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# The influence of iron, siderophores and refractory DOM on cyanobacterial biomass in oligotrophic lakes

RYAN J. SORICHETTI*, IRENA F. CREED* AND CHARLES G. TRICK*,†

*Department of Biology, Western University, London, ON, Canada

†Schulich School of Medicine and Dentistry, Western University, London, ON, Canada

## SUMMARY

1. Our conceptual understanding of factors that promote cyanobacterial growth is inadequate in the face of rising public concern about cyanobacterial blooms in oligotrophic freshwater lakes.
2. We hypothesised that cyanobacterial density would be highest in lakes with low levels of phosphorus (P), nitrogen (N), total dissolved iron (TDFe) and dissolved organic matter (DOM) with labile properties, where cyanobacteria use siderophores to scavenge Fe and overcome Fe limitation. We tested this hypothesis by measuring cyanobacterial density during peak biomass in 25 oligotrophic lakes representing gradients in total P (TP), nitrate, TDFe and DOM concentrations.
3. Total phytoplankton biomass, using chlorophyll-*a* (chl-*a*) as a proxy, was a function of TP ($r^2$ = 0.83, $P$ < 0.001). Cyanobacterial density was highest in lakes with low chl-*a*, low TP, variable (low and high) nitrate and low TDFe. Regression tree analysis confirmed that TDFe, specifically low concentrations (<3.2 μg $L^{-1}$), gave rise to the highest cyanobacterial densities in lakes.
4. All lakes had detectable concentrations of hydroxamate and/or catecholate siderophores. In lakes with relatively low TDFe (<3.2 μg $L^{-1}$), cyanobacterial density was positively correlated with hydroxamate siderophore concentration ($r^2$ = 0.77, $P$ = 0.01). In lakes with higher TDFe (≥3.2 μg $L^{-1}$), cyanobacterial density was positively correlated with nitrate ($r^2$ = 0.84, $P$ < 0.001) and ammonium ($r^2$ = 0.75, $P$ < 0.001) concentrations.
5. Dissolved organic matter may have an overriding control on cyanobacterial density, with cyanobacterial densities typically highest where DOM concentrations were low (<5 mg $L^{-1}$) and with a humification index <5. These findings suggest that DOM with labile properties may allow cyanobacteria to gain access to Fe complexed with DOM and thus to overcome Fe limitation, while DOM with refractory properties may bind Fe tightly so that Fe is not readily bioavailable to cyanobacteria.
6. A new conceptual model is presented that emphasises the potential influence of DOM quantity and quality on the functioning of siderophores and the provision of a supply of Fe to cyanobacteria in lakes with low macronutrient supply.

*Keywords*: cyanobacteria, dissolved organic matter, iron, oligotrophic, siderophore

## Introduction

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

Public concern about the emergence of cyanobacterial harmful blooms in oligotrophic lakes is rising. Cyanobacteria are often bloom forming, having the capacity to

Correspondence: Irena F. Creed, Department of Biology, Western University, London, ON, Canada, N6A 5B7.
E-mail: icreed@uwo.ca

 

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, and posing serious human, animal and ecosystem health concerns (Mur, Skulberg & Utkilen, 1999).

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

Studies investigating 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, Hudson and Price (1991) showed that $N_2$-fixing cyanobacteria require Fe at concentrations up to 20 times higher than 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. 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 specialised 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 and plays a critical role in N and P uptake. The bioavailable N pool is dependent on Fe supply required for N assimilation (NtR) and $N_2$ 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. We need to understand the use of siderophores by cyanobacteria in oligotrophic lakes and the potential for DOM to bind Fe and siderophores in lakes.

The purpose of this study was to determine whether the presence of siderophores correlates with elevated cyanobacterial biomass relative to the biomass of eukaryotic phytoplankton 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) and would correlate with 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 additionally 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 scavenge Fe from DOM complexes and overcome Fe limitation. Focusing on a set of oligotrophic lakes with similar physical characteristics but different macro- and micronutrient conditions, we aimed to determine whether Fe limits cyanobacterial growth and whether the presence of Fe-binding siderophores alleviates Fe limitation.

## Methods

### *Study sites*

For this study, 25 oligotrophic lakes were selected based on public concern for the potential of cyanobacterial harmful blooms in the lakes (Table 1). The lakes were characteristically shallow, thermally stratified during the warm summer months, and dimictic with major mixing events during spring snowmelt and autumn storms. These two major periods of hydrological connectivity between land and lake represent important episodes of terrestrial nutrient input (Creed & Beall, 2009; Mengistu, Quick & Creed, 2013).

### *Lake water chemistry sample collection*

In 2011, the lakes were sampled once during the period from late September to late October, corresponding to peak phytoplankton biomass according to previous growing season biomass data from the same lakes (Sorichetti, Creed & Trick, 2014). While on the lake, temperature and pH were measured at 1 m depth below the lake surface using a YSI 600 QS multiparameter sonde with a YSI 650 MDS display (YSI Incorporated, Yellow Springs, OH, U.S.A). 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 centre of the lake and stored in the dark on ice in a cooler until returning to the field laboratory. Best efforts were made to sample outside any phytoplankton bloom, if present, and not directly in the highest density to avoid sampling senescent cells and thus to capture actively growing biomass.

