cells L^-1); non-filled data points are lakes with low cyanobacterial density (<5.0 × 10^6 cells L^-1). Dashed horizontal line represents the separation point in lakes with high and low cyanobacterial density.
Figure 3.4 Regression trees depicting the chemical determinants of (a) phytoplankton biomass, using chlorophyll-\(a\) as a proxy, and (b) cyanobacterial density in lakes in the Algoma Highlands of central Ontario.

Chlorophyll-\(a\)

(a) \(< 12.2 \mu g \text{ L}^{-1}\)  TP  \(\geq 12.2 \mu g \text{ L}^{-1}\)

2.3 \(\mu g \text{ L}^{-1}\)  \(n = 15\)

8.9 \(\mu g \text{ L}^{-1}\)  \(n = 10\)

Cyanobacterial Density

(b) \(\geq 3.2 \mu g \text{ L}^{-1}\)  TDFe  \(< 3.2 \mu g \text{ L}^{-1}\)

2.0 \times 10^7 \text{ cells L}^{-1}\)  \(n = 7\)

2.7 \times 10^6 \text{ cells L}^{-1}\)  \(n = 18\)
Table 3.4 Pearson correlation matrix for nutrients, siderophores and cyanobacterial density in lakes with <3.2 μg L\(^{-1}\) TDFe (critical α = 0.05) in the Algoma Highlands of central Ontario. Hydroxamate siderophore concentration was positively and significantly correlated to cyanobacterial density. Data are presented as Pearson R (sample n). Positive Pearson R indicates a positive linear relationship between variables and a negative Pearson R indicates a negative linear relationship.

<table>
<thead>
<tr>
<th></th>
<th>DOC</th>
<th>TN</th>
<th>TP</th>
<th>TN/TP</th>
<th>NO(_3)</th>
<th>NH(_4^+)</th>
<th>TDFe</th>
<th>Hydroxamate</th>
<th>Catecholate</th>
<th>Cyanobacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>0.48</td>
<td>0.67</td>
<td>0.29</td>
<td>0.09</td>
<td>0.06</td>
<td>0.44</td>
<td>0.09</td>
<td>-0.61</td>
<td>0.24</td>
<td>-0.58</td>
</tr>
<tr>
<td>DOC</td>
<td><strong>0.83</strong></td>
<td>0.71</td>
<td>-0.38</td>
<td>-0.54</td>
<td>0.16</td>
<td>-0.53</td>
<td>-0.06</td>
<td>0.08</td>
<td>-0.24</td>
<td></td>
</tr>
<tr>
<td>TN</td>
<td><strong>0.79</strong></td>
<td></td>
<td>-0.16</td>
<td>0.26</td>
<td>-0.59</td>
<td>-0.31</td>
<td>0.07</td>
<td>-0.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TP</td>
<td>-0.84</td>
<td>0.04</td>
<td>-0.04</td>
<td><strong>-0.84</strong></td>
<td>0.18</td>
<td>-0.20</td>
<td>-0.27</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>TN/TP</td>
<td>-0.16</td>
<td>0.30</td>
<td>0.69</td>
<td>0.04</td>
<td>0.37</td>
<td>0.07</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO(_3)</td>
<td></td>
<td></td>
<td></td>
<td>0.21</td>
<td>-0.11</td>
<td>-0.46</td>
<td>-0.30</td>
<td></td>
<td>-0.11</td>
<td></td>
</tr>
<tr>
<td>NH(_4^+)</td>
<td></td>
<td></td>
<td></td>
<td><strong>-0.01</strong></td>
<td><strong>-0.76</strong></td>
<td><strong>-0.48</strong></td>
<td><strong>-0.60</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TDFe</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>0.88</strong></td>
</tr>
<tr>
<td>Hydroxamate</td>
<td><strong>0.57</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>0.52</strong></td>
<td></td>
</tr>
<tr>
<td>Catecholate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Pearson R values in bold indicate a statistically significant relationship between variables (P < 0.05) and italicized Pearson R indicates no significant relationship.
In lakes with TDFe concentration ≥3.2 μg L⁻¹, cyanobacterial density was positively (Pearson R = 0.92 and 0.86, \( P < 0.05 \); Table 3.5) and linearly (\( r^2 = 0.84 \) and 0.75, \( P < 0.001 \)) correlated to nitrate and ammonium concentrations respectively.

### 3.3.4 Dissolved organic matter

DOM ranged from c. 450 to 19600 μg L⁻¹. DOM composition was variable among the study lakes. EEMs revealed signatures characteristic of DOM with humic- and protein-like properties. HIX values ranged from 2.4 to 19.1, with a median value of 5.8 among all lakes.

### 3.3.5 Siderophores

Lakes with both high (\( >5.0 \times 10^6 \) cells L⁻¹) and low (\( <5.0 \times 10^6 \) cells L⁻¹) cyanobacterial density had hydroxamate and/or catecholate siderophores (Fig. 3.5a and 3.5b). Hydroxamate siderophore concentration in lakes ranged from 0 to 825 μg L⁻¹. Catecholate siderophore concentration in lakes ranged from 2455 to 8628 μg L⁻¹ (Table 3.3). Cyanobacterial density in lakes was highest when DOM concentration was relatively low (Fig. 3.6a) and when DOM HIX was relatively low (Fig. 3.6b). Catecholate siderophore concentration was positively correlated to DOM concentration (\( r^2 = 0.65, \ P < 0.001 \)), in particular the refractory component of DOM as indicated by HIX (\( r^2 = 0.72, \ P < 0.001 \)). No relationship was found between hydroxamate siderophore concentration and either DOM concentration or DOM HIX.

### 3.3.6 Cyanobacterial community composition and nitrogen use

The three study lakes with high cyanobacterial density and lowest nitrate concentration had cyanobacterial communities comprised of *Anabaena sp.* and *Microcystis sp.* (Table 3.6; Fig. 3.3b). The three study lakes with high cyanobacteria and highest nitrate concentration had cyanobacterial communities comprised of *Anabaena sp.*, *Gloecapsa sp.*, *Microcystis sp.* and *Nostoc sp.* (Table 3.6; Fig. 3.3b). The *Anabaena sp.* observed in the high cyanobacteria and lowest nitrate lakes had visible heterocysts while the *Anabaena sp.* and *Nostoc sp.* in the high cyanobacteria and highest nitrate lakes did not have visible heterocysts.
Table 3.5 Pearson correlation matrix for nutrients, siderophores and cyanobacterial density in lakes with $\geq 3.2 \mu g L^{-1}$ TDFe (critical $\alpha = 0.05$) in the Algoma Highlands of central Ontario. Inorganic N (nitrate and ammonium) was positively and significantly correlated to cyanobacterial density. Data are presented as Pearson $R$ (sample $n$). Positive Pearson $R$ indicates a positive linear relationship between variables and a negative Pearson $R$ indicates a negative linear relationship.

<table>
<thead>
<tr>
<th></th>
<th>DOC</th>
<th>TN</th>
<th>TP</th>
<th>TN/TP</th>
<th>NO$_3^-$</th>
<th>NH$_4^+$</th>
<th>TDFe</th>
<th>Hydroxamate</th>
<th>Catecholate</th>
<th>Cyanobacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>-0.72 (18)</td>
<td>-0.55 (18)</td>
<td>-0.01 (18)</td>
<td>0.01 (18)</td>
<td>0.14 (17)</td>
<td>-0.06 (17)</td>
<td>-0.56 (18)</td>
<td>0.22 (18)</td>
<td>-0.64 (18)</td>
<td>0.11 (18)</td>
</tr>
<tr>
<td>DOC</td>
<td>0.69 (18)</td>
<td>0.15 (18)</td>
<td>-0.23 (18)</td>
<td>-0.32 (17)</td>
<td>-0.17 (17)</td>
<td>0.64 (18)</td>
<td>0.22 (18)</td>
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<td>-0.15 (18)</td>
</tr>
<tr>
<td>TN</td>
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<td>0.00 (18)</td>
<td>-0.11 (17)</td>
<td>-0.01 (17)</td>
<td>0.34 (18)</td>
<td>0.23 (18)</td>
<td>0.54 (18)</td>
<td>-0.07 (18)</td>
<td>-0.40 (18)</td>
<td>-0.40 (18)</td>
</tr>
<tr>
<td>TP</td>
<td>-0.68 (18)</td>
<td>-0.47 (17)</td>
<td>-0.35 (17)</td>
<td>0.08 (18)</td>
<td>0.07 (18)</td>
<td>0.13 (18)</td>
<td>-0.40 (18)</td>
<td>-0.40 (18)</td>
<td>-0.40 (18)</td>
<td>-0.40 (18)</td>
</tr>
<tr>
<td>TN/TP</td>
<td>0.60 (17)</td>
<td>0.17 (17)</td>
<td>-0.20 (18)</td>
<td>-0.21 (18)</td>
<td>-0.30 (18)</td>
<td>0.40 (18)</td>
<td>0.40 (18)</td>
<td>0.40 (18)</td>
<td>0.40 (18)</td>
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</tr>
<tr>
<td>NO$_3^-$</td>
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<td>-0.17 (17)</td>
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<td>-0.38 (17)</td>
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<td>0.86 (17)</td>
<td>0.86 (17)</td>
<td>0.86 (17)</td>
<td>0.86 (17)</td>
</tr>
<tr>
<td>NH$_4^+$</td>
<td>-0.23 (17)</td>
<td>-0.20 (17)</td>
<td>-0.25 (17)</td>
<td>-0.25 (17)</td>
<td>-0.25 (17)</td>
<td>0.86 (17)</td>
<td>0.86 (17)</td>
<td>0.86 (17)</td>
<td>0.86 (17)</td>
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</tr>
<tr>
<td>TDFe</td>
<td>0.14 (18)</td>
<td>0.75 (18)</td>
<td>-0.19 (18)</td>
<td>-0.19 (18)</td>
<td>-0.19 (18)</td>
<td>0.86 (17)</td>
<td>0.86 (17)</td>
<td>0.86 (17)</td>
<td>0.86 (17)</td>
<td>0.86 (17)</td>
</tr>
<tr>
<td>Hydroxamate</td>
<td>0.26 (18)</td>
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<td>-0.10 (18)</td>
<td>-0.10 (18)</td>
<td>-0.10 (18)</td>
<td>0.86 (17)</td>
<td>0.86 (17)</td>
<td>0.86 (17)</td>
<td>0.86 (17)</td>
<td>0.86 (17)</td>
</tr>
<tr>
<td>Catecholate</td>
<td>-0.22 (18)</td>
<td>-0.22 (18)</td>
<td>-0.22 (18)</td>
<td>-0.22 (18)</td>
<td>-0.22 (18)</td>
<td>0.86 (17)</td>
<td>0.86 (17)</td>
<td>0.86 (17)</td>
<td>0.86 (17)</td>
<td>0.86 (17)</td>
</tr>
</tbody>
</table>

Pearson $R$ values in bold indicate a statistically significant relationship between variables ($P < 0.05$) and italicized Pearson $R$ indicates no significant relationship.
Figure 3.5 (a) Hydroxamate and (b) catecholate siderophore concentration versus cyanobacterial density during peak biomass in lakes in the Algoma Highlands of central Ontario. Black-filled data points are lakes with high cyanobacterial density (>5.0 × 10^6 cells L⁻¹); non-filled data points are lakes with low cyanobacterial density (<5.0 × 10^6 cells L⁻¹). Dashed horizontal line represents the separation point in lakes with high and low cyanobacterial density.
Figure 3.6 (a) Hydroxamate and (b) catecholate siderophore concentration versus cyanobacterial density during peak biomass in lakes in the Algoma Highlands of central Ontario. Black-filled data points are lakes with high cyanobacterial density (>5.0 × 10^6 cells L^-1); non-filled data points are lakes with low cyanobacterial density (<5.0 × 10^6 cells L^-1). Dashed horizontal line represents the separation point in lakes with high and low cyanobacterial density.
Table 3.6 Cyanobacterial community composition in three lakes with high cyanobacterial density and low nitrate concentrations and three lakes with high cyanobacterial density and highest nitrate concentration in the Algoma Highlands of central Ontario. High cyanobacteria and low nitrate lakes had N₂-fixing cyanobacterial genera with heterocysts while the high cyanobacteria and high nitrate lakes had non-N₂-fixing cyanobacterial genera or those with no heterocysts.

