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Iron and iron-binding ligands as cofactors that limit cyanobacterial biomass across a lake trophic gradient

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. The frequency and intensity of cyanobacterial blooms (cyanoblooms) is increasing globally. While cyanoblooms in eutrophic (nutrient-rich) freshwater lakes are expected to persist and worsen with climate change projections, many of the 'new' cyanobloom reports pertain to oligotrophic (nutrient-poor) freshwater lakes with no prior history of cyanobloom occurrence.
2. Iron (Fe) is required in nearly all pathways of cyanobacterial macronutrient use, although its precise role in regulating cyanobacterial biomass across a lake trophic gradient is not fully understood.
3. In all lakes sampled representing a gradient in trophic status from oligotrophic to hypereutrophic (2.2–561.2 μg L^{-1} total phosphorus), the relative cyanobacterial biomass was highest at low predicted Fe bioavailability in eutrophic Alberta lakes (<1.0 × 10^{-22} mol L^{-1}) and low Fe concentration in oligotrophic Ontario Lakes (<3.2 μg L^{-1}).
4. Fe-binding organic ligands were measured within this range of low bioavailable Fe. Concentrations of ligands with reactive hydroxamate moieties were positively correlated to cyanobacterial biomass in lakes with low Fe bioavailability and supply, suggesting a possible cellular origin (i.e. siderophores) mediated by low Fe.
5. These findings suggest that Fe serves as a possible cofactor that maintains cyanobacterial biomass across a lake trophic gradient and that cyanobacteria invoke a similar Fe-scavenging system to overcome Fe limitation in lakes of all trophic states.

Keywords: cyanobacteria, iron, lake, limitation, trophic status

Introduction

Amidst increasing global concern about cultural eutrophication and cyanobacterial blooms (cyanoblooms) in freshwater ecosystems over the past five decades (Smith & Schindler, 2009), there remains disparity in our understanding of lake physical and water chemical parameters that regulate cyanoblooms in lakes (Schindler, 2012). The urgency to formulate a complete conceptual understanding of cyanobacterial biomass controls is greater now than ever. The frequency and magnitude of cyanoblooms is expected to increase with future climate change predictions (Paerl & Huisman, 2008, 2009). Changes in spatial patterns of cyanobloom occurrence have also occurred over the past two decades. Whereas cyanobloom occurrence has traditionally been associated with eutrophic aquatic ecosystems (Schindler, 1974), cyanoblooms are now reported in oligotrophic and mesotrophic freshwater systems, including *Gloeotrichia echinulata* blooms in north-eastern U.S.A. (Carey, Weathers & Cottingham, 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.

Predicting cyanobacterial dominance in lakes has traditionally been associated with elevated total nitrogen (N) or total phosphorus (P) concentrations (Downing, Watson & McCauley, 2001) or low atomic ratios of total N : P (Smith, 1983). While the conceptual model regarding the specific macronutrient N and P regulatory factors on cyanobloom development remains debated (Conley *et al*., 2009; Schindler, 2012), it is well understood that iron (Fe) is essential in regulating the

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

efficiency of macronutrient use by cyanobacteria, as it plays a critical role in N and P uptake (Molot *et al.*, 2014). Nitrogen assimilation (Lin & Stewart, 1998) and N_2 fixation (Murphy, Lean & Nalewajko, 1976) by diazotrophic cyanobacteria are dependent on Fe supply. The bioavailable P pool is dependent on the potential for Fe to bind to phosphate and precipitate from oxic surface waters (Moore & Reddy, 1994). Extracellular Fe-binding siderophores have been shown to be important sources of Fe to cyanobacteria (Wilhelm & Trick, 1994; Neilands, 1995), in turn regulating important macronutrient uptake. Field-based investigations have provided evidence for the role of Fe in regulating cyanobacterial biomass in lakes in North America (Molot *et al.*, 2010; Sorichetti, Creed & Trick, 2014a) and the use of Fe-binding siderophores that give cyanobacteria a competitive advantage for Fe acquisition when the supply or bioavailability of Fe in lakes is low (Hassler *et al.*, 2009; Sorichetti, Creed & Trick, 2014b).

Considerable evidence indicates that cyanobacteria respond to low-Fe conditions by either altering the expression of Fe transporters or by producing siderophores and their concomitant Fe-siderophore transport system (Wilhelm, 1995). Early studies isolated and elucidated the structure of specific siderophores from *Anabaena* spp. (Simpson & Neilands, 1976; Itou, Okada & Murakami, 2001) and documented that the production of Fe-binding ligands by cyanobacteria can (i) provide a competitive advantage for cyanobacteria (Murphy *et al.*, 1976); (ii) vary with N-Fe status (Kerry, Laudenbach & Trick, 1988); (iii) directly influence Fe transport into cyanobacteria (Goldman *et al.*, 1983; Singh, McIntyre & Sherman, 2003; Fujii, Rose & Waite, 2011a; Fujii *et al.*, 2011b; Stevanovic *et al.*, 2012); and (iv) moderate the toxicity of other metals (McKnight & Morel, 1980). More recent ecophysiological studies have indicated that Fe availability is strongly influenced by non-siderophore dissolved organic matter (DOM) compounds with Fe-binding abilities (Imai, Fukushima & Matsushige, 1999; Nagai *et al.*, 2006; Fujii *et al.*, 2014).