### *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

**Table 1** Coordinates and physical characteristics of lakes in the Algoma Highlands of central Ontario

| Lake | Latitude (N) | Longitude (W) | Max depth (m) | Thermocline depth (m) | Surface temp. (ºC) |
|---|---|---|---|---|---|
| Negick | 47°12′20.35″ | 84°29′22.81″ | 5.3 | 3.3 | 14.0 |
| Caysee | 47°10′58.05″ | 84°39′22.84″ | 1.3 | 1.1 | 14.8 |
| Huff | 47°10′15.40″ | 84°33′56.25″ | 4.0 | 1.0 | 16.0 |
| Tay | 47°10′01.60″ | 84°17′13.23″ | 0.8 | 0.4 | 13.0 |
| Airport | 47°09′22.04″ | 84°17′34.80″ | 7.7 | 2.2 | 13.6 |
| Mystery | 47°07′24.18″ | 84°16′25.46″ | 0.6 | 0.4 | 13.0 |
| Upper Griffin | 47°05′09.96″ | 84°24′05.27″ | 8.1 | 2.5 | 16.5 |
| Lower Griffin | 47°04′53.95″ | 84°25′11.11″ | 7.8 | 2.2 | 16.3 |
| Big Turkey | 47°02′51.59″ | 84°25′10.39″ | 42.7 | 3.7 | 15.7 |
| Little Turkey | 47°02′31.15″ | 84°24′28.20″ | 7.3 | 2.4 | 15.8 |
| Upper Tilley | 47°00′56.46″ | 84°23′15.65″ | 6.1 | 1.4 | 16.6 |
| Lower Tilley | 46°59′59.39″ | 84°23′13.91″ | 1.5 | 0.5 | 15.5 |
| Carp | 46°58′13.61″ | 84°33′38.93″ | 1.5 | 0.3 | 15.2 |
| Sill | 46°46′20.22″ | 84°15′14.01″ | 7.3 | 2.6 | 16.2 |
| Cloudy | 46°26′19.92″ | 83°55′59.80″ | 7.4 | 2.0 | 17.2 |
| Constance | 46°25′53.21″ | 83°13′30.35″ | 7.8 | 4.0 | 17.6 |
| Appleby | 46°25′39.64″ | 83°20′55.55″ | 5.1 | 3.2 | 16.5 |
| Woodrow | 46°24′31.70″ | 83°19′57.14″ | 2.0 | 0.7 | 15.8 |
| Round | 46°23′26.86″ | 83°49′37.43″ | 3.1 | 1.1 | 15.5 |
| Desbarats | 46°23′11.83″ | 83°55′55.66″ | 9.1 | 2.2 | 16.8 |
| Ottertail | 46°22′48.61″ | 83°45′01.56″ | 3.0 | 0.7 | 14.8 |
| Hilton Beach | 46°15′54.49″ | 83°53′30.37″ | 6.2 | 1.5 | 15.1 |
| Bright | 46°15′16.00″ | 83°16′47.00″ | 3.1 | 1.1 | 16.4 |
| Twin | 46°13′51.80″ | 83°55′43.38″ | 3.5 | 1.6 | 16.1 |
| Dean | 46°13′39.17″ | 83°10′43.87″ | 14.9 | n.a. | 16.8 |

n.a., Data not collected.

lakes upon return to shore and 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.
4. 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.

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

### *Phytoplankton*

A 500 mL subsample of lake water was filtered through 0.7-μm Whatman GF/F filters (GE Healthcare Life Sciences, Baie d'Urfe, QC, Canada) and analysed for chlorophyll-*a* (chl-*a*) using a Turner 10-AU field fluorometer (Turner Designs, Sunnyvale, CA, U.S.A) according to EPA Method 445.0 (Arar & Collins, 1997). A 3.5 mL subsample of unfiltered lake water was preserved with 1% buffered formaldehyde (v/v) in sterile 5-mL cryule vials (Wheaton, Millville, NJ, U.S.A), and phytoplankton community composition was assessed using a BD FACSCalibur flow cytometer (BD Biosciences, Sparks, MD, U.S.A) 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, U.S.A).

### *Nutrients*

A 90 mL subsample of unfiltered lake water was preserved with 10% $H_2SO_4$ (v/v) in screw-top borosilicate tubes. Total phosphorus (TP) concentration was assessed by autoclaving for 30 min in sulphuric acid–persulphate media to convert all P to orthophosphate at 121°C and presented to a Technicon AutoAnalyzer (AAII) System with a method detection limit (MDL) of 0.02 μM (SEAL Analytical, Mequon, WI, U.S.A).

Total N was assessed using a Shimadzu TOC-$V_{CPH}$ with TNM-1 and ASI-V auto-sampler (MDL = 100 μg $L^{-1}$) (Shimadzu, Kyoto, Japan). A 300 mL subsample of lake water was filtered through 0.45-μm Pall Life Sciences (Mississauga, ON, Canada) polysulphonate membrane disc filters and analysed 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).