<table>
<thead>
<tr>
<th>Lake</th>
<th>Cyanobacterial Density ($\times 10^6$ cells L⁻¹)</th>
<th>Nitrate (μg L⁻¹)</th>
<th>Genera Present</th>
<th>% Cyanobacteria (by density)</th>
<th>Heterocysts Observed</th>
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n.a. = Cyanobacterial genera that do not produce heterocysts for N₂ fixation.
3.4 Discussion

3.4.1 Phytoplankton and nutrients during peak biomass

We investigated nutrient regimes that promote cyanobacterial growth in lakes with low macronutrient supply. According to Wetzel’s (2001) definition of trophic status that is based on TP, 12 of the 25 study lakes were ultra-oligotrophic, 12 were oligotrophic, and one was mesotrophic. We found that TP limited phytoplankton biomass within and across lakes of various trophic status as indicated by the correlation between TP and chl-a concentration ($r^2 = 0.83$, $P < 0.001$). The relationship between TP and phytoplankton biomass has been well supported in previous research (e.g., Schindler, 1978; Downing et al., 2001). Although TP served as a relatively strong predictor variable, explaining 83% of phytoplankton biomass, there was considerable variation in the achieved phytoplankton biomass level for any given concentration of TP. A possible explanation for this observed variability is that Fe may be limiting the conversion of P to biomass by binding P and thereby restricting phytoplankton access to the entire P pool in aerobic surface waters (Moore & Reddy, 1994). In other words, Fe may be a regulator of macronutrient (P) use efficiency.

We investigated the relationship between chl-a concentration and phytoplankton density to establish whether chl-a predicted the dominance of cyanobacteria or eukaryotes in lakes. We found that cyanobacterial density was highest when chl-a concentration was low and eukaryote density was highest when chl-a concentration was high. Watson, McCauley & Downing (1997) found that in temperate oligotrophic lakes, phytoplankton biomass and species diversity is low and cyanobacteria can dominate the picoplankton community, but with nutrient enrichment, phytoplankton species diversity increases. These observations support our understanding of the competitive advantage that cyanobacteria have over other phytoplankton for nutrients in oligotrophic lakes with low N and P supply (Downing et al., 2001).

The competitive advantage of cyanobacteria under low macronutrient conditions is further supported when we investigated the relationships between TP, nitrate and TDFe versus cyanobacterial density in the lakes. TP was chosen since it was the only
measurable P form in lakes (SRP below MDL in all lakes) and nitrate was chosen because of the role of Fe in nitrate assimilation by phytoplankton (NtR activity). We found that cyanobacterial density was highest when TP concentration was low and when nitrate concentration was variably low and high. The relationship between TP versus cyanobacterial density was expected since cyanobacteria have more efficient P transport affinity and uptake systems compared to eukaryotes (Molot & Brown, 1986; Mur et al., 1999), particularly at low P concentrations, resulting in selection for cyanobacteria. Similarly, study lakes with the lowest nitrate concentration and highest cyanobacterial density were expected since N-limited lakes favor the dominance of N2-fixing cyanobacteria that compensate for the reduced N loading by fixing atmospheric N2 (Schindler et al., 2008). However, three study lakes with the highest nitrate concentration also had relatively high cyanobacteria density, which was an unexpected finding. These three study lakes were also found to have the lowest Fe concentrations among all study lakes (median TDFe concentration = 3.5 μg L⁻¹).

Among all study lakes, cyanobacterial density was highest when TDFe concentration was low. This finding is consistent with laboratory (Kerry et al., 1988) and lake (Sorichetti et al., 2013) research showing that cyanobacteria are competitive over eukaryotes at low Fe concentrations. Previous research has attributed this competitive advantage to the ability for cyanobacteria to use Fe-binding ligands, hydroxamate and catecholate siderophores, when Fe-limited. The use of siderophores by cyanobacteria is well documented in laboratory studies (Kerry et al., 1988; Wilhelm & Trick, 1994; Wilhelm, Maxwell & Trick, 1996; Wilhelm, MacCauley & Trick, 1998) and in marine environments (Barbeau et al., 2003; Eldridge et al., 2004). However, few field studies have been conducted in freshwater environments (e.g., Murphy et al., 1976).

3.4.2 Siderophores in the study lakes during peak biomass

This study represents the first documented record of siderophores in lakes in oligotrophic lakes on the Canadian Shield to the best of our knowledge. We found measurable concentrations of hydroxamate and/or catecholate siderophores in all lakes. The presence of siderophores in lakes may be considered a ‘fingerprint’ of water chemistry that indicates Fe limitation. A linear relationship between siderophore concentration and
cyanobacterial biomass should not be expected since siderophores are not drawn down, but can rather be re-used for additional Fe scavenging (Neilands, 1995). However, the variability observed in the capacity for cyanobacteria to achieve high levels of biomass in the presence of siderophores suggests that there may be additional factors regulating the bioavailability of Fe to cyanobacteria.

Regression tree analysis clearly documented the factor primarily correlating to relatively high cyanobacterial density in lakes was the capacity for cyanobacteria to thrive in low TDFe environments. In lakes with low TDFe concentrations (<3.2 μg L\(^{-1}\)), cyanobacterial density was significantly correlated to the concentration of hydroxamate siderophores (\(r^2 = 0.77, P = 0.01\)). This provides evidence that cyanobacteria use hydroxamate siderophores in lakes to scavenge Fe and overcome Fe limitation. In lakes with relatively higher TDFe concentrations (≥3.2 μg L\(^{-1}\)), cyanobacterial density was significantly correlated to nitrate (\(r^2 = 0.84, P < 0.001\)) and ammonium (\(r^2 = 0.75, P < 0.001\)) concentrations. With Fe required for NtR activity, there may have been sufficient Fe for nitrate assimilation by all phytoplankton in lakes with relatively higher TDFe concentrations and so the supply of inorganic N may have then limited cyanobacterial biomass.

### 3.4.3 Potential influence of DOM on Fe bioavailability

We investigated whether light limitation could explain why highest cyanobacterial density occurred in lakes with low DOM concentration. Our metric of light in lakes was Secchi depth and we found no relationship with cyanobacterial density (Sorichetti, Creed & Trick, unpublished data). We attributed this lack of a relationship to some species of cyanobacteria having the potential to regulate their buoyancy in the vertical water column for positioning to obtain optimal light levels. This suggests that DOM influences cyanobacterial biomass by some other process.

DOM can be considered a natural Fe-binding ligand when refractory due to its humic acid composition having high affinity for metal ions (Baken et al., 2011). DOM also has the potential to bind organic molecules such as siderophores directly or indirectly through mixed-ligand complexation with bound Fe (Chen & Wang, 2008). Lakes with high
cyanobacterial density had relatively low DOM concentration. This finding indicates that lakes with large DOM concentrations may have a greater potential to tightly bind Fe, thereby limiting Fe scavenging potential and cyanobacterial proliferation. In contrast, lakes with lower DOM concentrations may have a lower potential to tightly bind Fe making it more bioavailable to cyanobacteria.

Not only the quantity but also the quality of DOM may have influenced the bioavailability of Fe to cyanobacteria. Lakes with high cyanobacterial density had DOM with labile properties (lower HIX). These findings support our hypothesis that lakes with DOM that has refractory properties (higher HIX) have a higher binding capacity for Fe, limiting the bioavailability of Fe to cyanobacteria. In contrast, lakes with DOM that has labile properties may have a lower potential to bind Fe or will bind weakly allowing cyanobacteria to scavenge Fe from DOM complexes.

Catecholate siderophore concentration was significantly correlated to DOM concentration ($r^2 = 0.65$, $P < 0.001$) and HIX ($r^2 = 0.72$, $P < 0.001$) while no such relationships were found with hydroxamate siderophore concentration. Additionally, catecholate siderophore concentrations in lakes were over 30× higher than hydroxamate siderophore concentrations. We are unaware of studies available to compare the measured magnitude of catecholate siderophore concentration in lakes. It may be possible that the method used for quantitative analysis of catecholate siderophores (Arnow, 1937) measured phenolic groups of aromatic compounds on DOM complexes. Previous research has shown that DOM in freshwater lakes is often comprised of aromatic compounds with phenolic and benzene ring structures (Gondar et al., 2005), which are the target compounds in catechol colorimetric analysis (Arnow, 1937).

3.4.4 Evidence for iron regulation of nitrogen use efficiency

We investigated patterns and correlations that may point to mechanisms that explain high cyanobacterial density in lakes with low and high nitrate concentration. We compared the cyanobacterial community composition in three lakes with highest nitrate concentrations (median = 270 μg L$^{-1}$) to three lakes with lowest nitrate concentrations (median = 1.8 μg L$^{-1}$). Both groups of lakes had relatively high cyanobacterial density, detectable
hydroxamate and catecholate siderophore concentrations and the lowest TDFe concentrations among all lakes. The differences in nitrate concentration among lakes may have been due to differences in the ability for phytoplankton to draw down nitrate while Fe-limited. Study lakes with low nitrate and high cyanobacterial density had communities comprised of *Anabaena sp.* (heterocysts present) and *Microcystis sp.* Study lakes with high nitrate and high cyanobacterial density had communities comprised of *Anabaena sp.*, *Gloeocapsa sp.*, *Microcystis sp.* and *Nostoc sp.* with no visible heterocysts. Since the heterocyst is the site of N\(_2\) fixation in cyanobacteria (Stewart, Rowell & Telor, 1975), cells with heterocysts are considered to be N-limited and fix N\(_2\) as a primary N source when present. This suggests that in low nitrate lakes, siderophores were used to scavenge Fe for N\(_2\) fixation (NiR activity), whereas in high nitrate lakes, siderophores were used to scavenge Fe for N assimilation (NtR activity). The presence of heterocyst N\(_2\)-fixers versus non-fixers in the cyanobacterial community supports this suggestion. These findings provide evidence that Fe may be a regulator of macronutrient (N) use efficiency.

### 3.4.5 A new conceptual model

DOM appears to have a critical role in regulating Fe supply to cyanobacteria and there are several possible, but presently untested, scenarios describing this potential influence (Fig. 3.7). DOM may serve as a ligand, biding Fe directly, with binding capacity dependent on the quality of DOM (labile or refractory) in lakes and ranging from a ligand that has weak to strong Fe-binding affinities. DOM may additionally have the capacity to bind siderophores directly or indirectly through mixed-ligand complexation with bound Fe, which would render the bound Fe not readily bioavailable to cyanobacteria and limit Fe scavenging. The number of active binding sites on DOM could therefore determine the magnitude in which DOM binds Fe directly, and siderophores directly or indirectly, limiting Fe bioavailability to cyanobacteria.