Most comparative field studies that have investigated nutrient regulatory factors and cyanobacterial biomass across a lake trophic gradient have focused on N and P (e.g. Jeppesen *et al.*, 2005; Smith & Schindler, 2009; Rigosi *et al.*, 2014). It remains unclear whether the role of Fe in regulating cyanobacterial biomass is dependent on lake trophic status or whether cyanobacterial communities in lakes of various trophic states respond similarly to Fe stress. The role of Fe in regulating cyanobacterial biomass and Fe-binding ligand utilisation is well documented in isolated laboratory studies (Kerry *et al.*, 1988; Wilhelm & Trick, 1994; Wilhelm, Maxwell & Trick, 1996; Wilhelm, MacCauley & Trick, 1998) and in marine field studies (Barbeau *et al.*, 2003; Eldridge *et al.*, 2004). However, few field studies have been conducted in freshwater environments (e.g. Murphy *et al.*, 1976; Sorichetti *et al.*, 2014a,b), and to the best of our knowledge, none compares the role of Fe in regulating cyanobacterial biomass across a lake trophic gradient. This study built upon the limited findings available from oligotrophic freshwater environments to explore the relationship between Fe supply and bioavailability to cyanobacterial biomass and Fe-binding ligand concentrations in temperate lakes that span a range of trophic states from ultra oligotrophic to hypereutrophic. We hypothesised that (i) cyanobacteria occur at higher biomass in lakes of all trophic states when bioavailable Fe concentration is low; (ii) cyanobacterial biomass is positively correlated to Fe-binding ligand concentration in lakes of all trophic states when bioavailable Fe concentration is low, suggesting cyanobacterial use of ligands to overcome Fe limitation; and (iii) despite differences in the supply (concentration) of DOM among lakes of all trophic states, the bioavailability of Fe is dependent on the potential for DOM to bind Fe.

Methods

Study sites

For this study, 25 lakes in the Algoma Highlands of central Ontario and 30 lakes in the Beaverhill catchment in central Alberta were selected to represent contrasting hydrologic and climatic regions of Canada (Fig. 1). Lakes with minimal direct anthropogenic influence, other than atmospheric deposition of potential contaminants, were chosen. Annual precipitation–potential evapotranspiration (P-PET) in the relatively wet Algoma Highlands in Ontario is 400–799 mm and P-PET in the relatively dry Beaverhill catchment is 100–199 mm (I. F. Creed, unpubl. data). Lakes in the Algoma Highlands in Ontario are characteristically shallow (<9 m), thermally stratify during the warm summer months, are dimictic with major mixing events occurring during the spring snowmelt and autumn storms, and experience variable hydrologic connectivity between landscape and lake throughout the growing season. Lakes in the Beaverhill catchment are characteristically shallow (<2 m) with a stable vertical water column, experience little to no mixing and are hydrologically disconnected from the surrounding landscape during summer months (July and August).

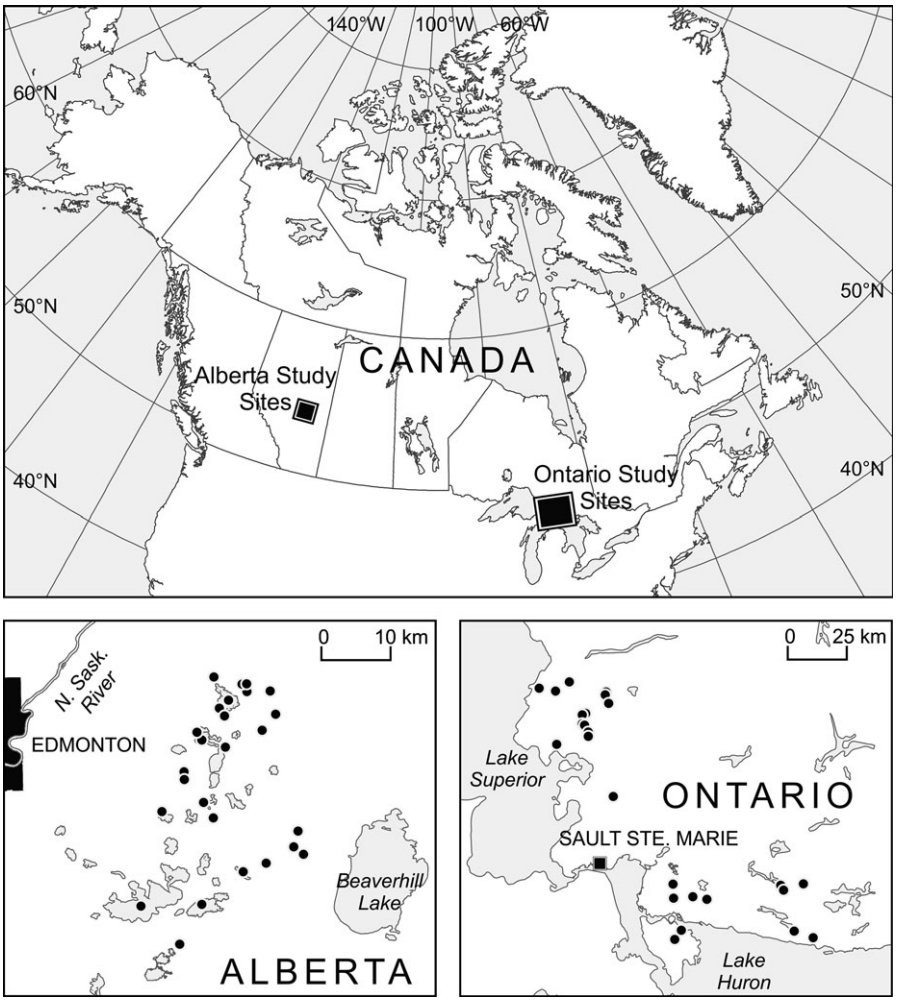

Fig. 1 Location of study lakes in Alberta and Ontario, Canada.