### *DOM*

Dissolved organic matter (0.45-μm filtered) was assessed using a Shimadzu TOC-$V_{CPH}$ with TNM-1 and ASI-V auto-sampler (MDL = 4 μg $L^{-1}$) (Shimadzu, Kyoto, Japan). A 100 mL subsample 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 and McKnight (2005) on a Cary Eclipse spectrofluorometer (Agilent Technologies, Santa Clara, CA, U.S.A) 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 240–450 nm at 2-nm intervals and with an emissions wavelength range of 300–600 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 to 800 nm on a Cary 300 UV-Vis spectrophotometer (Agilent Technologies). PARAFAC analysis on EEMs was conducted in MATLAB R2009B (MathWorks,

Natick, MA, U.S.A) according to Cory and McKnight (2005) to evaluate the degree of DOM humification in the study lakes.

### *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).

### *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 the relationships between TP and chl-*a* concentrations; DOM and the concentration of siderophores; the degree of DOM humification and the concentration of siderophores; the concentration of hydroxamate siderophores and cyanobacterial density; and the concentration of nitrate and cyanobacterial density. Pearson's correlation analysis (critical $\alpha = 0.05$) was used to investigate autocorrelation among lake nutrient concentrations, siderophore concentrations and cyanobacterial density. Statistical measures were performed in SigmaPlot (v.11.0, SYSTAT Software, Chicago, IL, U.S.A).

Regression tree analysis was performed in R (v.2.15.3, Lucent Technologies, Murray Hill, NJ, U.S.A) using the 'rpart' package to investigate the chemical determinants of chl-*a* and cyanobacterial density. Chemical parameters incorporated into the regression tree model included TP, TN, TN/TP, nitrate, ammonium, TDFe, sulphate, DOC, calcium, magnesium, chloride, pH, and surface water temperature.

## Results

### *Phytoplankton community structure during peak biomass*

Lake phytoplankton community characteristics including chl-*a* concentration, total phytoplankton density and the density of cyanobacteria and eukaryotes that comprised total density are presented in Table 2.

Among the study lakes, total phytoplankton biomass (estimated by the concentration of chl-*a*) 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 $>5.0 \times 10^6$ cells $L^{-1}$ as lakes having high cyanobacteria and $<5.0 \times 10^6$ cells $L^{-1}$ as lakes having low cyanobacteria.

### *Nutrient ranges during peak biomass*

Lake chemical characteristics are presented in Table 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

**Table 2** Lake phytoplankton community characteristics (median, range, 25th and 75th percentile) for chl-*a*, eukaryotes, cyanobacterial and total phytoplankton density in lakes in the Algoma Highlands of central Ontario

| $N = 25$ | Chl-*a* (μg $L^{-1}$) | Eukaryotes (cells $L^{-1}$) | Cyanobacteria (cells $L^{-1}$) | Total density (cells $L^{-1}$) |
|---|---|---|---|---|
| Median | 3.5 | $1.6 \times 10^8$ | $2.1 \times 10^6$ | $1.6 \times 10^8$ |
| Range | 19.8 | $4.5 \times 10^8$ | $4.6 \times 10^7$ | $4.5 \times 10^8$ |
| 25% | 1.8 | $1.1 \times 10^8$ | $1.0 \times 10^6$ | $1.1 \times 10^8$ |
| 75% | 6.3 | $2.2 \times 10^8$ | $7.1 \times 10^6$ | $2.2 \times 10^8$ |

 

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.

### *Phytoplankton and nutrients during peak biomass*

Total P was positively correlated total phytoplankton biomass, as estimated by chl-*a* ($r^2 = 0.83$, $P < 0.001$) (Fig. 1). Cyanobacteria had highest density when total phytoplankton biomass was relatively low and lowest density when total phytoplankton biomass was relatively high (Fig. 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. 2b). Nutrient conditions that occurred when cyanobacterial density was highest included low concentrations of TP (Fig. 3a), variable (low and high) nitrate (Fig. 3b) and low TDFe (Fig. 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. 4a,b).

Pearson's 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's $r = 0.88$, $P < 0.05$; Table 4) and linearly ($r^2 = 0.77$, $P = 0.01$) correlated with the concentration of hydroxamate siderophores. In lakes with TDFe concentration ≥3.2 μg $L^{-1}$, cyanobacterial density was positively (Pearson's $r = 0.92$ and 0.86, $P < 0.05$; Table 5) and linearly ($r^2 = 0.84$ and 0.75, $P < 0.001$) correlated with nitrate and ammonium concentrations, respectively.

### *Dissolved organic matter*

Dissolved organic matter ranged from *c*. 450 to 19 600 μg $L^{-1}$. DOM composition was variable among the study lakes. EEMs revealed signatures characteristic of DOM with humic- and protein-like properties. The degree of DOM humification ranged from 2.4 to 19.1, with a median value of 5.8 among all lakes.