We found that cyanobacteria thrived in lakes with a low Fe supply. Siderophores provide cyanobacteria a strategy for Fe scavenging when Fe-limited. However, the bioavailability of siderophores to cyanobacteria appear to be regulated by DOM quantity and quality and therefore must be incorporated into any conceptual model considering Fe and
Figure 3.7 Conceptual model for macronutrient (P and N) assimilation by cyanobacteria with the influence of DOM and siderophores on Fe bioavailability. Thickness of the feedback lines indicates the strength of potential binding between DOM, hydroxamate siderophores or catecholate siderophores with Fe. Black circles represent enzymatic processes in which Fe is involved for nutrient assimilation by nitrate reductase (NtR), nitrogenase (NiF) and ferric reductase (FeR).
macronutrient (N and P) uptake mechanisms (Fig. 3.7). We suggest that cyanobacteria use hydroxamate siderophores as a means to scavenge Fe in lakes where DOM has labile properties. An improved understanding of the potential for DOM to bind siderophores and Fe will improve our ability to predict cyanoHAB formation in lakes with a relatively low macronutrient supply. Future research is needed on factors that influence DOM properties and the conditions under which it serves as a siderophore and/or Fe source versus sink to phytoplankton in lakes.
3.5 References


4 Suitability of a cytotoxicity assay for detection of potentially harmful compounds produced by freshwater bloom-forming phytoplankton

This chapter was published in *Harmful Algae* (Sorichetti et al., 2014).

4.1 Introduction

Algal blooms are marked by significant increases in the population of pelagic algae resulting in the aesthetic, odorous and/or biochemical fouling of surface waters (Reynolds and Walsby, 1975). Harmful algal blooms (HABs) are a sub-category of these events with most being distinguished by the presence of particular taxa at high levels of biomass that have the ability to produce toxins, irritants and/or noxious secondary metabolites in addition to other harmful effects including fish gill clogging, shading light access at depths and creating anoxic waters (Carmichael, 1992, 2001; Watson, 2003). Freshwater HABs can have serious ecological, toxicological and physiological effects on aquatic and terrestrial biota and increased occurrence of these events in North American over the past three decades poses cause for concern (Skulberg et al., 1984; Falconer, 1999; Carmichael, 2001, 2008; Paerl et al., 2001; Winter et al., 2011; Huber et al., 2012).

Blooms of high biomass are commonly attributed to excess nutrients entering waterbodies (Heisler et al., 2008; Paerl and Huisman, 2008). HABs are formed when these nutrients are preferentially assimilated by harmful algal species that accumulate in biomass (Reynolds and Walsby, 1975; Paerl, 1988). Despite the multiple factors that promote HAB formation, accelerated eutrophication of surface waters by human inputs is the leading cause of HABs in freshwater systems (Schindler, 1987; Smith, 2003). Macronutrient loading of nitrogen (N) and phosphorus (P) from atmospheric and terrestrial sources is of particular concern and have been implicated as key nutrients for bloom development (Schindler, 1977; Guildford and Hecky, 2000).

Additional work has illustrated the importance of micronutrients in algal growth (Klausmeier et al., 2004; Molot et al., 2010; Fujii et al., 2011; Sorichetti et al., 2013). Iron (Fe) has been
identified as a critical micronutrient for bloom development and maintenance because it is required for essential cellular processes such as photosynthesis, pigment biosynthesis and (in some cyanobacteria) nitrogen fixation (Guikema and Sherman, 1983; Raven et al., 1999; Sterner et al., 2004). Fe has also been identified as a regulator of microcystin production, a potent cyanobacterial hepatotoxin (Kaplan et al., 2012). However, unlike most cellular functions, the production of microcystin increases when cyanobacteria are Fe-limited (Utkilen and Gjølme, 1995; Alexova et al., 2011; Kaplan et al., 2012). The role of Fe in chrysophyte noxious compound production is unknown.

Cyanobacteria are the most common group of algae associated with freshwater HABs. The most common genera in lakes include: *Microcystis, Anabaena, Aphanizomenon* and *Gloeotrichia*, all of which have the potential to produce toxins, noxious and/or bioactive compounds (Carmichael, 2001; Paerl et al., 2001; Watson et al., 2008; Molot et al., 2010; Winter et al., 2011). The incidence of blooms dominated by chrysophyte algae may also be increasing in North America (Winter et al., 2011). Chrysophyte genera that are commonly observed dominating bloom events in lakes include: *Dinobryon, Synura, Uroglena* and *Mallomonas* (Nicholls, 1995; Watson, 2003; Paterson et al., 2004, 2008). While chrysophyte algae common to freshwaters do not produce toxins, they are infamous for their ability to produce a variety of noxious compounds that act as irritants or offensive taste and odor causing substances (Jüttner et al., 1986; Nicholls, 1995; Watson et al., 2008; Paterson et al., 2004).

This study addresses the need for quick, reliable and cost effective tools to detect and evaluate the cytotoxicity of freshwater samples that may be contaminated with toxic, noxious and/or bioactive algal metabolites. In 2008, Environment Canada released a report addressing the increase in HAB occurrence (Charlton et al., 2008). Three important research needs were outlined in this report, including: improving detection, characterization and modeling of toxic and noxious algal metabolites produced during HABs (Charlton et al., 2008). Recent research has investigated the ability of mammalian cell lines to detect the cytotoxic effects of compounds produced by marine algal isolates (Dorantes-Aranda et al., 2011) and this work has illustrated the potential for the adaptation of these assays to detect compounds produced by freshwater algae (Burkholder et al., 2005; Dorantes-Aranda et al., 2011). Adapting existing
cell-based assays to quantify the toxicity of natural lake water samples is one way to improve detection and may offer a solution to the first step in the line of research needs. The low volume of sample required for analysis, the large number of samples that can be tested, the rapid exposure time, ease of sample preparation and avoiding the sacrifice of whole organisms are the main advantages of cell-based assays (Dayeh et al., 2005).

A cell-based assay of particular interest is the rainbow trout gill (RTgill-W1) cytotoxicity assay (RCA) that has been used in conjunction with various in vitro and in vivo methods of analysis to serve as a proxy and assess the effects of a wide range of environmental pollutants and toxicants on aquatic organisms such as fish and invertebrates (Lee et al., 2009). The RCA considers viability of cell metabolism using the reducing environment of cells to measure changes in cellular metabolic activity. Reduction in the rezasurin-containing fluorescent dye PrestoBlue™ is measured spectrofluorometrically as recovery from suspected cellular metabolic impairment can be evaluated by applying this dye to living cell lines. A decline in PrestoBlue™ reduction is thus indicative of cellular metabolic impairment. The adaptation of the RCA for investigations into polycyclic aromatic hydrocarbons, industrial effluents, petrochemicals, jellyfish venom and many other compounds occurring in both freshwater and marine systems has made it a prominent candidate for further use in freshwater research (Schirmer et al., 1998, 2001; Dayeh et al., 2005; Helmholz et al., 2010).

In this study, the effectiveness of the RCA is assessed by applying it to natural lake water samples, analytical standards of toxins and noxious compounds produced by freshwater algae and analysis of complex mixtures of metabolites produced by cultured freshwater algae, including both toxic and/or noxious compound-producing cyanobacteria and chrysophyte algae. To the best of our knowledge, this study represents the first application of the RCA using algal toxins and noxious compounds with related studies focusing primarily on the cellular effects of microcystins on fish liver cell lines (Boaru et al., 2006). A fish cell based bioassay is appropriate for this investigation because the toxic, noxious and bioactive compounds produced by cyanobacteria and chrysophytes are known to affect growth rate, modify behavior and exert histopathological effects in the liver, intestine, kidneys, heart, spleen and gills of fish and other aquatic organisms (Malbrouck and Kestemont, 2006).
The following hypotheses are tested:

\( H_1 \): The RCA is effective in assessing cytotoxicity in natural lake water samples collected as a part of a routine water quality sampling strategy.

\( H_2 \): The application of analytical standards of toxins and/or noxious compounds produced by freshwater algae will result in a concentration dependent decrease in the viability of cells in the RCA.

\( H_3 \): The application of complex mixtures of algal metabolites extracted from cultures of toxic and/or noxious compound-producing cyanobacteria and chrysophyte algae will result in a concentration dependent decrease in viability as measured by the RCA where: (a) exposure solutions derived from exponential growth phases will result in significant reductions in cell viability relative to those of stationary growth phase; (b) exposure to solutions derived from lysed cultures (intracellular + extracellular compounds) will result in significant reductions in cell viability relative to solutions derived from non-lysed samples (extracellular compounds only); and (c) exposure solutions extracted from low Fe treatments (0.1 μM) will result in significant reductions in cell viability in relative to the high Fe treatments (10 μM).

Adapting the RCA to detect toxins, noxious and/or bioactive compounds produced by cyanobacteria and chrysophytes will provide insight into the additive, synergistic and/or antagonistic biological effects that these compounds may exhibit while produced in complex mixtures by various cyanobacteria and chrysophyte algae (Dayeh et al., 2005).

4.2 Methods

4.2.1 Development of cytotoxicity assays

RTgill-W1 cells were obtained from the American Type Culture Collection (ATCC CRL-2523) (Bols et al., 1994). The cell line culture was maintained in the dark at 20°C in sterile, plug sealed, tissue culture treated flasks containing a sterile hydrophilic surface that promotes cell attachment on the inside walls of the flask.
Cells were cryopreserved by suspending cell solutions in L-15 Complete-5% (v/v) dimethylsulfoxide (DMSO) medium at a density of 10^6 cells mL^{-1}. Aliquots (1 mL) were pipetted into 2 mL polypropylene Cryule® vials (985746, Wheaton) and immediately sealed. A -1°C minute^{-1} ‘Mr. Frosty’ freezing container (5100-0001, Thermo Fischer Scientific Inc.) filled with isopropyl alcohol was used to hold the vials and was immediately placed into a -80°C freezer. Cells remained viable in the freezer for a minimum of one year following initial freezing, as evidenced by successful culturing of cryopreserved cells. The absence of cell culture contamination by mycoplasma was confirmed through the use of a MycoAlert™ assay kit (LT07-118, Lonza) every two to three weeks. A 1 mL aliquot taken from the supernatant after the cell suspension was centrifuged at 200 × g for five minutes. The sample was combined with buffer solution and pipetted into a 96-micro well plate (MWP) in triplicate. Positive and negative control solutions were loaded into the plate and all wells were analyzed with the Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, Santa Clara, CA) set to read chemi/bio-luminescence from 540 nm to 700 nm.

All solutions and equipment were sterilized via autoclave, 70% ethanol, or 0.2 μm filtration prior to use and handling occurred in a laminar flow hood with the appropriate aseptic techniques employed to maintain the sterility of the culture. Small culture flasks (25 cm^2) were used for initial sub-culturing from cryopreserved samples and large culture flasks (75 cm^2) were used for the subsequent sub-cultures (353109/353110, BD Biosciences). This sub-culturing sequence ensured that an ample volume of cells at the appropriate density was available for use in multiple assays. Cells were grown in 0.2 μm filter-sterilized L-15 Complete (Leibovitz’s L-15 Complete) medium (MT10-045-CV Mediatech), 10% (v/v) fetal bovine serum (FBS, A15-204, PAA Laboratories) and 2% (v/v) antibiotic/antimycotic solution (17-745E, Lonza), which was renewed twice weekly (old medium removed and replaced with fresh medium). Sub-culturing and/or cell cultivation was carried out when a confluent monolayer of cells was observed over the majority of growth surface via inverted microscopy (Axiovert 100 TV, Zeiss). Cell detachment was achieved by applying a phosphate buffered saline (PBS)-0.53 mM EDTA solution rinse (one minute) followed by incubating (five minutes) with 0.25% Trypsin-2.21 mM EDTA in Hank’s balanced salt solution (CA45000-664, VWR Canada). Cells were collected by pipet and concentrated by
centrifugation at 200 × g for five minutes. The sub-culturing ratio was commonly 1:4, although this varied according to the degree of confluence observed prior to detachment.