Field sampling

Water for chemical analyses was collected once during the algal bloom period, which was identified by visible surface algal blooms (scums) on lakes. Ontario lakes were sampled in September 2011, and Alberta lakes were sampled in August 2012. 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, U.S.A.). Lake surface water samples integrated to 1 m depth were collected in 500-mL pre-rinsed polyethylene bottles near the centres of the lakes, outside of algal blooms 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 algal blooms and not directly in the highest density to avoid sampling of senescent cells and capturing actively growing biomass.

Water sample collection and filtration methodology for the Fe-binding ligands was adapted from Macrellis *et al.* (2001). Large volume water filtration was conducted lakeside at all lakes immediately upon returning to shore within 20 min of water sample collection. A total of 40 L of bulk lake water was collected by manually lowering two 20-L cleaned and pre-rinsed polyethylene carboys to a maximum depth of 1 m from the lake surface then transferred into a 60-L cleaned and pre-rinsed polyethylene bin when arriving at shore. 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 conducted at the low-speed setting using a stepwise methodology to ensure filtration efficiency. Bulk lake water was first filtered through a sponge pre-filter to exclude large particulate matter then successively through 60-, 20-, then 1-μm cartridge filters. A total of 40 L of final filtered water for all lakes was transported back to the field laboratory for further processing.

Laboratory work

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

For 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 analysed for chlorophyll-*a* (chl-*a*) concentration using a Turner 10-AU field fluorometer (Turner Designs, Sunnyvale, CA, U.S.A.) according to EPA Method 445.0 (Arar & Collins, 1997). Chlorophyll-*a* was used as a proxy for total algal biomass in all lakes (Jeffrey & Humphrey, 1975). Phycocyanin (PC) was used as a proxy for total cyanobacterial biomass in all lakes (Gregor & Maršálek, 2005) and was measured directly in the Alberta lakes and indirectly in the Ontario lakes.

For Alberta lakes, a 200 mL sub-sample of lake water was filtered through 0.7-μm Whatman GF/F filters (GE Healthcare Life Sciences) and analysed for PC. Extraction of PC from GF/F filters was conducted according to Lawrenz, Fedewa & Richardson (2011). To lyse cells and extract PC, 1 mL of phosphate butter (10 mM, pH 6) was added to samples, which were then subjected to freeze-thawing (3×) followed by sonication (30 s: six 5 s pulses at 10 magnitude using a VerSonic 100 sonicator (VirTis, Gardiner, NY, U.S.A.)). Sonicate was then immersed in 5 mL of phosphate buffer and incubated in the dark for 24 h. Extracted samples were filtered through 0.45-μm Acrodisc Supor Membrane syringe filters (Pall Life Sciences, Port Washington, NY, U.S.A.) to clear extracted solution of filter particles and cellular debris. Absorbance readings were performed using a Beckman-Coulter DU60 spectrophotometer (Beckman-Coulter, Mississauga, ON, U.S.A.). Samples were read on the spectrophotometer using a 1 mL glass cuvette with 1 cm path length at 620 nm for PC.

For Ontario lakes, 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, U.S.A.), and density of cyanobacteria and eukaryotic algae (cells L^{-1}) 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. Phycocyanin concentrations for Ontario lakes were estimated using cyanobacterial density (as cells L^{-1}) according to the linear model ($y = 1.7x + 1.7$) by Brient *et al.* (2008) for monitoring cyanobacterial biomass in freshwater systems. The linear regression model proposed by Brient *et al.* (2008) was generated using 800 observations from 35 eutrophic lakes in western France. Brient *et al.* (2008) found a 10 to 15% range of variation in the number of cyanobacterial cells correlated to PC concentration with a confidence interval of 95% around a linear regression with $r^2 = 0.73$.

For nutrient analyses, a 90 mL sub-sample of unfiltered lake water was preserved with 10% H_2SO_4 (v/v) in screw-top borosilicate tubes. Total P concentration was determined by autoclaving for 30 min in sulphuric acid-persulphate medium 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 determined using a Shimadzu TOC-V_{CPH} with TNM-1 and ASI-V autosampler (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 analysed for nitrate and ammonium (colorimetry, MDL = 3.5 and 5.9 μg L^{-1}, respectively) and total dissolved Fe (TDFe, inductively coupled plasma spectrometry, MDL = 0.83 μg L^{-1}) according to the Ontario Ministry of the Environment and Energy Standards Development Branch (1996). Dissolved organic carbon (DOC, 0.45 μm filtered) concentration was determined using a Shimadzu TOC-V_{CPH} with TNM-1 and ASI-V autosampler (MDL = 4 μg L^{-1}) (Shimadzu, Kyoto, Japan). We used the lake trophic status definitions based on total P as outlined by Wetzel (2001) to determine the trophic states of lakes.

Ferric ion concentration in lakes was modelled in Visual MINTEQ (v.3.0, KTH Royal Institute of Technology, Stockholm, Sweden). Concentrations of the following nutrients and lake parameters were incorporated into the model for Alberta and Ontario lakes: DOC, total P, nitrate, ammonium, sulphate, TDFe, calcium, magnesium, chloride, pH and surface water temperature. The Stockholm Humic Model of humic organic matter and metal complexation in Visual MINTEQ was used (Gustafsson, 2001). The Stockholm Humic Model was run with an assumed ratio of fulvic : humic acids of 36% fulvic to 64% humic acids for Alberta lakes (Curtis & Adams, 1995) and 50% for Ontario lakes (Thurman & Malcolm, 1981). The ferric ion concentrations reported represent non-complexed ferric ions that are readily bioavailable for cellular uptake and expressed as a pFe value calculated as $\log_{10}$ [modelled ferric ion] (dimensionless).