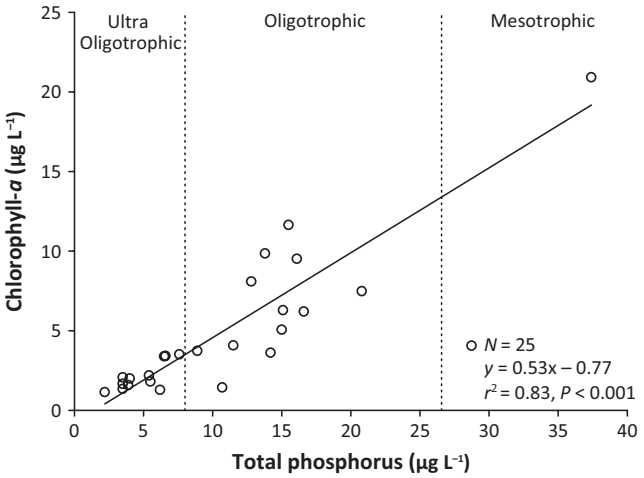


**Fig. 1** Total phosphorus versus phytoplankton biomass, using chl-*a* as a proxy, during peak biomass in lakes in the Algoma Highlands of central Ontario. Vertical lines represent trophic status definitions based on TP for ultra-oligotrophic (TP = 0–8 μg $L^{-1}$), oligotrophic (TP = 8–26.7 μg $L^{-1}$) and mesotrophic (TP = 26.7–84.4 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 ($r^2 = 0.65$, $P < 0.001$).

### *Siderophores*

Lakes with both high (>5.0 × $10^6$ cells $L^{-1}$) and low (<5.0 × $10^6$ cells $L^{-1}$) cyanobacterial density had hydroxamate and/or catecholate siderophores (Fig. 5a,b). 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). Cyanobacterial density in lakes was highest when DOM concentration was relatively low (Fig. 6a) and when the degree of DOM humification was relatively low (Fig. 6b). Catecholate siderophore concentration was positively correlated with DOM concentration ($r^2 = 0.65$, $P < 0.001$) and in particular the refractory

**Table 3** Lake chemical characteristics (median, range, 25th and 75th percentile) for chemical characteristics of lakes in the Algoma Highlands of central Ontario

| $N = 25$ | pH | DOM (μg $L^{-1}$) | TP (μg $L^{-1}$) | TN (μg $L^{-1}$) | $NO_3^-$ (μg $L^{-1}$) | $NH_4^+$ (μg $L^{-1}$) | TDFe (μg $L^{-1}$) | Hydroxamate (μg $L^{-1}$) | Catecholate (μg $L^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| Median | 7.4 | 3075.6 | 8.9 | 364.6 | 4.0 | 8.0 | 7.7 | 111.3 | 4109.3 |
| Range | 1.9 | 19117.9 | 35.2 | 316.8 | 310.3 | 61.1 | 151.1 | 825.7 | 6172.8 |
| 25% | 7.3 | 2318.7 | 5.4 | 305.3 | 1.8 | 3.0 | 3.2 | 0.0 | 3782.2 |
| 75% | 7.5 | 4666.8 | 15.0 | 426.0 | 43.5 | 12.0 | 35.8 | 327 | 5279.0 |

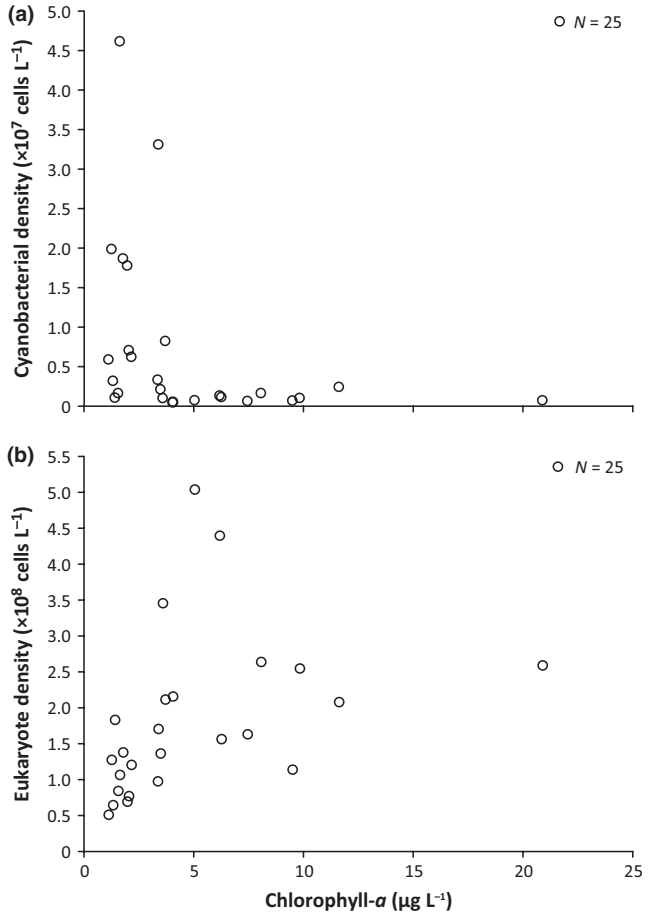


**Fig. 2** Chlorophyll-*a* concentration versus (a) cyanobacterial and (b) eukaryote density during peak biomass in lakes in the Algoma Highlands of central Ontario.

component of DOM as indicated by the degree of DOM humification ($r^2 = 0.72$, $P < 0.001$). No relationship was found between hydroxamate siderophore concentration and either DOM concentration or the degree of DOM humification.