The RCAs were conducted in 96-MWP. Cell concentrations were adjusted to a density of 1.5×10^5 cells mL^(-1) in L-15 Complete medium based on haemocytometer counts. Aliquots (200 μL) of the cell solution were transferred into the wells of a sterile, tissue culture treated 96-MWP (353075, BD Biosciences). Wells on the perimeter of the 96-MWP were not seeded with the cell suspension to avoid edge effects. Plates were immediately covered with a lid, wrapped with Parafilm M® and incubated in the dark at 20°C until a confluent monolayer was observed in each well via microscopy (~ three days). Plates were moved into the laminar flow hood, inverted over a waste basin to drain the L-15 Complete medium overlying the monolayer at the bottom of each seeded well, then kept inverted and placed onto a stack of paper towels to drain any remaining medium. Once the L-15 Complete medium was drained, wells were rinsed with 200 μL of PBS followed by another inversion over a waste basin and drying on paper towel.

A 200 μL aliquot of the exposure solutions, controls (L-15 Complete and BG-11 medium) and blanks (Barnstead Nanopure Infinity Ultrapure water) were loaded into the appropriate wells of the 96-MWP. Silicone sealing-mats (521-01-151, Axygen) were used to prevent cross contamination among wells. Plates were wrapped in Parafilm M® to limit evaporative loss. Inverted microscopy was used to minimize cell detachment during the rinse and application steps and then incubated in the dark at 20°C. Following removal of exposure solutions (via inversion as described previously) and a rinse with PBS (200 μL), 100 μL aliquots of exposure medium (5% (v/v) PrestoBlue™ L-15 Complete solution) were applied to all wells. PrestoBlue™ (A-13262, Life Technologies), a resazurin-based compound that uses the reducing environment of cells to measure metabolic activity, was used to quantitatively assess cell viability. The plate was wrapped in Parafilm M® and left to incubate in the dark at 20°C for two hours. The plate was read using a fluorescence spectrophotometer (Agilent Technologies, Santa Clara, CA) with multi-well plate attachment set to read excitation/emission of 540 nm/590 nm. Raw fluorescence (RFU) was converted into cell viability as a percentage of the control.
4.2.2 Preparation of algal metabolites for RCA

4.2.2.1 Natural lake water assessment

Six oligotrophic lakes (Hilton Beach, Desbarats Lake, Bright Lake, Dean Lake, Woodrow Lake and Constance Lake) located within a 150 km radius around Sault Ste. Marie, Ontario were selected based on minimal direct anthropogenic influence and public concern about the potential of cyanobacterial HABs (cHABs) in the lakes (Sorichetti et al., 2013; Table 4.1). The lakes covered a wide range of algal biomass and relative cyanobacterial contributions to the biomass. The lakes were sampled during peak biomass in October 2011. Surface water temperature was measured at 1 m depth below the lake surface using the YSI 600 QS multi-parameter sonde with the YSI 650 MDS display (YSI Incorporated, Yellow Springs, OH).

Lake surface water samples were collected with a custom built 1 m long PVC tube water grab sampler near the centre of the lake, outside of an algal bloom if present and stored in 1 L pre-rinsed polyethylene bottles in a dark cooler on ice until returning to the field laboratory. Best efforts were made to sample outside of an algal bloom and not directly in the highest algal biomass to avoid sampling senescent cells and to capture those actively growing.

All lake water samples were processed in the field within 12 hours of sample collection and analyzed immediately upon returning to the laboratory or frozen at -80°C before analysis. A 90 mL subsample of lake water was preserved with 10% H$_2$SO$_4$ (v/v) in screw-top borosilicate tubes for total P and measured with the Technicon AutoAnalyzer (AAII) System (SEAL Analytical, Mequon, WI). A 300 mL subsample of lake water was filtered through 0.45 µm Pall Life Sciences polysulfonate membrane disc filters and analyzed for dissolved Fe with inductively coupled plasma spectrometry. A 300 mL subsample of lake water was filtered through 0.7 µm Whatman GF/F filters and analyzed for chlorophyll-a (chl-a) using the Turner 10-AU field fluorometer (Turner Designs, Sunnyvale, CA) according to EPA Method 445.0 (Arar & Collins, 1997).

Subsamples of lake water (15 mL) were centrifuged at 5,000 × g for 10 minutes, re-suspended in 5 mL of L-15 Complete buffer solution and sonicated using a Virsonic 100
Table 4.1 Lake name, coordinates (lat/long) and chemical characteristics (surface water temperature, chl-a, Fe and TP concentration) of the six natural lakes in the Algoma Highlands of central Ontario, Canada.

<table>
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<th>Lake Name</th>
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<th>Longitude (W)</th>
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<th>Chl-a (μg L⁻¹)</th>
<th>Fe (μg L⁻¹)</th>
<th>TP (μg L⁻¹)</th>
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<td>Bright</td>
<td>09/10/2011</td>
<td>46°15'16.00&quot;</td>
<td>83°16'47.00&quot;</td>
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<td>6.2</td>
<td>3.2</td>
<td>16.6</td>
</tr>
<tr>
<td>Dean</td>
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<td>46°13'39.17&quot;</td>
<td>83°10'43.87&quot;</td>
<td>16.8</td>
<td>9.8</td>
<td>35.8</td>
<td>13.8</td>
</tr>
<tr>
<td>Constance</td>
<td>09/10/2011</td>
<td>46°25'53.21&quot;</td>
<td>83°13'30.35&quot;</td>
<td>17.6</td>
<td>2.2</td>
<td>3.2</td>
<td>5.4</td>
</tr>
<tr>
<td>Hilton Beach</td>
<td>10/10/2011</td>
<td>46°15'54.49&quot;</td>
<td>83°53'30.37&quot;</td>
<td>15.1</td>
<td>1.3</td>
<td>1.5</td>
<td>6.2</td>
</tr>
<tr>
<td>Desbarats</td>
<td>10/10/2011</td>
<td>46°23'11.83&quot;</td>
<td>83°55'55.66&quot;</td>
<td>16.8</td>
<td>20.9</td>
<td>50.9</td>
<td>37.4</td>
</tr>
</tbody>
</table>
ultrasonic cell disruptor (SP Scientific) at a continuous power output of 10 for five minutes per 10 mL of sample to lyse cells. This resulted in a 3× concentration of the sample prior to application to the RCA in best efforts to elicit a measurable signal in a limited timeframe while lakes were blooming during peak biomass. Two exposure periods (36 hour and 60 hour) were selected to assess the impact lake water samples had on cell viability.

4.2.2.2 Analytical standards of algal metabolite assessment

Thirteen compounds were selected for analysis with the RCA (Table 4.2). Analytical standards of nodularin and seven microcystin variants (LA, LF, LR, LW, LY, RR and YR) were obtained (ALX-850-325-KI01, Alexis Biochemicals) and stored in the dark at -20°C. Analytical grade solutions of (E,E)-2,4-decadienal (W313505, Sigma), (E,E)-2,4-heptadienal (W316407, Sigma), β-cyclocitrinal (2,6,6-Trimethyl-1-cyclohexene-1-carboxaldehyde; W363928, Sigma), (±)-geosmin (G5908, Sigma) and 2-methylisoborneol (743364, Sigma) were obtained and stored in the dark at 4°C. Exposure solutions consisted of single compounds dissolved in L-15 Complete medium for use in the RCA (Eschbach et al., 2001; Dayeh et al., 2003). Nodularin and all seven microcystin variants (LA, LF, LR, LW, LY, RR and YR) were incubated for 24, 48 and 72 hours at each of the following concentrations: 0.1 nM, 1.0 nM, 10 nM, 100 nM and 1000 nM. The noxious metabolites (E,E)-2,4-decadienal, (E,E)-2,4-heptadienal, β-cyclocitrinal (2,6,6-Trimethyl-1-cyclohexene-1-carboxaldehyde), (±)-geosmin and 2-methylisoborneol were incubated for 24, 48 and 72 hours at each of the following concentrations: 10 nM, 100 nM, 1,000 nM, 10,000 nM, 100,000 nM and 1,000,000 nM.

4.2.2.3 Algal culture mixed metabolite assessment

Nine algal cultures were obtained from the Canadian Phycological Culture Collection (CPCC) in Waterloo, ON, Canada (Table 4.3).

Five cyanobacterial cultures, including: *Anabaena flos-aquae* (CPCC 64 and 543), *Aphanizomenon schindlerii* (CPCC 631) and *Microcystis aeruginosa* (CPCC 124 and CPCC 299) were used as representative algae that produce freshwater HABs (Molot et al., 2010;
Table 4.2 Chemical information, origin and associated hazards of toxins and noxious compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical Formula</th>
<th>Molecular Weight (g mol(^{-1}))</th>
<th>Algal Origin</th>
<th>Associated Hazards</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microcystin-LA</td>
<td>C(<em>{46})H(</em>{67})N(<em>{7})O(</em>{12})</td>
<td>910.0</td>
<td>Cyanobacteria</td>
<td>Hepatotoxic; skin, eye irritant</td>
</tr>
<tr>
<td>Microcystin-LF</td>
<td>C(<em>{52})H(</em>{71})N(<em>{7})O(</em>{12})</td>
<td>986.2</td>
<td>Cyanobacteria</td>
<td>Hepatotoxic; skin, eye irritant</td>
</tr>
<tr>
<td>Microcystin-LR</td>
<td>C(<em>{49})H(</em>{74})N(<em>{10})O(</em>{12})</td>
<td>995.2</td>
<td>Cyanobacteria</td>
<td>Hepatotoxic; skin, eye irritant</td>
</tr>
<tr>
<td>Microcystin-LW</td>
<td>C(<em>{54})H(</em>{72})N(<em>{8})O(</em>{12})</td>
<td>1,025.2</td>
<td>Cyanobacteria</td>
<td>Hepatotoxic; skin, eye irritant</td>
</tr>
<tr>
<td>Microcystin-LY</td>
<td>C(<em>{52})H(</em>{71})N(<em>{7})O(</em>{13})</td>
<td>1,002.2</td>
<td>Cyanobacteria</td>
<td>Hepatotoxic; skin, eye irritant</td>
</tr>
<tr>
<td>Microcystin-RR</td>
<td>C(<em>{49})H(</em>{75})N(<em>{13})O(</em>{12})</td>
<td>1,038.2</td>
<td>Cyanobacteria</td>
<td>Hepatotoxic; skin, eye irritant</td>
</tr>
<tr>
<td>Microcystin-YR</td>
<td>C(<em>{52})H(</em>{72})N(<em>{10})O(</em>{13})</td>
<td>1,045.2</td>
<td>Cyanobacteria</td>
<td>Hepatotoxic; skin, eye irritant</td>
</tr>
<tr>
<td>Nodularin</td>
<td>C(<em>{41})H(</em>{60})N(<em>{8})O(</em>{10})</td>
<td>825.0</td>
<td>Cyanobacteria</td>
<td>Hepatotoxic; skin, eye irritant</td>
</tr>
<tr>
<td>(E,E)-2,4-Decadienal</td>
<td>C(<em>{10})H(</em>{16})O</td>
<td>152.2</td>
<td>Chrysophyte</td>
<td>Skin, eye irritant; fatty/citrus odour</td>
</tr>
<tr>
<td>(E,E)-2,4-Heptadienal</td>
<td>C(<em>{7})H(</em>{10})O</td>
<td>110.2</td>
<td>Chrysophyte</td>
<td>Skin, eye irritant; rancid fish odour</td>
</tr>
<tr>
<td>β-Cyclocitral</td>
<td>C(<em>{10})H(</em>{16})O</td>
<td>152.2</td>
<td>Chrysophyte</td>
<td>Skin, eye irritant; sweet tobacco odour</td>
</tr>
<tr>
<td>2-Methylisoborneol</td>
<td>C(<em>{11})H(</em>{20})O</td>
<td>168.3</td>
<td>Chrysophyte</td>
<td>Skin, eye irritant; musty odour</td>
</tr>
<tr>
<td>Geosmin</td>
<td>C(<em>{12})H(</em>{22})O</td>
<td>182.3</td>
<td>Chrysophyte</td>
<td>Skin, eye irritant; earthy odour</td>
</tr>
</tbody>
</table>
Table 4.3 Cyanobacteria and chrysophyte isolates obtained from the Canadian Phycological Culture Collection (CPCC) in Waterloo Ontario, Canada.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Strain</th>
<th>Isolation location</th>
<th>Year of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanobacteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Anabaena flos-aquae</em></td>
<td>CPCC 64</td>
<td>Lake Ontario, ON, Canada</td>
<td>1987</td>
</tr>
<tr>
<td><em>Anabaena flos-aquae</em></td>
<td>CPCC 543</td>
<td>Burton Lake, SK, Canada</td>
<td>2001</td>
</tr>
<tr>
<td><em>Aphanizomenon schindleri</em></td>
<td>CPCC 631</td>
<td>Experimental Lakes Area, ON, Canada</td>
<td>2005</td>
</tr>
<tr>
<td><em>Microcystis aeruginosa</em></td>
<td>CPCC 124</td>
<td>Heart Lake, ON, Canada</td>
<td>1987</td>
</tr>
<tr>
<td><em>Microcystis aeruginosa</em></td>
<td>CPCC 299</td>
<td>Pretzlaff Pond, AB, Canada</td>
<td>1990</td>
</tr>
<tr>
<td>Chrysophyceae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Dinobryon sp.</em></td>
<td>CPCC 392</td>
<td>Sippewisset, MA, USA</td>
<td>1986</td>
</tr>
<tr>
<td><em>Synura petersenii</em></td>
<td>CPCC 495</td>
<td>Hwy 410, NL, Canada</td>
<td>1982</td>
</tr>
<tr>
<td><em>Synura uvella</em></td>
<td>CPCC 422</td>
<td>Arrowwood Lake, ND, USA</td>
<td>1995</td>
</tr>
<tr>
<td><em>Uroglena sp.</em></td>
<td>CPCC 276</td>
<td>Dickie Lake, ON, Canada</td>
<td>1991</td>
</tr>
</tbody>
</table>
Winter et al., 2011; Sorichetti et al., 2013). Two cultures, *Anabaena flos-aquae* (CPCC 64) and *Microcystis aeruginosa* (CPCC 299), are confirmed microcystin producers.