Final filtered water for the analysis of Fe-binding ligands was processed using column chromatography to isolate the ligands. 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 the ligands to 200 mL of XAD-16 (amberlite) resin (Macrellis *et al.*, 2001). Once all final filtered water 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 the ligand sample. Finally, 500 mL of methanol was passed through the column to elute the isolated ligand 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 quantify 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 quantify 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

Mann–Whitney *U* rank sum analyses (critical $\alpha = 0.05$) to compare lake water chemistry and algal parameters between Alberta and Ontario were conducted in SigmaPlot (v.11.0; SYSTAT Software, Chicago, IL, U.S.A.). Linear regression analysis (critical $\alpha = 0.05$) was conducted in SigmaPlot (v.11.0, SYSTAT Software) to investigate the relationship between total P and total algal biomass. Non-metric multidimensional scaling (NMDS) ordination analysis was conducted in PC-ORD (v. 6.0; MjM Software Design, Gleneden Beach, OR, U.S.A.) to investigate the multidimensional relationships between lake water chemistry and total algal and cyanobacterial biomass. Regression tree analysis was conducted in R (v.2.15.3; Lucent Technologies, Murray Hill, NJ, U.S.A.) using the 'rpart' package to investigate lake water chemical determinants of total algal and cyanobacterial biomass. Lake water chemical parameters incorporated into the regression tree model included total P, soluble reactive P (SRP), total N, total N : P, nitrate, ammonium, TDFe, sulphate, DOC, calcium, magnesium, chloride, pH and surface water temperature. Statistical outliers in the data set were identified using the median absolute deviation nonparametric outlier test with a critical value of 5 (Barnett & Lewis, 1984).

Results

Lake chemical and algal conditions

Median absolute deviation nonparametric estimates identified three Alberta lakes as statistically significant outliers and were removed from all statistical analyses, figures and tables. Outlier status was driven by exceptionally high DOC (>146 mg L^{-1}) and low modelled ferric ion concentrations (pFe = < −36). The outlier lakes were relatively large and deep compared with the other 27 Alberta lakes. These outlier lakes had a surface area >1 200 000 m^2, while all other Alberta lakes had a surface area < 1 000 000 m^2. The maximum depths of the outlier lakes were >4.6 m, while all other Alberta lakes had a maximum depth < 2.0 m. Therefore, the final sample size was 27 lakes in Alberta.

Lakes in Alberta and Ontario spanned the entire range along the trophic gradient from oligotrophic to hypereutrophic (Fig. 2). Lakes in Ontario were oligotrophic to mesotrophic (except for one Ontario lake that was classified as eutrophic), whereas lakes in Alberta were eutrophic to hypereutrophic. Alberta lakes had significantly higher pH, DOC, total N, total P, total N : P, SRP, ammonium and TDFe concentrations than Ontario lakes ($P < 0.05$, Table 1). No significant differences were found between Ontario and Alberta lakes in their values for nitrate, modelled ferric ion bioavailability (as pFe), or the concentrations of hydroxamate and catecholate siderophores.

The results of the chemical equilibrium model used to estimate ferric ion concentration in lakes indicated that DOM in Alberta lakes bound significantly more ferric

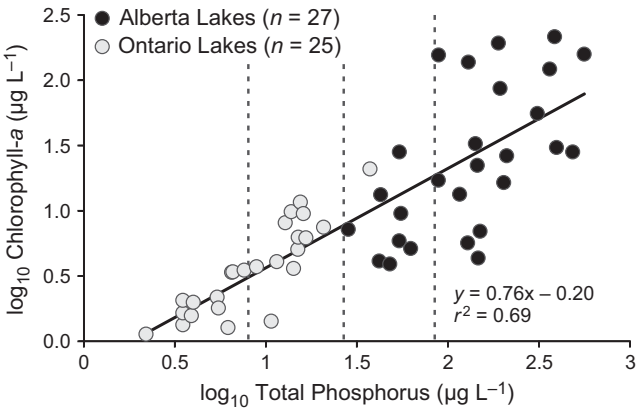

Fig. 2 Algal biomass, using chlorophyll-*a* as a proxy, versus total phosphorus in Alberta and Ontario lakes. Hashed vertical lines represent lake trophic classifications based on total phosphorus defined by Wetzel (2001).

Table 1 Median, maximum, minimum and range of all chemical parameters measured in Alberta and Ontario lakes [pH, dissolved organic carbon (DOC), total nitrogen (TN), total phosphorus (TP) TN : TP, nitrate (NO_3^-), ammonium (NH_4^+), soluble reactive P (SRP), total dissolved iron (TDFe), modelled ferric iron (pFe), hydroxamates and catecholates]