### *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 6; Fig. 3b). The three study lakes with high cyanobacteria and highest nitrate concentration had cyanobacterial communities comprised of *Anabaena* sp., *Gloeocapsa* sp., *Microcystis* sp. and *Nostoc* sp. (Table 6; Fig. 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.

## Discussion

### *Phytoplankton and nutrients during peak biomass*

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 statuses as indicated by the correlation between TP and chl-*a* concentration. 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 and explained 83% of phytoplankton

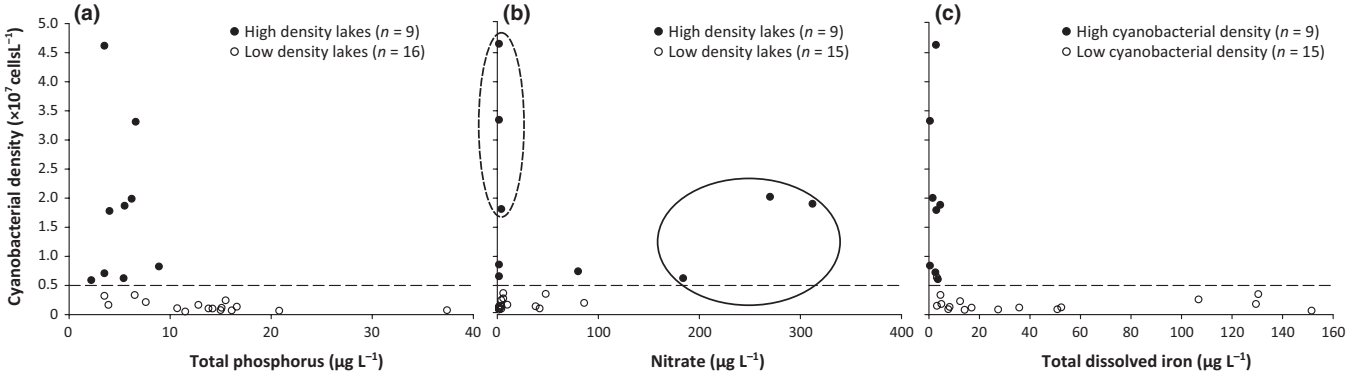


**Fig. 3** (a) Total phosphorus, (b) nitrate and (c) total dissolved iron concentration versus cyanobacterial density during peak biomass in lakes in the Algoma Highlands of central Ontario. Three study lakes (solid circle) deviated from the hypothesised trend, having high cyanobacterial density and 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.

 

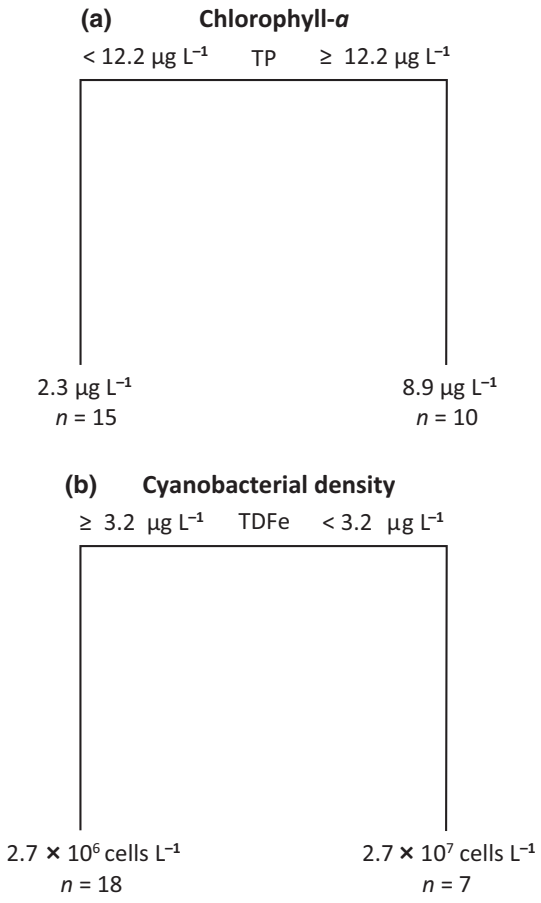


**Fig. 4** Regression trees depicting the chemical determinants of (a) phytoplankton biomass, using chlorophyll-*a* as a proxy and (b) cyanobacterial density in lakes in the Algoma Highlands of central Ontario.

biomass, there was considerable variation in the achieved phytoplankton biomass 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 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 and 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 the species diversity of phytoplankton 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 was further supported by 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 was below the 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 and 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