Four chrysophyte cultures, *Dinobryon sp.* (CCMP 392), *Synura uvella* (CPCC 422), *Synura petersenii* (CPCC 495) and *Uroglena sp.* (CPCC 276) were selected to be used as representative algae that cause taste and odor bloom events (Nichols 1995; Watson and Satchwill, 2003; Paterson et al., 2008).

Cyanobacterial cultures were maintained in modified BG-11 medium (Fe concentration=30 μM as ferric ammonium citrate) and chrysophyte cultures were maintained in WC medium (Fe concentration=11.7 μM as FeCl₃·6H₂O) (Andersen et al., 2005). All culture glassware was prepared trace metal clean and stored in acid washed containers (soaked in 10% HCl for ≥ 24 hours). Major nutrient additions were passed through a Chelex® 100 ion exchange resin (C7901, Sigma) to remove excess trace metals prior to addition to the medium (Price et al., 1988, 1989). All media were prepared using Barnstead Nanopure Infinity Ultrapure water and adjusted to a pH of 7.5. Media (2.0 L) were then microwave-sterilized on ‘High’ (1000 W) power twice for 10 minutes prior to use and stored at 4°C. Media were warmed to room temperature (~20 °C) prior to addition to any culture.

Cyanobacterial cultures were grown at 24°C under constant light conditions of 70 ± 10 μmol photons m⁻² s⁻¹ as recommended by the CPCC. Chrysophyte cultures were grown under light conditions consisting of a 12-hour light-dark photoperiod and an irradiance of 20 ± 5 μmol photons m⁻² s⁻¹ as recommended by the CPCC. Three of the chrysophyte cultures were grown at a temperature of 20°C, while *Synura petersenii* (CPCC 495) was maintained at 10°C as recommended by the CPCC. Algal culture growth was monitored by optical density at 720 nm with a DU 640 spectrophotometer (Beckman Coulter). Using optical density as a proxy for the biomass of samples avoided counting complications for cells that either formed clumps, colonies or filaments.

For exponential growth phase experiments, a continuous culture apparatus was used to maintain cyanobacterial and chrysophyte cultures at a constant exponential growth rate with a fixed concentration of nutrients in the medium. High-Fe treatments were maintained at 10 μM
Fe and low-Fe treatments were maintained at 0.1 μM Fe. Modified aquaria (25 cm × 50 cm × 30 cm) were placed ~15 cm from a side-mounted fluorescent light source. Aquaria were angled to reduce the incoming flux of light to 70 ± 10 μmol photons m⁻² s⁻¹ in each of the culture tubes. Tanks were filled near to the top with tap water and wall-mounted heaters maintained a constant water temperature of 24°C. Glass culture tubes (250 mL) were arranged haphazardly, suspended vertically in each tank and capped with a modified rubber stopper to exclude ambient dust and to also support glass tubing that delivered fresh medium to the culture tubes and allow spent medium to flow out of the culture. All glass culture tubes were placed the same distance away from the light source in best effort to ensure equal light exposure among all cultures. An adjustable electric pump provided sterile medium to the culture tubes, allowing the biomass of each culture to be controlled via dilution. All parts of the culture apparatus were acid-washed, rinsed in ultrapure water and ethanol-sterilized prior to inoculation.

For stationary growth phase experiments, all algal cultures were maintained in a batch culture for five days beyond the cessation of exponential growth, which were monitored daily. High-Fe treatments were maintained at 10 μM Fe and low-Fe treatments were maintained at 0.1 μM Fe.

Following termination of experiments, cyanobacterial and chrysophyte samples in their culture medium were stored in trace metal-cleaned bottles in the dark at -20°C. These samples were kept in storage until immediately before application to the RCA when they were thawed to room temperature. Algae in their culture medium proved to be resistant to lysis during freezing, which made this storage method ideal for the preservation of cells and bioactive compounds (data not shown) (Furtula et al., 2004; Kim et al., 2009).

Subsamples of algal cultures were split into equal parts prior to RCA application. Half of the sample was centrifuged at 10,000 × g for 10 minutes for cyanobacteria and 1,000 × g for 10 minutes for chrysophytes; higher speeds were required to pelletize physically smaller cultures of cyanobacteria like M. aeruginosa 124 and 299. The other half of the sample was lysed using a Virsonic 100 ultrasonic cell disrupter (SP Scientific) at a continuous power output of 10 for five minutes per 10 mL of sample. During sonication,
placing the sample tube in an ice bath allowed the sample to stay cool in best efforts to avoid denaturing organics. Lysed samples were then centrifuged as described previously. The resulting solutions were then mixed with buffer solution for the RCA. A serial dilution of each exposure solution in L-15 Complete medium allowed for the assessment of the solutions over a range of concentrations; 0%, 20%, 40%, 60%, 80% and 100% concentration relative to the original culture density. These concentrations were expressed in terms of optical density at 720 nm.

4.2.3 Statistical Measures

Significant differences in % viability between controls and natural lake water samples for each exposure time were tested using a one-way ANOVA with a Dunnett test. Significant differences in % viability between controls and each algal metabolite standard and exposure time were tested using a one-way ANOVA with a Dunnett test. Significant differences in % viability between controls and cell density, Fe concentration and exposure time for algal culture mixed metabolites was tested using a one-way ANOVA with a Dunnett test. In any test where normality and/or equal variance failed, the one-way ANOVA was replaced with ANOVA on Ranks. When applicable, EC_{50} values were calculated for each algal metabolite standard tested according to Alexander et al. (1999). Critical α=0.05 for all ANOVA and Dunnett tests performed.

4.3 Results

4.3.1 RCA exposure to natural lake waters

Through application of the RCA to natural lake waters, resultant percent viability measured after the 36-hour exposure trial was: Hilton Beach (82.2%), Desbarats Lake (74.0%), Woodrow Lake (73.6%), Constance Lake (50.9%), Bright Lake (46.7%) and Dean Lake (30.6%). Within the 60-hour exposure trial, resultant percent viability was: Hilton Beach (89.8%), Bright Lake (70.0%), Woodrow Lake (67.6%), Desbarats Lake (67.3%), Constance Lake (58.3%) and Dean Lake (24.7%). Three lakes showed significant reductions in viability relative to the control for the 36 hour exposure trial (Fig. 4.1) including: Constance Lake, Bright Lake and Dean Lake (all p<0.001). Dean Lake showed a significant reduction in viability relative to the control (p=0.002) for the
Figure 4.1 RCA viability (%) for each of the six natural lake samples from central Ontario, Canada. Numbers in parentheses on x-axis indicate chl-a concentrations for each lake. Lettering indicates significant differences in viability between each lake sample and control for each exposure time.
60-hour exposure trial (Fig. 4.1). The reduction in viability observed in both 36-hour and 60-hour exposure trials was not correlated to phytoplankton biomass \((p=0.88\) and 0.39, respectively), as represented by chl-\(a\) concentration (Fig. 4.1), or the proportion of cyanobacterial contribution to phytoplankton biomass. However, when Desbarats Lake was removed from the analysis (the lake with more than twice the chl-\(a\) concentration of any other lake), there was a significant negative correlation between viability and chl-\(a\) concentration \((p=0.003\) and \(r=-0.52\) for the 36-hour exposure trials; \(p=0.004, r=-0.51\) for the 60-hour exposure trials).

4.3.2 RCA exposure to analytical standards of algal metabolites

The RCA was insensitive to concentrations of individual microcystin variants and nodularin at or below environmental averages or at concentrations that were three orders of magnitude higher than environmental averages (Fig. 4.2). Reductions in viability greater than 50% were not observed in any standard cyanobacterial metabolite tested, therefore the EC\(_{50}\) values for all microcystin variants and nodularin were outside the tested range limit and were many orders of magnitude higher than concentrations that would be measured in natural aquatic systems. Significant reductions in viability relative to controls were observed in the microcystin-LR \((p<0.001)\) and microcystin-RR \((p<0.001)\) standards at the highest concentration tested (1,000 nM) for the 24 hour, 48 hour and 60 hour exposure trials (Fig. 4.2). These correspond to concentrations higher than relevant maximum environmental concentrations (Table 4.4).