	pH	DOC (μg L^{-1})	TP (μg L^{-1})	TN (μg L^{-1})	TN : TP (Molar)	SRP (μg L^{-1})	NO_3^- (μg L^{-1})	NH_4^+ (μg L^{-1})	TDFe (μg L^{-1})	pFe (log Fe^{3+})	Hydroxamates (μg L^{-1})	Catecholates (μg L^{-1})
Alberta (n = 27)												
Med.	7.8[b]	34 200.0[b]	141.7[b]	1990.0[b]	41.8[b]	35.3[b]	2.0	24.0[b]	180.0[b]	−19.2	220.9	5059.0
Max.	9.7	63 600.0	561.2	10 900.0	133.6	192.1	18.0	214.0	2000.0	−14.9	1001.6	13 854.3
Min.	7.1	146.0	28.4	1040.0	1.3	0.6	2.0	4.0	0.04	−35.3	0.0	2614.2
Range	2.6	63 454.0	532.8	9860.0	132.3	191.5	16.0	210.0	2000.0	20.5	1001.6	11 240.1
Ontario (n = 25)												
Med.	7.4[a]	3075.6[a]	8.9[a]	364.6[a]	20.9[a]	0.1[a]	4.0	8.0[a]	7.7[a]	−21.6	111.3	4109.3
Max.	8.0	19 567.4	37.4	547.2	83.1	0.1	312.0	64.0	151.5	−17.2	825.7	8628.2
Min.	6.1	449.5	2.2	230.4	4.1	0.1	1.8	3.0	0.4	−25.1	0.0	2455.5
Range	1.9	19 117.9	35.2	316.8	79.0	0.0	310.3	61.1	151.1	7.9	825.7	6172.8

Superscript letters indicate significant differences in chemical parameters between Alberta and Ontario lakes based on Mann–Whitney U rank sum test (critical $\alpha = 0.05$).

ions than DOM in Ontario lakes at a median molar concentration of 3.2×10^{-6} versus 1.4×10^{-7} M, respectively ($P < 0.001$).

Alberta lakes were found to have significantly higher total algal biomass (chl-*a*) and cyanobacterial biomass (PC) compared with Ontario lakes ($P < 0.05$, Table 2).

Water chemistry, total algal and cyanobacterial biomass

A positive, significant linear relationship was found between chl-*a* concentration and total P among lakes of all trophic states ($r^2 = 0.69$, $P < 0.05$, Fig. 2).

The primary water chemical parameters that contributed positively to chl-*a* and PC in Alberta lakes were total P, SRP, total N, pH, DOC and hydroxamate concentration (Fig. 3a); Axis 1 explained 81.5% and Axis 2 explained 13.2% (94.7% total) of the variability observed in chl-*a* and PC in Alberta lakes. Alberta lake NMDS stress was 3.89 and instability was 0.00. The primary water chemical parameters that contributed positively to cyanobacterial biomass in Ontario lakes were total N : P and dissolved inorganic N (DIN, Fig. 3b); Axis 1 explained 86.0% and Axis 2 explained 12.2% (98.1% total) of the variability observed in chl-*a* and PC in Ontario lakes. Ontario lake NMDS stress was 3.90 and instability was 0.00.

For both sets of lakes, the following Fe was considered: TDFe and modelled bioavailable ferric ion concentration (as pFe). Modelled ferric ion bioavailability (as pFe) was negatively correlated to chl-*a* and PC (Fig. 3). In Alberta lakes, pH significantly influenced total algal biomass (Fig. 4a, top). Lakes with pH >9 had the highest total algal biomass (median = 114.3 μg L^{-1} chl-*a*). In Ontario lakes, total P significantly influenced total algal

Table 2 Median, maximum, minimum and range of all algal biomass parameters measured in Alberta and Ontario lakes [chlorophyll-*a* (chl-*a*) and phycocyanin (PC)]

	Chl-*a* (μg L^{-1})	PC (μg L^{-1})
Alberta (n = 27)		
Med.	22.3[b]	40.3[b]
Max.	215.7	256.9
Min.	3.9	9.7
Range	211.8	247.1
Ontario (n = 25)		
Med.	3.5[a]	8.3[a]
Max.	20.9	61.2
Min.	1.1	6.4
Range	19.8	54.8

Superscript letters indicate significant differences in algal biomass parameters between Alberta and Ontario lakes based on Mann–Whitney U rank sum test (critical $\alpha = 0.05$).

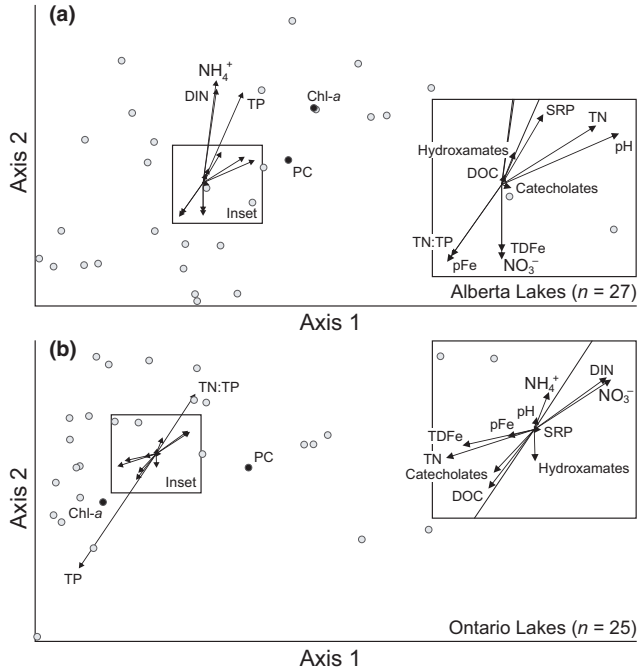

Fig. 3 Non-metric multidimensional scaling (NMDS) ordination plots presenting the chemical parameters in (a) Alberta and (b) Ontario lakes correlated to algal [as chlorophyll-*a* (chl-*a*)] and cyanobacterial biomass [as phycocyanin (PC).] Open circles represent the 27 Alberta and 25 Ontario study lakes. Axes are rotated to PC. Length and angle of the chemical parameter vectors indicate the strength and direction of the relationship between chemical parameters, algal and cyanobacterial biomass in lakes. Alberta lake NMDS stress = 3.89; instability = 0.00. Ontario lake NMDS stress = 3.90; instability = 0.00