**Table 4** Pearson's correlation matrix for nutrients, siderophores and cyanobacterial density in lakes with <3.2 µg $L^{-1}$ TDFe (critical $\alpha = 0.05$) in the Algoma Highlands of central Ontario. Hydroxamate siderophore concentration was positively and significantly correlated with cyanobacterial density. Data are presented as Pearson's *r* (sample *n*)

| | DOC | TN | TP | TN/TP | $NO_3^-$ | $NH_4^+$ | TDFe | Hydroxamate | Catecholate | Cyanobacteria |
|---|---|---|---|---|---|---|---|---|---|---|
| pH | *0.48* (7) | *0.67* (7) | *0.29* (7) | *0.09* (7) | *0.06* (7) | *0.44* (7) | *0.09* (7) | *−0.61* (7) | *0.24* (7) | *−0.58* (7) |
| DOC | | **0.83** (7) | *0.71* (7) | *−0.38* (7) | *−0.54* (7) | *0.16* (7) | *−0.53* (7) | *−0.06* (7) | *0.08* (7) | *−0.24* (7) |
| TN | | | **0.79** (7) | *−0.34* (7) | *−0.16* (7) | *0.26* (7) | *−0.59* (7) | *−0.31* (7) | *0.07* (7) | *−0.48* (7) |
| TP | | | | **−0.84** (7) | *0.04* (7) | *−0.04* (7) | **−0.84** (7) | *−0.18* (7) | *−0.20* (7) | *−0.27* (7) |
| TN/TP | | | | | *−0.16* (7) | *0.30* (7) | *0.69* (7) | *0.04* (7) | *0.37* (7) | *0.07* (7) |
| $NO_3^-$ | | | | | | *0.21* (7) | *−0.11* (7) | *−0.46* (7) | *−0.30* (7) | *−0.11* (7) |
| $NH_4^+$ | | | | | | | *−0.01* (7) | **−0.76** (7) | *−0.48* (7) | *−0.60* (7) |
| TDFe | | | | | | | | *−0.07* (7) | *0.24* (7) | *−0.06* (7) |
| Hydroxamate | | | | | | | | | *0.57* (7) | **0.88** (7) |
| Catecholate | | | | | | | | | | *0.52* (7) |

Pearson's *r* values in boldface indicate a statistically significant relationship between variables ($P < 0.05$) and italicised Pearson's *r* indicates no significant relationship.

**Table 5** Pearson's correlation matrix for nutrients, siderophores and cyanobacterial density in lakes with ≥3.2 μg $L^{-1}$ TDFe (critical $\alpha = 0.05$) in the Algoma Highlands of central Ontario. Inorganic N (nitrate and ammonium) was positively and significantly correlated with cyanobacterial density. Data are presented as Pearson's *r* (sample *n*). Pearson's *r* values in boldface indicate a statistically significant relationship between variables ($P < 0.05$), and italicised Pearson's *r* indicates no significant relationship

| | DOC | TN | TP | TN/TP | $NO_3^-$ | $NH_4^+$ | TDFe | Hydroxamate | Catecholate | Cyanobacteria |
|---|---|---|---|---|---|---|---|---|---|---|
| pH | **−0.72** (18) | **−0.55** (18) | *−0.01* (18) | *0.01* (18) | *0.14* (17) | *−0.06* (17) | **−0.56** (18) | *0.22* (18) | **−0.64** (18) | *0.11* (18) |
| DOC | | **0.69** (18) | *0.15* (18) | *−0.23* (18) | *−0.32* (17) | *−0.17* (17) | **0.64** (18) | *0.22* (18) | **0.91** (18) | *−0.15* (18) |
| TN | | | *0.07* (18) | *0.00* (18) | *−0.11* (17) | *−0.01* (17) | *0.34* (18) | *0.23* (18) | **0.54** (18) | *−0.07* (18) |
| TP | | | | **−0.68** (18) | *−0.47* (17) | *−0.35* (17) | *0.08* (18) | *0.07* (18) | *0.13* (18) | *−0.40* (18) |
| TN/TP | | | | | **0.60** (17) | *0.17* (17) | *−0.20* (18) | *−0.21* (18) | *−0.30* (18) | *0.40* (18) |
| $NO_3^-$ | | | | | | **0.81** (17) | *−0.32* (17) | *−0.17* (17) | *−0.38* (17) | **0.92** (17) |
| $NH_4^+$ | | | | | | | *−0.23* (17) | *−0.20* (17) | *−0.25* (17) | **0.86** (17) |
| TDFe | | | | | | | | *0.14* (18) | **0.75** (18) | *−0.19* (18) |
| Hydroxamate | | | | | | | | | *0.26* (18) | *−0.10* (18) |
| Catecholate | | | | | | | | | | *−0.22* (18) |

lakes favour the dominance of $N_2$-fixing cyanobacteria that compensate for reduced N loading by fixing atmospheric $N_2$ (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 lakes also had the lowest Fe concentrations of all study lakes (median TDFe concentration = 3.5 μg $L^{-1}$).

Among our set of lakes, cyanobacterial density was highest when TDFe concentration was low. This is consistent with laboratory (Kerry *et al.*, 1988) and lake (Sorichetti *et al.*, 2014) research showing that cyanobacteria are competitive over eukaryotes at low Fe concentrations. Previous research has attributed this competitive advantage to the ability of cyanobacteria when Fe-limited to use Fe-binding ligands, hydroxamate and catecholate siderophores. 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).