The noxious taste and odour causing compounds tested with the RCA showed more significant reductions in viability compared to the microcystin and nodularin toxins tested (Fig. 4.3). Significant reductions in viability relative to controls were observed in all noxious taste and odour causing compounds at the highest concentration tested (1,000,000 nM) for the 24 hour, 48 hour and 60 hour exposure time trials (all \(p<0.001\)). No significant reductions in viability relative to controls were observed at the two lowest concentrations tested (10 nM and 100 nM) for any exposure time trial. A notable reduction in viability was observed in heptadienal, where a 100% reduction in viability relative to controls was observed at a concentration of 1,000 nM during the 72-hour trial \((p<0.001)\). This suggests that heptadienal may be detectable at environmentally relevant
Figure 4.2 The effect of cyanotoxins on cell viability using the RCA. Toxicity was assessed by incubating seven microcystin (MC) variants and nodularin for 24, 48 and 72 hours at each of the following concentrations: 0.1 nM, 1.0 nM, 10 nM, 100 nM and 1000 nM. Mean viability (%) ± SEM, n=10; grey-filled points indicate statistically significant differences in viability for each concentration and incubation time tested. Non-filled points indicate no significant difference between treatment and control. X-axes are plotted on a log$_{10}$ scale.
Table 4.4 Algal metabolites, RCA EC$_{50}$ values calculated from 72 hour exposure trials [using the formula in Alexander et al., (1999)] and mean and maximum environmental concentrations for toxins and noxious compounds (from Watson, 2003).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mean Environmental Concentration (nM)</th>
<th>Maximum Environmental Concentration (nM)</th>
<th>RCA EC$_{50}$ Values (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microcystin-LA</td>
<td>$&lt;10^0$</td>
<td>-</td>
<td>$&gt;10^3$</td>
</tr>
<tr>
<td>Microcystin-LF</td>
<td>$&lt;10^0$</td>
<td>-</td>
<td>$&gt;10^3$</td>
</tr>
<tr>
<td>Microcystin-LR</td>
<td>$&lt;10^0$</td>
<td>$&lt;10^2$</td>
<td>$&gt;10^3$</td>
</tr>
<tr>
<td>Microcystin-LW</td>
<td>$&lt;10^0$</td>
<td>-</td>
<td>$&gt;10^5$</td>
</tr>
<tr>
<td>Microcystin-LY</td>
<td>$&lt;10^0$</td>
<td>-</td>
<td>$&gt;10^3$</td>
</tr>
<tr>
<td>Microcystin-RR</td>
<td>$&lt;10^0$</td>
<td>-</td>
<td>$&gt;10^3$</td>
</tr>
<tr>
<td>Microcystin-YR</td>
<td>$&lt;10^0$</td>
<td>-</td>
<td>$&gt;10^3$</td>
</tr>
<tr>
<td>Nodularin</td>
<td>$&lt;10^0$</td>
<td>-</td>
<td>$&gt;10^3$</td>
</tr>
<tr>
<td>(E,E)-2,4-Decadienal</td>
<td>$&lt;10^1$</td>
<td>$&lt;10^2$</td>
<td>$2\times10^3$</td>
</tr>
<tr>
<td>(E,E)-2,4-Heptadienal</td>
<td>$&lt;10^1$</td>
<td>$&lt;10^2$</td>
<td>$5\times10^3$</td>
</tr>
<tr>
<td>β-Cyclocitral</td>
<td>$&lt;10^1$</td>
<td>$&lt;10^2$</td>
<td>$3\times10^6$</td>
</tr>
<tr>
<td>Geosmin</td>
<td>$&lt;10^{-1}$</td>
<td>$&lt;10^1$</td>
<td>$7\times10^2$</td>
</tr>
<tr>
<td>2-Methylisoborneol</td>
<td>$&lt;10^0$</td>
<td>$&lt;10^0$</td>
<td>$6\times10^3$</td>
</tr>
</tbody>
</table>
Figure 4.3 The effect of noxious taste and odor compounds on cell viability using the RCA. Toxicity was assessed by incubating five compounds for 24, 48 and 72 hours at each of the following concentrations: 10 nM, 100 nM, 1,000 nM, 10,000 nM, 100,000 nM and 1,000,000 nM. Mean viability (%) ± SEM, n=10; grey-filled points indicate statistically significant differences in viability for each concentration and incubation time tested. Non-filled points indicate no significant difference between treatment and control. X-axes are plotted on a log$_{10}$ scale.
concentrations. For the remainder of the metabolites tested, reductions in viability were observed at concentrations exceeding environmental averages found in natural aquatic systems (Table 4.4).

### 4.3.3 RCA exposure to algal culture mixed metabolites

The RCA assessment of algal metabolites produced during exponential growth phase by cyanobacteria showed no significant reductions in viability with one exception being *A. flos-aquae* (CPCC 64), which showed significant reductions in viability relative to controls in 10 μM Fe (*p*<0.001) and 10 μM Fe lysed (*p*<0.001) treatments at 40%, 60%, 80% and 100% cell density (Fig. 4.4). Conversely, algal metabolites accumulated (or present) during stationary growth phase by cyanobacteria all showed significant reductions in viability relative to controls in the 10 μM Fe lysed treatment (*p*<0.001) at 40%, 60%, 80% and 100% cell density (Fig. 4.4). *A. flos-aquae* (CPCC 64) and *A. flos-aquae* (CPCC 543) showed significant reductions in viability relative to controls in the stationary growth phase 10 μM Fe lysed treatment (*p*<0.001) at all cell densities tested (Fig. 4.4). *M. aeruginosa* (CPCC 299) resulted in 100% reductions in viability across all treatments during stationary growth phase tests (all *p*<0.001). This result was not observed in any other cyanobacterial or chrysophyte culture tested. Significant reductions in viability in the stationary growth phase tests were additionally observed in 0.1 μM Fe, 10 μM Fe and 0.1 μM Fe lysed treatments with significant differences relative to controls indicated in Figure 4.4 for each cyanobacterial culture tested.

Four trends were observed across all stationary growth phase tests of cyanobacterial cultures: (1) in treatments that resulted in significant reductions in viability at low Fe treatments (0.1 μM Fe), equal or greater reductions in viability were observed at high Fe treatments (10 μM Fe); (2) 10 μM Fe treatments showed reductions in viability more frequently than the 0.1 μM Fe treatments; (3) exposure solutions composed of intracellular and extracellular (lysed) material resulted in greater reductions in viability than their counterparts containing only extracellular (non-lysed) material; and (4) optical densities of cultures that resulted in mild reductions (≥50%) in viability ranged from ~0.1 a.u. to 0.6 a.u. (720 nm), with large variation among algal cultures and treatments.
The effect of metabolites from cyanobacterial cultures produced in exponential (left, A-E) and stationary (right, A'-E') growth phase on cell viability using the RCA. Extracts from five cultures: [A,A'] *Microcystis aeruginosa* (CPCC 124) [B,B'] *Microcystis aeruginosa* (CPC 299) [C,C'] *Anabaena flos-aquae* (CPCC 64) [D,D'] *Anabaena flos-aquae* (CPCC 543) [E,E'] *Aphanizomenon schindleri* (CPCC 631) and four treatments: Fe 10 μM, Fe 0.1 μM, Fe 10 μM lysed and Fe 0.1 μM lysed were tested. Mean viability (%) ± SEM, n=3; grey-filled points indicate statistically significant differences in viability for each treatment cell density relative to the control. Non-filled points indicate no significant difference between treatment and control. Exposure solutions were evaluated at concentrations equivalent to 0%, 20%, 40%, 60%, 80% and 100% of the culture density, as expressed by optical density at 720 nm.
The RCA assessment of algal metabolites produced during exponential and stationary growth phase by chrysophytes showed no reductions in viability, with one exception. *S. petersenii* (CPCC 495) resulted in significant reductions in viability relative to controls in stationary growth phase 10 μM Fe lysed treatment (*p*<0.001) at 40%, 60%, 80% and 100% cell density (Fig. 4.5).

### 4.4 Discussion

Freshwater algae are known to produce a large number of toxic, noxious and bioactive metabolites, thus rendering it difficult to select one specific test to quantify environmental effects. Using a bioassay rather than an extraction and analytical test may provide useful information on both the general toxicity of a sample and on the direct regulation of toxins (Fent, 2001). The suitability of an assay can be assessed by its ability to detect compounds at relatively low concentrations (Fent, 2001).

Samples of natural lake water from a region where community members have raised concerns for public health and drinking water quality due to observed blooms resulted in variation in the levels of toxicity based on the RCA. From these samples we concluded that (1) the RCA was variably sensitive to the compounds in lakes potentially produced by cyanobacteria and/or chrysophyte algae; and (2) in contrast to our expectations, there was no significant correlation between the algal biomass in the sample or, alternatively, the percentage of either cyanobacteria or chrysophytes with any measure of toxicity. However, there was a significant correlation between cell viability and chl-*a* concentrations when the lake with the highest chl-*a* concentration (Desbarats Lake) was removed from the analysis. Desbarats Lake blooms annually with cyanobacterial communities primarily comprised of *Aphanizomenon* sp. while blooms in the other five lakes are mixed with *Microcystis* sp., *Anabaena* sp. and *Aphanizomenon* sp. All six lakes are otherwise known to have cyanobacterial blooms. The variation in lake water toxicity suggests a research need to verify if the RCA is a suitable tool for use in algal sampling and subsequent factors that may negatively affect gill cell survival.

Despite the application of seven microcystin variant standards produced by cyanobacteria at concentrations that exceed environmentally relevant averages, the RCA proved to be insensitive to detecting these compounds (Fig. 4.2). Previous work has shown that fish and
Figure 4.5 The effect of metabolites from chrysophyte cultures produced in exponential (left, A-D) and stationary (right, A’-D’) growth phase on cell viability using the RCA. Extracts from four isolates: [A,A’] *Synura petersenii* (CPCC 495) [B,B’] *Synura uvella* (CPCC 422) [C,C’] *Dinobryon sp.* (CPCC 392) [D,D’] *Uroglena sp.* (CPCC 276) and four treatments: Fe 10 μM, Fe 0.1 μM, Fe 10 μM lysed and Fe 0.1 μM lysed were tested. Mean viability (%) ± SEM, n=3; grey-filled points indicate statistically significant differences in viability for each treatment cell density relative to the control. Non-filled points indicate no significant difference between treatment and control. Exposure solutions were evaluated at concentrations equivalent to 0%, 20%, 40%, 60%, 80% and 100% of the culture density, as expressed by optical density at 720 nm.
mammalian cell lines display a wide range of sensitivity to these hepatotoxins, although no mammalian or fish cell line has been shown to be sensitive to microcystins at environmentally relevant concentrations (McDermott et al., 1998; Chong et al., 2000; Pichardo et al., 2005, 2006, 2007). This repeated insensitivity may be due to the nature of the cell-based assays, which are primarily tests for acute cytotoxic effects; while microcystins, nodularins and a variety of other cyanotoxins generally show effects over periods of long-term chronic exposure dependent on dosage (Chen et al., 2009; El Ghazali et al., 2010).

The RCA provided evidence that it may be an effective evaluator of noxious taste and odour compounds with further investigation warranted (Fig. 4.3). For most of the tested compounds a dose-dependent reduction in cell viability was documented, particularly at relatively high concentrations of metabolites. With the exception of the reduction in viability observed in the heptadienal trials, the investigated compounds caused negligible reductions in viability at average environmental concentrations (Table 4.4). Although these compounds do not have an extensive history in bioassay research as is the case with microcystins, the documented characteristics of these compounds as potential dermal irritants makes these results unexpected (Graham et al., 2008). However, these compounds are generally recognized for their ability to produce foul taste and odors, rather than their ability to adversely affect aquatic biota (Watson, 2003).

The significant reductions in viability observed in the RCA trials involving bioactive metabolites from cyanobacterial cultures have shed light on several key areas of cyanobacterial metabolite production and storage. The most consistent result observed was the lack of response observed from exposure solutions derived from exponential growth phase relative to those from stationary growth phase (Fig. 4.4). Despite a reduction in viability observed in the high Fe (10 μM) treatments in the exponential growth phase sample from A. flos-aquae (CPCC 64), the remaining four cultures resulted in no significant reductions in viability from any sample derived from exponential growth phase. These findings suggest that either the cytotoxic/bioactive compounds are not being produced in the exponential growth phase, or that they are being produced but are not present at high enough concentrations to have a significant impact on the RCA. It may also be possible that A. flos-aquae (CPCC 64) produced a toxin variant detectable by RCA that was not tested for in the cyanobacterial
analytical standard toxin test or that *A. flos-aquae* (CPCC 64) may have produced a consortium of toxins giving rise to an RCA signal that we did not otherwise achieve in analyzing individual toxins independently. This result is contradictory to our predictions and does not support findings from similar studies that found elevated cyanobacterial toxin production rates in exponential growth phase (Schatz et al., 2007; Kaplan et al., 2012).

Another result from cyanobacterial testing was the propensity for lysed samples (intracellular + extracellular material) to result in greater reductions in viability relative to the non-lysed samples (extracellular material only) of the same Fe treatment (Fig. 4.4) in the RCA. This observation is supported by previous research documenting that the proportion of bioactive material retained inside the cell over the course of population growth is greater than the proportion that is excreted to the extracellular environment prior to senescence (Park et al., 1998). This may explain the differences observed between cyanobacterial exponential and stationary growth phase tests, as the accumulation of intracellular compound would increase proportionally with the age of the cells prior to entering senescence.