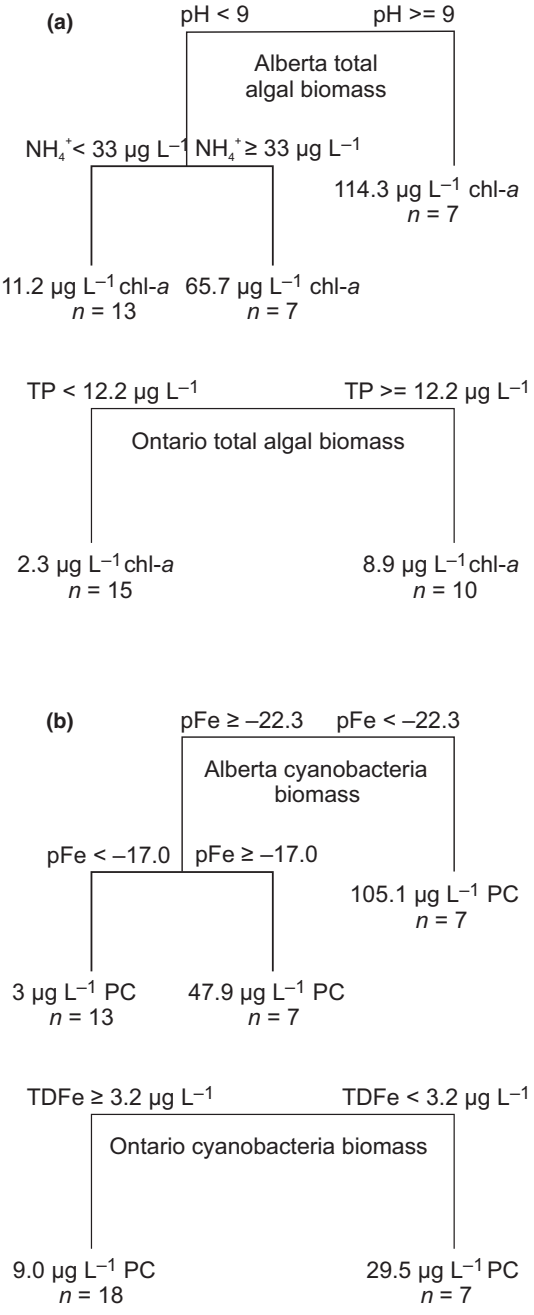

Fig. 4 Regression trees depicting the lake water chemical determinants of (a) total algal biomass [as chlorophyll-*a* (chl-*a*)] and (b) cyanobacterial biomass [as phycocyanin (PC)] in Alberta (top) and Ontario (bottom) lakes. The terminal branches of the regression tree are median values.

biomass (Fig. 4a, bottom). Lakes with total P concentrations ≥ 12.2 µg L^{-1} had significantly higher total algal biomass (median = 8.9 µg L^{-1} chl-*a*) compared with lakes with relatively lower concentrations of total P.

In Alberta lakes, modelled bioavailable ferric ion concentration (as pFe) was significantly correlated to cyanobacterial biomass (Fig. 4b, top). Lakes with pFe < −22.3 (relatively low modelled ferric ion bioavailability) had the highest cyanobacterial biomass (median = 105.1 µg L^{-1} PC). In Ontario lakes, TDFe was significantly correlated to cyanobacterial biomass (Fig. 4b, bottom). Lakes with TDFe concentrations < 3.2 µg L^{-1} had significantly higher cyanobacterial biomass (median = 29.5 µg L^{-1} PC) compared with lakes with relatively higher concentrations of TDFe (≥3.2 µg L^{-1}) with 9.0 µg L^{-1} PC.

Fe stress, Fe-binding ligands and cyanobacterial biomass

In the lakes identified in Fig. 4b to have the lowest Fe (TDFe in Ontario or pFe in Alberta) and highest cyanobacterial biomass [seven lakes in Ontario (28%) and seven lakes in Alberta (26%)], the concentration of hydroxamates was significantly and positively correlated to cyanobacterial biomass as PC (Table 3). The concentration of catecholates was not correlated to any water chemical or algal parameter.

Table 3 Spearman correlation matrix for lake chemical [pH, dissolved organic carbon (DOC), total nitrogen (TN), total phosphorus (TP) TN : TP, nitrate (NO_3^-), ammonium (NH_4^+), soluble reactive P (SRP), total dissolved iron (TDFe), modelled ferric iron (pFe), hydroxamates and catecholates] and algal parameters [chlorophyll-*a* (chl-*a*) and phycocyanin (PC)] in the seven Alberta and seven Ontario lakes identified by regression tree analysis (Fig. 4) with highest cyanobacterial biomass and lowest Fe bioavailability and concentration (critical $\alpha = 0.05$)