### *Siderophores in the study lakes during peak biomass*

This study represents the first documented record of siderophores 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 indicated that the factor primarily correlated with 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 with the concentration of hydroxamate siderophores. This provides a physiological indication that hydroxamate siderophores benefit the cyanobacterial population in lakes to overcome Fe

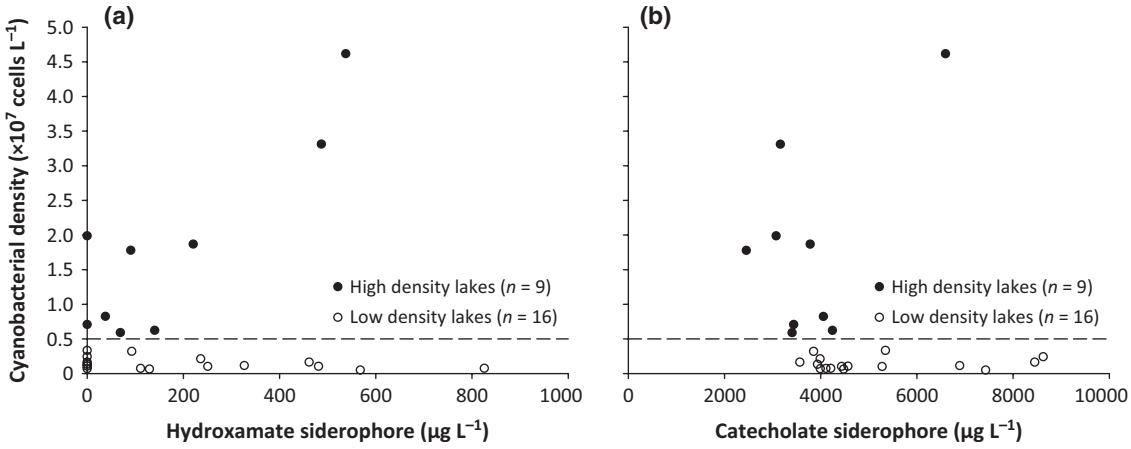


**Fig. 5** (a) Hydroxamate and (b) catecholate siderophore concentration versus cyanobacterial density during peak biomass in lakes in the Algoma Highlands of central Ontario. Black-filled data points are lakes with high cyanobacterial density (>5.0 × $10^6$ cells $L^{-1}$); non-filled data points are 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.

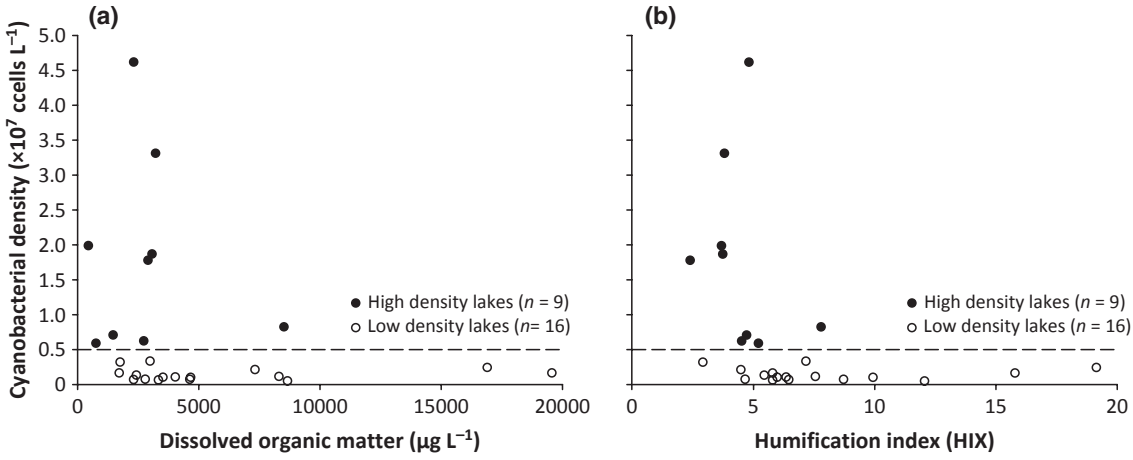


**Fig. 6** (a) Dissolved organic matter and (b) humification index versus cyanobacterial density during peak biomass in lakes in the Algoma Highlands of central Ontario. Black-filled data points are lakes with high cyanobacterial density (>5.0 × $10^6$ cells $L^{-1}$); non-filled data points are lakes 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.

limitation. In lakes with relatively higher TDFe concentrations (≥3.2 µg $L^{-1}$), cyanobacterial density was significantly correlated with nitrate and ammonium 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.

### *Potential influence of DOM on Fe bioavailability*

Using an analysis of Secchi depth measurements, we found no correlational relationship with cyanobacterial density (R.J. Sorichetti, I.F. Creed & C.G. Trick, unpublished data). We attributed this lack of a relationship between DOM concentration and the vertical distribution of cyanobacteria to some species of cyanobacteria having the potential to regulate their buoyancy in the water column to obtain optimal light levels. This suggests that DOM influences cyanobacterial biomass by some other process other than light attenuation.