The role of Fe was observed to be a contributing factor in the cytotoxicity of algal metabolites investigated in cyanobacterial cultures. While Fe is commonly limiting in freshwater lakes, the experimental levels chosen here reflect natural levels of Fe (0.1 μM) and a treatment in which Fe was in excess (10 μM). The latter treatment resulted in the greatest reductions in viability observed with the RCA (Fig. 4.4). Cells grown under the high Fe (10 μM) treatment repeatedly resulted in the most cytotoxic samples, providing support for the claim that it is under optimum growth conditions, rather than nutrient limitation, that bioactive compound synthesis peaks in cyanobacteria (Kaebernick and Neilan, 2001). This result suggests that the Fe-limited conditions that have been reported to favor microcystin production are not necessarily ideal for the production of a wider variety of bioactive compounds as has been suggested by previous work (Alexova et al., 2011; Kaplan et al., 2012). One possible explanation is that the bioactive products responsible for reductions in viability observed with the RCA are derivatives of molecules produced by photosynthetic processes (Kaebernick and Neilan, 2001), though this was not specifically measured. Fe-limited treatments may decrease the cell’s photosynthetic efficiency and, therefore, limit the production of these metabolites.
Aside from the reductions in viability observed in the stationary growth phase, high Fe (10 μM) and lysed treatment of *S. petersenii* (CPCC 495), no significant reduction in viability was observed with the RCA in any other chrysophyte-derived treatment or culture (Fig. 4.5). The reduction in viability observed in CPCC 495 suggests similar patterns to that of the cyanobacterial tests: high Fe (10 μM) conditions and intracellular storage may be important factors in the production of bioactive compounds. However, these findings are not supported by any of the other three chrysophyte culture mixed metabolites investigated. The overall lack of response observed in the RCA when exposed to metabolite mixtures from chrysophyte cultures was likely a result of the inability to grow cultures to sufficient cell densities. An additional step to concentrate chrysophyte cell cultures and obtain higher cell density for the experiment was considered but this additional methodology would be contradictory to the rapid assessment characteristic and intent for this bioassay.

4.5 Conclusions

It is possible that the RCA measured toxicity of bioactive metabolites in lake samples and in cyanobacterial cultures that were not tested as isolated analytical standards. For example, β-methyl amino alanine is a cyanotoxin with the potential for production by all cyanobacterial genera, but isolated standards for this cyanotoxin could not be acquired for experimentation. Other metabolites not tested as isolated standards, though could have been present and detected in the lake samples and algal cultures include: cylindrospermopsins, anatoxins, saxitoxins, alysiatoxins and lyngbyatoxins. Further research into the applicability of the RCA to measure cytotoxicity in lake samples should focus on testing a wider range of known cyanotoxins that are most likely to occur, or those previously identified, in specific study lakes of interest. An additional next step in the methodological aspect of the RCA is to test a gradient in concentration factors of lake water samples. This study concentrated all lake water samples 3× and achieved variable responses to cell viability as measured by the RCA. Testing a gradient in concentration factors would identify whether the RCA is suitable for use in lakes with low or extremely high biomass accumulation.
In spite of the induction of cytotoxic responses to natural lake samples, the RCA was relatively insensitive to known or putative toxins and/or noxious compounds. The selected test did respond to the analytical standards of metabolites but only at excessively high levels compared to average environmental concentrations. Analysis of noxious compounds produced by chrysophyte algae revealed similar insensitivity with the RCA, although reductions in viability were observed at concentrations greater than two orders of magnitude higher than average environmental concentrations. Cyanobacterial cultures grown in relatively high Fe (10 μM) conditions were responsible for greater reductions in cell viability based on the RCA, suggesting that the production of cytotoxins and bioactive compounds responsible for observed reductions is greater in these conditions compared to low Fe (0.1 μM) conditions. Comparing this finding to the established link between low Fe conditions and the stimulation of microcystin production suggests that the monitoring of microcystin levels in natural systems is likely not a good indicator of the overall levels of cytotoxic and/or bioactive compounds present in a sample, although it is still an important parameter to monitor with regards to drinking water quality.
4.6 References


Chapter 5

5 Conclusions

The objectives of this thesis were to: determine if P or N limits cyanobacterial biomass; determine the role Fe may play in alleviating P or N limitation on cyanobacterial biomass; investigate the presence of hydroxamate and catecholate siderophores in lakes; determine whether the presence of siderophores alleviates Fe limitation on cyanobacterial biomass; investigate the role DOM may play in regulating the bioavailability of Fe and siderophores to cyanobacteria; and investigate the effectiveness and adaptability of the RCA by applying it to analytical standards, complex mixtures (extracted from laboratory culture) of phytoplankton secondary metabolites and natural lake water samples.

The three year oligotrophic lake sampling campaign revealed that none of the lakes experienced what would constitute a mono-specific phytoplankton community or “cyanobloom” during peak biomass, > 50% cyanobacteria within the phytoplankton community, with the highest measured proportion being 36% cyanobacteria. This thesis provides critical information regarding the initial stages of cyanobloom development, that being the switch from picocyanobacteria dominated communities to diverse “transitionary” communities (Figure 1.2).

It was found that the role of P and N (individually or as a ratio) on cyanobacterial biomass was negligible and the single most important factor regulating cyanobacterial biomass in the oligotrophic lakes was Fe. From this thesis, it is evident that Fe regulates cyanobacterial macronutrient use efficiency in lakes and the measuring (or modeling) of bioavailable Fe should be part of routine lake water sampling campaigns related to cyanobacteria. This finding provides new insights and avenues of exploration for research scientists and lake managers interested in understanding cyanobloom formation in lakes with low P and N supply. Future research programs should focus on landscape controls on the mobilization of Fe from landscapes to lakes and the fate of Fe in lakes with changing P and N dynamics due to global climate change.
Hydroxamate and/or catecholate siderophores were detected in all 25 oligotrophic lakes and this thesis represents the first documented record of measured siderophores in central Ontario lakes. The role of hydroxamate siderophores in lakes was clear – within a range of low Fe availability, cyanobacterial density was positively correlated to hydroxamate siderophore concentration. A negative relationship (although not significant) between catecholate siderophore concentration and cyanobacterial density was found in lakes and the role of catecholate siderophores is less clear. The concentration of catecholate siderophores was 10× higher than that for hydroxamate siderophores. This may indicate that the catecholate signal measured in lakes may be a combination of cyanobacterial catecholate siderophores and catechol compounds from terrestrial sources (DOM) in lakes. This is also evidenced by the significant relationship between DOM concentration and catecholate siderophore concentration (Figure 3.8 B). It is also unclear whether cyanobacteria can use one and/or both siderophore types simultaneously to scavenge Fe in Fe-limited conditions. Future research programs should focus on the metabolic cost for the production of hydroxamate vs. catecholate siderophores when cells are Fe-limited to address the discrepancy observed in the relationship between hydroxamate and catecholate siderophores vs. cyanobacterial density in low Fe conditions. Further research is also needed on method development to identify the specific source of siderophores measured, whether derived from cyanobacterial or allochthonous sources as no such methodology currently exists.

EEMs were used to indicate the quality of DOM in lakes and it was found that labile DOM may serve as an Fe and siderophore source while refractory DOM may serve as an Fe and siderophore sink. This finding provides new insight into the exploration of cyanobloom development in lakes along a gradient of DOM (or water colour), which can be further associated to the degree of hydrological connectivity between landscapes with various degrees of wetland contribution within lake catchments. The collection of EEMs and associated data (e.g., degree of humification) should be incorporated into routine lake water sampling campaigns related to cyanobacteria. The EEM data investigated in this thesis yield potential for this rapid yet data-rich methodology to serve as a quick indicator for cyanobloom risk in lakes.
The RCA has potential for applicability as a useful tool in natural lake water samples to detect toxic and/or noxious bioactive compounds. The RCA did provide a measure of toxicity in samples from oligotrophic lakes but the assay was insensitive to analytical standards of the cyanotoxins tested and was only sensitive to noxious compound standards at concentrations higher than environmental averages. The RCA may have measured the toxicity of cyanotoxins in lake samples that were not tested as analytical standards, or the possible consortium of cyanotoxins present in lakes may have contributed to a cumulative cytotoxicity effect resulting in reduced viability using the RCA. A wider range of cyanotoxins should be further tested with the RCA to further explore its suitability as a reliable indicator of lake toxicity.

This thesis has provided evidence for the critical role of Fe in the regulation of cyanobacterial biomass and has supported the hypothesis that cyanobacteria have the ability to induce the competitive siderophore Fe acquisition strategy to overcome Fe limitation in oligotrophic lakes, dependent of the quantity and quality of DOM. Further evidence has been provided to support the RCA as a potentially practical tool to assess the cytotoxicity of lakes and improve our ability to rapidly detect and characterize the potentially harmful effects of cyanoblooms in freshwater lakes.
Appendix A: Peak biomass season raw data (2009 to 2011)

This appendix presents the surface water temperature, pH, macronutrient and Fe raw data as well as all phytoplankton metrics collected in each of the 25 oligotrophic study lakes during peak biomass from 2009 to 2011.
Table A.1 Surface water temperature, macronutrient and Fe concentrations for each of the 25 oligotrophic study lakes during peak biomass season in 2009.

<table>
<thead>
<tr>
<th>Lake</th>
<th>Date (2009)</th>
<th>Surface Temp (°C)</th>
<th>pH</th>
<th>DOC (µg L$^{-1}$)</th>
<th>TN (µg L$^{-1}$)</th>
<th>TP (µg L$^{-1}$)</th>
<th>N:P (Molar)</th>
<th>NO$_3^-$ (µg L$^{-1}$)</th>
<th>NH$_4^+$ (µg L$^{-1}$)</th>
<th>TDFe (µg L$^{-1}$)</th>
<th>Fe$^{3+}$ (M)</th>
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<td>7.93</td>
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<td>59.73</td>
<td>23.10</td>
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Table A.2 Phytoplankton metrics for each of the 25 oligotrophic study lakes during peak biomass season in 2009.

<table>
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<tr>
<th>Lake</th>
<th>Date (2009)</th>
<th>Chl-a (µg L(^{-1}))</th>
<th>Eukaryote (cells L(^{-1}))</th>
<th>Cyanobacteria (cells L(^{-1}))</th>
<th>Total Biomass (cells L(^{-1}))</th>
<th>Cyanobacteria (%)</th>
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Table A.3 Surface water temperature, macronutrient and Fe concentrations for each of the 25 oligotrophic study lakes during peak biomass season in 2010.

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<th>Lake</th>
<th>Date (2010)</th>
<th>Surface Temp (°C)</th>
<th>pH</th>
<th>DOC (µg L⁻¹)</th>
<th>TN (µg L⁻¹)</th>
<th>TP (µg L⁻¹)</th>
<th>N:P (Molar)</th>
<th>NO₃⁻ (µg L⁻¹)</th>
<th>NH₄⁺ (µg L⁻¹)</th>
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**Table A.4** Phytoplankton metrics for each of the 25 oligotrophic study lakes during peak biomass season in 2010.

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<th>Eukaryote (cells L⁻¹)</th>
<th>Cyanobacteria (cells L⁻¹)</th>
<th>Total Biomass (cells L⁻¹)</th>
<th>Cyanobacteria (%)</th>
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Table A.5 Surface water temperature, macronutrient and Fe concentrations for each of the 25 oligotrophic study lakes during peak biomass season in 2011.