$n = 14$	DOC	TN	TP	TN : TP	NO_3^-	NH_4^+	SRP	TDFe	pFe	Hydroxamates	Catecholates	Chl-*a*	PC
pH	**0.63**	**0.76**	**0.78**	*0.059*	*0.11*	**0.79**	**0.78**	**0.54**	**−0.65**	*0.50*	*−0.20*	**0.79**	*0.52*
DOC		*0.52*	*0.43*	*0.17*	*−0.049*	*0.43*	*0.38*	*0.17*	**−0.62**	**0.76**	*−0.47*	**0.67**	**0.60**
TN			**0.82**	*0.40*	*0.037*	**0.80**	**0.81**	*0.35*	**−0.79**	*0.31*	*0.073*	**0.81**	*0.40*
TP				*−0.080*	*0.020*	**0.82**	**0.92**	*0.51*	*−0.54*	*0.30*	*−0.079*	**0.75**	*0.45*
TN : TP					*0.27*	*0.23*	*0.10*	*−0.12*	*−0.48*	*0.029*	*0.0022*	*0.24*	*0.19*
NO_3^-						*0.41*	*0.12*	*−0.044*	*0.19*	*−0.25*	*−0.46*	*−0.13*	*−0.10*
NH_4^+							**0.89**	*0.39*	**−0.56**	*0.20*	*−0.12*	**0.63**	*0.32*
SRP								**0.65**	*−0.51*	*0.29*	*−0.021*	**0.74**	*0.52*
TDFe									*−0.082*	*0.30*	*−0.16*	**0.60**	*0.53*
pFe										*−0.31*	*−0.077*	**−0.68**	*−0.29*
Hydroxamates											*−0.31*	*0.49*	**0.70**
Catecholates												*−0.26*	*−0.41*
Chl-*a*													**0.70**

Data are presented as Spearman ρ. Positive Spearman ρ indicates an increasing monotonic trend between parameters and negative Spearman ρ indicates a decreasing monotonic trend. Spearman ρ values in bold indicate a statistically significant relationship between parameters ($P < 0.05$) and italicised Spearman ρ indicates no significant relationship.

Discussion

The primary objective of this study was to investigate whether lake trophic status determines how cyanobacterial biomass responds to Fe stress and the availability of Fe-binding ligands within lakes. Fe supply (as TDFe) and modelled bioavailability (as pFe) were the primary determinants of cyanobacterial biomass in lakes of all trophic states. The concentration of Fe-binding hydroxamates in lakes with relatively low-Fe supply or modelled bioavailability was correlated to cyanobacterial biomass, suggesting that these Fe-binding ligands provide cyanobacteria with a competitive means to obtain Fe.

Lake chemical and algal conditions

We found that total P regulated total algal biomass (as chl-*a*) across lakes of various trophic states. The relationship between total P and total algal biomass has been well supported in previous field-based research (e.g. Schindler, 1978; Downing *et al.*, 2001). While total P served as a relatively strong predictor variable for total algal biomass, explaining 69% of the variability in total algal biomass among lakes, there was considerable variation in the achieved algal biomass for any given concentration of total P. One explanation for this observed variability is that Fe may have been limiting the conversion of P to biomass by binding P and restricting algal access to the entire P pool in oxic surface waters (Moore & Reddy, 1994), which would support a role for Fe as a regulator of macronutrient (P)-use efficiency.

The NMDS analysis teased apart the multivariate relationships among lake water chemical parameters and total algal and cyanobacterial biomass in lakes. The close proximity of total algal and cyanobacterial biomass in the Alberta lakes ordination plot suggests that the algal community was primarily comprised of cyanobacteria with similar regulatory water chemical parameters. In contrast, the distant proximity of total algal and cyanobacterial biomass in Ontario lakes suggested a separation in lake algal communities from those primarily comprised by cyanobacteria and those by other algae with different regulatory water chemical parameters. Although NMDS successfully explained the combined nutrient factors that regulated total algal and cyanobacterial biomass in Alberta (94.7%) and Ontario lakes (98.1%), further investigation was required to identify a specific primary nutrient factor that regulated total algal and cyanobacterial biomass in lakes.

Water chemistry, total algal and cyanobacterial biomass

The lakes selected for investigation in this study were representative of all trophic states from oligotrophic to hypereutrophic, based on Wetzel's (2001) total P definitions of lake trophic states. Macronutrient concentrations in lakes of all trophic states were characteristic of those found in other lake field studies. Downing & McCauley (1992) reported that total N : P is typically highest in

oligotrophic lakes and lowest in hypereutrophic lakes. This was attributed to the fact that oligotrophic lakes typically receive N and P from natural undisturbed catchments, which export less P than N. In contrast, mesotrophic and eutrophic lakes typically receive nutrients from various sources, often on managed landscapes, that have relatively lower N inputs compared to P, while hypereutrophic lakes have N and P inputs corresponding to that of sewage. In contrast to this, we found that total N : P was significantly higher in the eutrophic and hypereutrophic lakes in Alberta than in the oligotrophic and mesotrophic lakes in Ontario. This may be because all lakes selected for this study were largely removed from direct anthropogenic influence, including forest harvesting, agriculture and sewage inputs. The N and P mixtures entering the 'naturally' eutrophic and hypereutrophic Alberta lakes would be of different composition, more similar to that of oligotrophic systems and less similar to that reported by Downing & McCauley (1992).

Although TDFe concentrations in Alberta lakes were significantly higher than in Ontario lakes, the modelled bioavailable ferric ion fraction (represented as pFe) of this TDFe pool was not significantly different between Alberta and Ontario lakes. This implies that, despite Alberta lakes having a significantly higher Fe supply than Ontario lakes, the amount of the TDFe fraction that is bioavailable for cellular use does not differ between Alberta and Ontario lakes. This may be due to the tendency for Fe to bind to DOM in oxic surface waters, which results in Fe-DOM complexes that render Fe non-bioavailable for cellular use while bound (Fujii *et al.*, 2014). Sorichetti *et al.* (2014a) and I.F. Creed (unpubl. data) showed evidence to suggest that catecholate ligands, whether produced by cyanobacteria or bound in DOM complexes, can bind Fe tightly, rendering the Fe non-bioavailable. Since DOC concentrations in Alberta lakes were an order of magnitude higher than in Ontario lakes, it is likely that much of the TDFe in Alberta lakes is bound in Fe-DOM complexes and is not bioavailable for cellular use. This argument is strongly supported by our finding that, compared with DOM in Ontario lakes, DOM in Alberta lakes bound significantly more ferric ions in DOM complexes rendering this Fe non-bioavailable. Despite differences in TDFe concentrations among lakes, the bioavailability of Fe for cellular use can be similar among lakes of various trophic states. These findings provide a unique opportunity to explore how cyanobacteria respond to Fe stress in lakes of various trophic states with different macronutrient and micronutrient conditions but similar Fe bioavailability.