Dissolved organic matter 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

**Table 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 $N_2$-fixing cyanobacterial genera with heterocysts, while the high cyanobacteria and high-nitrate lakes had non-$N_2$-fixing cyanobacterial genera or those with no heterocysts

| Lake | Cyanobacterial Density ($\times 10^6$ cells $L^{-1}$) | Nitrate ($\mu g\ L^{-1}$) | Genera Present | % Cyanobacteria (by density) | Heterocysts Observed |
|---|---|---|---|---|---|
| High cyanobacteria and high-nitrate lakes | | | | | |
| Cloudy | 18.7 | 312 | *Nostoc* sp. | 77.4 | No |
| | | | *Gloeocapsa* sp. | 12.9 | n.a. |
| | | | *Microcystis* sp. | 9.7 | n.a. |
| Hilton Beach | 20.0 | 270 | *Gloeocapsa* sp. | 58.3 | n.a. |
| | | | *Microcystis* sp. | 41.7 | n.a. |
| Upper Griffin | 5.9 | 184 | *Gloeocapsa* sp. | 94.4 | n.a. |
| | | | *Anabaena* sp. | 5.6 | No |
| High cyanobacteria and low-nitrate lakes | | | | | |
| Huff | 17.8 | 4.0 | *Microcystis* sp. | 77.8 | n.a. |
| | | | *Anabaena* sp. | 22.2 | Yes |
| Sill | 33.1 | 1.8 | *Microcystis* sp. | 50.0 | n.a. |
| | | | *Anabaena* sp. | 50.0 | Yes |
| Woodrow | 46.1 | 1.8 | *Anabaena* sp. | 100 | Yes |

n.a., Cyanobacterial genera that do not produce heterocysts for $N_2$ fixation.

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 high 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 degree of DOM humification). These findings support our hypothesis that lakes with DOM that has refractory properties (higher degree of DOM humification) 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 with DOM concentration ($r^2 = 0.65$, $P < 0.001$) and the degree of DOM humification ($r^2 = 0.72$, $P < 0.001$), while no such relationships were found with hydroxamate siderophore concentration. Additionally, catecholate siderophore concentrations in lakes were over 30 times higher than hydroxamate siderophore concentrations. We are unaware of studies available to compare the measured magnitude of catecholate siderophore concentration in lakes. It is reasonable to conclude 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).

### *Evidence for Fe regulation of nitrogen-use efficiency*

We compared cyanobacterial community composition in three lakes with highest nitrate concentrations (median = 270 $\mu g\ L^{-1}$) to three lakes with lowest nitrate concentrations (median = 1.8 $\mu g\ L^{-1}$). Both groups 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 of 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 to 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.

### *A new conceptual model*

Dissolved organic matter appears to have a critical role in regulating Fe availability to cyanobacteria and there are several possible, but presently untested, scenarios describing this potential influence (Fig. 7). DOM may serve as a ligand, binding 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 extent to which DOM binds Fe directly, and siderophores directly or indirectly, limiting Fe bioavailability to cyanobacteria.

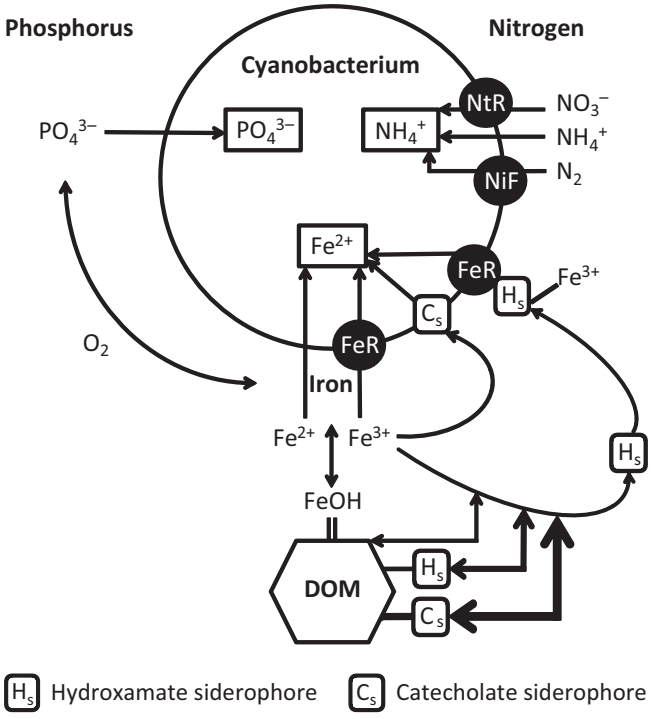


**Fig. 7** Conceptual model for macronutrient (P and N) assimilation by cyanobacteria with the influence of dissolved organic matter (DOM) and siderophores on Fe bioavailability. Thickness of the feedback lines indicates the strength of potential binding between DOM, hydroxamate siderophores or catecholate 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).

We found that cyanobacteria thrived in lakes with low Fe availability. Siderophores provide a strategy in cyanobacteria for Fe scavenging when Fe-limited. However, the bioavailability of siderophores to cyanobacteria appears to be regulated by DOM quantity and quality and therefore must be incorporated into any conceptual model considering Fe and macronutrient (N and P) uptake mechanisms (Fig. 7). We suggest that cyanobacteria use hydroxamate siderophores as a means of scavenging 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 cyanobacterial harmful bloom formation in lakes with a relatively low macronutrient supply. Future research is needed into the 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.

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