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<th>Date (2011)</th>
<th>Surface Temp (°C)</th>
<th>pH</th>
<th>DOC (µg L(^{-1}))</th>
<th>TN (µg L(^{-1}))</th>
<th>TP (µg L(^{-1}))</th>
<th>N:P (Molar)</th>
<th>NO(_3^-) (µg L(^{-1}))</th>
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Table A.6 Phytoplankton metrics and siderophore concentrations for each of the 25 oligotrophic study lakes during peak biomass season in 2011.

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<th>Cyano (cells L⁻¹)</th>
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Appendix B: Protocol for the collection and determination of hydroxamate and catecholate siderophores in freshwater lakes

This protocol was written by Ryan J. Sorichetti and Xue L. Du and completed on December 7th, 2011.

This protocol was developed for the collection and determination of hydroxamate type siderophores in freshwater lake samples. This protocol can also be applied to additional experimental studies (such as mesocosm, grow-out and chemostat experiments) with varying quantities of water available for sample collection and analyses. Appropriate corrections to volumes for sample filtering, processing, chromatography and analyses are required for applications with limited sample volumes. This protocol outlines a combination of previously published methodology. Water collection, filtration, and characterization methodology is adapted from Macrellis et al. (2001). Methodology for the quantification of hydroxamate siderophore concentration is adapted from Gilliam et al. (1981).

PREPARATION AND APPARATUS

1) Read all instructions outlined in this protocol prior to entering the field and always have a printed copy on your possession while in the field during sample collection.

2) Ensure all sample collection bottles, vials, and tubing are soaked with Contrad 70 detergent (www.deconlabs.com) for up to 24 hours, rinsed well with de-ionized water (DIW), dried, capped, and labeled prior to entering the field for sample collection.

3) Ensure the following apparatus’ and materials are available and operational prior to entering the field for sample collection:
   - **Submersible water pump filtration apparatus**: submersible filter pump, gas powered generator, extra fuel, hoses, Y-valve, 6 male-to-male piping connectors, plastic tubing, pre-filter sponge, 3 filter canisters, filter cartridges (60 μm, 30 μm, and 1 μm), and two clean garbage bins (or similar).
   - **Column apparatus #1**: 4.5 cm i.d. x 24 cm plexiglass column with 50mL(GO)/200 mL of XAD-16 resin. Bottom of column is closed off with glass wool and cheesecloth to allow liquid through but not resin beads.
   - **Column apparatus #2**: 2.5 cm i.d. x 42 cm plexiglass column with Biogel P6 resin (800-6,000 dalton (Da) particle sizes).
   - **Column apparatus #3**: 1.5 cm i.d. x 77 cm plexiglass column with Biogel P4 resin (300-4,000 Da particle sizes).
- **Spectrophotometer**: with 1 mm and 10 mm path length cuvette cell and holder.
- **Rotary evapourator**: with temperature controller.
- **Autoclave**: with timer and temperature controller.

4) Ensure all chemicals, reagents, and standards are available and ready for use.

**WATER COLLECTION AND FILTRATION**

1) Determine the desired volume of lake water to be filtered for siderophore analysis. If possible, it is best to conduct sample filtration on the lake shore using the submersible water pump filtration apparatus. If filtration will be conducted on the lake shore where sample volume is not a limiting factor, filtration and sample collection of up to 1 L (GO)/40 L is recommended. If it is not possible for filtration to occur on the lake shore, collect as much water volume as possible in clean carboys for transport to the laboratory.

2) Water filtration is conducted in a stepwise method using the submersible water pump filtration apparatus in the following order of filtration steps;
   - Pour raw lake water collected in the clean carboys from the center of the lake into the first pre-cleaned garbage can through a steel mesh sieve lined with double-layered cheesecloth to filter out large organisms.
   - Filter water directly from the lake (or carboy) into a pre-cleaned garbage bin (or similar sized large container) first through the sponge pre-filter with no filter cartridge yet installed to exclude large particulate matter – this is now your primary filtered water.
   - Insert a 60 μm filter cartridge into the first canister; insert a 30 μm filter cartridge into the subsequent canister; insert a 1 μm filter cartridge into the final canister and place the submersible water pump into the garbage bin containing your primary filtered water and filter into a second pre-cleaned garbage bin to obtain your secondary/final filtered water.

The final filtered water will be retained for further processing and analysis.

**COLUMN CHROMATOGRAPHY**

Following filtration, the final filtered water is then subjected to column chromatography to isolate siderophores in the final filtered water.

1) Pass all final filtered water through column apparatus #1 (as described in the PREPARATION AND APPARATUS section) at a rate no faster than 1.2 L/min to ensure maximum adsorption of siderophores to XAD-16 resin. Be sure to note the exact volume of final filtered water passed through column apparatus #1.

2) Once all final filtered water has been passed through column apparatus #1, pass Milli-Q water (MQW) through column apparatus #1 to thoroughly rinse the XAD-16 resin loaded with sample.
3) Pass 500 ml of methanol (three resin bed volumes) through column apparatus #1 to elude the isolated siderophores sample.

4) Methanol eluent containing isolated siderophores is retained and stored in the dark and in a freezer until further processing and analysis. If a freezer is not immediately available, methanol eluent containing isolated siderophores can be stored in the dark at no more than 4 °C.

5) XAD-16 resin in column apparatus #1 can be regenerated for multiple sample use by flushing with methanol and rinsing with MQW.

**ABSORBANCE SCAN**

This scan will act as a pre-screening method to detect the initial presence of siderophores and its binding effect to iron.

1) Concentrate the methanol eluent for each sample using rotary evapouration at a temperature less than 60°C until approximately 10 mL of methanol remains.

2) Extract 2 mL of concentrated eluent and deposit into 10 mm path length cuvette cell and place into holder. Run a full spectrum absorbance scan.

3) Add 3 mL of 1.0 mg L⁻¹ ferric chloride solution (FeCl₃) to the concentrated eluent and wait for 5 minutes to allow for reactions to occur. Complete scan again.

4) Compare absorbance values to determine whether siderophores are present in solution and binding to iron based on standard absorbance rates for methanol and iron and note arbitrary intensity (a.u.)

**ARNOW ANALYSIS**

The Arnow test (Arnow, 1937) is employed to quantitatively determine the concentration of catecholate compounds (catecholate siderophores) in lake water with a method detection limit of 0.02 µM.

1) Pipette 1.0 mL of concentrated sample in methanol into a 5 mL glass screw capped test tube (loosely screwed). Pipette 1.0 mL of each working standard solution into separate 5 mL glass screw capped test tubes (loosely screwed).

2) Add to each test tube (samples and standard solutions), in the order given, the following reagents, mixing well after each addition:
   - 1 mL of 0.5 N hydrochloric acid (HCl).
   - 1 mL of nitrite molybdate reagent (dissolve 10 g NaNO₂ and 10 g Na₂MoO₄ in 100 mL of MQW), a yellow colour will result.
   - 1 mL of 1 N sodium hydroxide (NaOH), a red colour will result.
- Dilute with MQW to a final volume of 5 mL and mix well.

3) Measure sample absorbance on a spectrophotometer in a 10 mm path length cuvette cell at $\lambda = 500$ nm.

4) The concentration of the unknown sample can be determine by generating a standard curve with 2,3-dihydroxybenzoic acid standards as described in Arnow (1937).

CZAKY ANALYSIS

The Czaky test (Gillam et al., 1981) is employed to quantitatively determine the concentration of hydroxamic acids (hydroxamate siderophores) in lake water with a method detection limit of 0.02 µM. Lee and Roughan (1971) recommend the Czaky test be conducted in the absence of light as upwards of a 10% decrease in measured hydroxylamine can be expected due to a light initiated free radical mechanism for the oxidation of hydroxylamine with iodine.

1) Pipette 2.0 mL of concentrated sample in methanol into a 15 mL glass screw capped test tube (loosely screwed). Add 2 mL of 3 M sulfuric acid (H$_2$SO$_4$) and hydrolyze the solution by autoclaving at 100°C for six hours.

2) After cooling to room temperature, transfer the hydrolyzed solution into a 50 mL volumetric flask and add in the following order:
   - 7 mL of 2 M sodium acetate solution (NaC$_2$H$_3$O$_2$).
   - 2 mL of sulfanilamide solution (C$_6$H$_8$N$_2$O$_2$S, 1% w/v in 30% v/v acetic acid).
   - 2 mL of iodine solution (I, 0.65% w/v in 1 w/v KI solution). 
   Swirl after each addition and allow the mixture to react for five minutes.

3) Remove any excess iodine by adding 4 mL of sodium arsenite solution (0.0065% w/v in MQW).

4) Add 2 mL of N-(1-Naphthyl)ethylenediamine solution (C$_{12}$H$_{14}$N$_2$·2HCl, 0.05% w/v in MQW) and let the reaction mixture sit for 30 minutes to complete colour development. After 30 minutes, dilute the sample to the 50 mL mark with MQW.

5) Measure sample absorbance on a spectrophotometer in a 100 mm path length cuvette cell at $\lambda = 543$ nm.

6) The concentration of the unknown sample can be determine by generating a standard curve with hydroxylamine standards as described in Gilliam et al. (1981), under the experimental section’s list of reagents, apparatus, and procedure.
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Curriculum Vitae

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Post-secondary Education and Degrees:
The University of Western Ontario
London, Ontario, Canada
2008-2013 Ph.D.

University of Waterloo
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Honours and Awards:
NSERC PGS-D3, 2010-2013
Great Lakes Futures Project Honorarium, 2012
Western Graduate Thesis Research Fund, 2012
Western Environment & Sustainability Excellence Award, 2012
Western Environment & Sustainability Travel Award, 2012
Gordon Research Conference Travel Award, 2011
Western Graduate Thesis Research Fund, 2011
Western Biology Student Travel Award, 2010
Western Environment & Sustainability Travel Award, 2009
Gordon Research Conference Travel Award, 2009
Western Environment & Sustainability Student Bursary, 2009
Western Graduate Student Teaching Award, 2009
CIDA/AUCC International Internship Program, 2007

Related Work Experience
Graduate Research Assistant
The University of Western Ontario Watershed Research Facility
2013-present

Graduate Teaching Assistant
The University of Western Ontario
2008-2013

GRA, Lake Naivasha Sustainability Project, Kenya
The University of Western Ontario
2011

Undergraduate Honours Thesis Committee Member
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2009-2010
CIDA/AUCC Intern, Lake Naivasha Sustainability Project, Kenya
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2007-2008

Field and Laboratory Technician
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Field and Laboratory Technician
The University of Waterloo Aquatic Ecology Group
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Published Manuscripts:

Sorichetti R.J., McLaughlin J.T., Creed I.F. & Trick C.G. (2014) Suitability of a
cytotoxicity assay for detection of potentially harmful compounds produced by

cyanobacterial predominance in oligotrophic lakes. Freshwater Biology. DOI:
10.1111/fwb.12295.

community composition, and silica content of diatoms epiphytic on Cladophora

Manuscripts in Submission:

Cornwell E.R., Goyette J-O., Sorichetti R.J., Allan J.D., Kashian D.R., Sibley P.K.,
Taylor W.D. & Trick C.G. Biological and chemical contaminants as a driver of
change in the Great Lakes – St. Lawrence River basin. Submitted to Journal of
Great lakes Research.

Molot L.A., Watson S.B., Creed I.F., Trick C.G., McCabe S.K., Verschoor M.J.,
Sorichetti R.J., Powe C., Venkiteswan J.J. & Schiff S.L. A novel model for
cyanobacteria bloom formation: the critical role of anoxia and ferrous iron.
Submitted to Freshwater Biology.

Sorichetti R.J., Creed I.F. & Trick C.G. Evidence for iron-limited cyanobacterial
siderophore use and the potential role of DOM influencing siderophore
bioavailability in oligotrophic lakes. Submitted to Freshwater Biology.