Regression tree analysis of lake chemical and biological parameters confirmed that the prime nutrient factor regulating cyanobacterial biomass in Alberta and Ontario lakes was Fe. This finding supports our hypothesis that cyanobacterial biomass in lakes of all trophic states will be highest when ferric ion concentration, and thus bioavailability, is low. Although Alberta lakes had significantly higher TDFe concentrations than the Ontario lakes, the significantly higher DOC in Alberta lakes bound more ferric ions in DOM complexes, so that modelled bioavailability (as pFe) was the primary chemical parameter that influenced cyanobacterial biomass in these Alberta lakes. In contrast, the Ontario lakes had significantly lower DOC concentrations that bound significantly lower amounts of ferric ions, and so the supply of Fe (as TDFe) to lakes rather than modelled bioavailability was of primary importance when comparing the role of Fe in regulating cyanobacterial biomass between these two hydrologic and climatic regions of Canada.

Total algal biomass in Alberta and Ontario lakes had a different set of regulating parameters. The primary parameter that regulated total algal biomass in Alberta lakes was pH. Since P has the potential to bind to Fe and DOM in oxic surface waters, rendering P non-bioavailable for cellular use (Moore & Reddy, 1994; Baken *et al.*, 2011), pH may be an important water chemical parameter that regulates the release of P from P-Fe or P-DOM complexes so the P is readily available for cellular uptake. Despite the significantly higher total P and SRP concentrations in Alberta lakes, much of this P pool may be locked up in Fe or DOM complexes and dependent on pH for geochemical release. The prime nutrient factor that regulated total algal biomass in Ontario lakes was total P, as has been reported elsewhere (e.g. Schindler, 1978; Downing *et al.*, 2001).

Fe stress, Fe-binding ligands and cyanobacterial biomass

Hydroxamates are water-soluble ligands that have relatively weak Fe-binding capacity, whereas catecholates are fat-soluble ligands that have relatively strong Fe-binding capacity (Neilands, 1995). The mechanism of Fe-binding to these ligand types differs as described by Neilands (1995). Hydroxamates are produced within the cell and are transported to the external environment *via* specialised membrane-bound protein channels where they bind soluble ferric ions. Upon contact of the Fe-ligand complex at the cell surface, ferric ions are reduced to ferrous ions and assimilated. Cate-

cholates are cell membrane-bound and Fe binding occurs at the cell surface where ferric ions are reduced to ferrous ions and assimilated. Cyanobacteria are the only algal group that possess the Fe-ligand uptake system and so have a competitive advantage for Fe scavenging over eukaryotic algae in Fe-limited conditions (Wilhelm & Trick, 1994).

Cyanobacteria in our studied lakes were able to thrive in lake surface waters when the supply and modelled bioavailability of Fe was low. Spearman correlation analysis confirmed that when lakes of all trophic states with low-Fe and high cyanobacterial biomass (according to regression tree analysis) were combined, cyanobacterial biomass was positively correlated to hydroxamate siderophore concentration. These findings suggest that when Fe supply and bioavailability is low in lakes, cyanobacteria in lakes of all trophic states may respond similarly to Fe stress and may utilise hydroxamate siderophores to scavenge Fe and satisfy Fe demand.

Catecholate siderophore concentrations were 10× higher than those of hydroxamate siderophores. There are no known studies available to compare the concentrations of catecholate siderophores in lakes to those observed in this study. One potential explanation for the high catecholate siderophore concentrations is that the analytical procedure did not measure only siderophores, but also the aromatic material comprising the DOC. Organic matter compounds contain aromatic phenolic groups, which are sensitive to the colorimetric assay for catechol compounds, or 1,2-dihydroxybenzenes (Arnow, 1937). The phenol and carboxyl chemical structures within DOC allow this organic compound to chelate ferric ions, as well as siderophores present in the sample with strong binding capacity that would further reduce bioavailable Fe (Nagai *et al*., 2006; Misumi *et al*., 2013). Catecholate siderophore concentration was also not significantly correlated to cyanobacterial biomass according to any analysis. We could not definitively rule out analytical interference by DOC and concluded that catecholate siderophores, in this study, were not produced by cyanobacteria and did not have an influence on cyanobacterial biomass maintenance in lakes.

While total P is an important macronutrient that regulates total algal biomass in lakes of all trophic states, we found Fe to be an important micronutrient that regulates cyanobacterial biomass in lakes of all trophic states. Cyanobacteria in lakes of all trophic states appear to respond similarly to Fe stress through the utilisation of Fe-binding hydroxamate Fe-binding ligands as a competitive strategy to overcome Fe stress in lakes. The mechanistic relationship among Fe stress, siderophore concentration and cyanobacterial biomass is remarkably similar across a broad range of lake trophic states and further advances our knowledge on the pervasiveness of cyanobacterial growth in lakes of all trophic conditions.

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