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Bioavailable iron in oligotrophic Lake Superior assessed using biological reporters

R. M. L. MCKAY[1]*, DAVID PORTA[1†], GEORGE S. BULLERJAHN[1], MAMOON M. D. AL-RSHAIDAT[1], JEFFREY A. KLIMOWICZ[1], ROBERT W. STERNER[2], TANYA M. SMUTKA[2], ERIK T. BROWN[3] AND ROBERT M. SHERRELL[4,5]

[1]DEPARTMENT OF BIOLOGICAL SCIENCES, BOWLING GREEN STATE UNIVERSITY, BOWLING GREEN, OH 43403, USA, [2]DEPARTMENT OF ECOLOGY, EVOLUTION AND BEHAVIOR, UNIVERSITY OF MINNESOTA, ST. PAUL, MN 55328, USA, [3]LARGE LAKES OBSERVATORY, UNIVERSITY OF MINNESOTA DULUTH, DULUTH, MN 55812, USA, [4]INSTITUTE OF MARINE AND COASTAL SCIENCES, RUTGERS UNIVERSITY, NEW BRUNSWICK, NJ 08901, USA AND [5]DEPARTMENT OF GEOLOGICAL SCIENCES, RUTGERS UNIVERSITY, NEW BRUNSWICK, NJ 08901, USA

[†]PRESENT ADDRESS: GREAT LAKES INSTITUTE FOR ENVIRONMENTAL RESEARCH, UNIVERSITY OF WINDSOR, WINDSOR, ONTARIO, CANADA N9B 3P4

*CORRESPONDING AUTHOR: rmmckay@bgnet.bgsu.edu

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Iron bioavailability in Lake Superior was assessed during field surveys conducted in 2001–2002. Dissolved iron (Fe_d) ranged between 1 and 4 nM at offshore stations and >10 nM at most nearshore sites. Iron availability was assessed using a luminescent Synechococcus bioreporter comprising a luciferase reporter controlled by an iron-responsive promoter isiAB. Bioreporter luminescence was negatively correlated to Fe_d measured in the samples. Distance from shore was a better predictor of iron bioavailability than was season. Water collected from most offshore stations sampled during spring and summer elicited higher bioreporter luminescence than did nearshore sites. Iron availability did not vary with depth during summer, despite the presence of elevated levels of Fe_d in the hypolimnion at most stations. Ultrafiltration (0.02 μm) of Fe_d at two offshore sites demonstrated Fe_d to be present mainly in a colloidal phase, yet the bioreporter responded solely to iron contained in the soluble phase. During spring, a parallel immunochemical assay of diatoms resulted in the detection of ferredoxin (Fd) but not flavodoxin (Flvd) at five stations indicating the presence of an iron sufficient assemblage of diatoms at these sites. Whereas neither bioreporter nor immunochemical approaches conducted during spring supported physiological iron deficiency among Lake Superior phytoplankton, the results did point to differences in the availability of iron to prokaryotes and eukaryotes.

INTRODUCTION

The role of iron in physiological processes such as photosynthesis, respiration, and nitrogen assimilation makes it one of the most important nutritive factors for phytoplankton growth. Despite its high abundance in the Earth's crust, low iron availability has been shown to limit phytoplankton growth in diverse marine environments (Coale *et al.*, 1996; Boyd *et al.*, 2000, 2004). This apparent contradiction is attributed to features of iron biogeochemistry that lead to precipitation or complexation of iron species in oxic waters. These considerations, combined with regionally low aeolian input of iron to many marine environments, result in iron limitation to the endemic phytoplankton.

Phytoplankton production in lakes is often viewed as being limited initially by phosphorus (Schindler, 1977) along with the secondary influence of nitrogen (Hecky and Kilham, 1988; Elser *et al.*, 1990). In addition, several studies have documented the existence of iron limitation in some freshwater environments (Schelske, 1962; Wetzel, 1966; Jackson and Hecky, 1980; Sterner, 1994; Twiss *et al.*, 2000). In a companion study to that presented here, iron was also identified as deficient in Lake Superior, since

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on at least four occasions, low iron availability constrained phosphorus-stimulated growth in bioassays (Sterner *et al.*, 2004). The possibility that iron can be a limiting element in freshwater environments, particularly in oligotrophic bodies such as Lake Superior, is consistent with recent perspectives on the abundance of trace metals in the Great Lakes by researchers adopting metal-clean sampling techniques (Nriagu *et al.*, 1996; Field and Sherrell, 2003). Dissolved bioactive trace metals, including iron, are present in low nanomolar to picomolar concentrations throughout most of the Laurentian Great Lakes system.

Enrichment bioassays, though offering direct experimental evidence for growth limitation, do not completely mimic the undisturbed natural environment: grazing is disrupted, physical mixing is decreased and the phytoplankton are isolated at a fixed optical depth (Banse, 1991; Carpenter, 1996). Further, whereas chemical measures of total dissolved iron (Fe_d) can provide an estimate of the potential for iron limitation, Fe_d is not synonymous with bioavailable iron. Some forms of particulate iron appear to be bioavailable (Mioni *et al.*, 2003), whereas some iron associated with the operationally defined dissolved phase (<0.45 μm) is not immediately available for uptake (Hutchins *et al.*, 1999). Without good information on which chemical pools are actually accessed by organisms *in situ*, knowledge of pool size does not directly address the question of bioavailability.

We have developed a luminescent cyanobacterial bioreporter as a tool to assess iron availability in freshwaters (Durham *et al.*, 2002; Porta *et al.*, 2003). The bioreporter, constructed in *Synechococcus* sp. PCC7942, features the iron-regulated *isiAB* promoter fused to *luxAB* encoding bacterial luciferase. Physiological characterization of the bioreporter demonstrated a dynamic range of response between $-\log(Fe^{3+})$ (pFe) 21.1 (30 nM Fe) and pFe 20.6 (100 nM Fe). Within this range, the bioreporter emits increasing amounts of light as bioluminescence as the concentration of iron is decreased. Although a contained incubation is still required with the bioreporter, incubation times are decreased markedly (6–12 h) compared with conventional bioassays (>24 h) because of the rapid induction and ease of detection of the bioluminescent response. The bioreporter thus provides a means to assess iron availability from the perspective of a living cyanobacterium that is an ecologically important member of the phytoplankton in many lakes (Munawar and Fahnenstiel, 1982; Fahnenstiel *et al.*, 1986, 1991; Callieri and Stockner, 2002).

However, this approach does not specifically gauge iron availability to eukaryotic cells in Lake Superior. The cyanobacterial iron bioreporter is a prokaryotic organism possessing an iron acquisition strategy distinct from eukaryotes. Whereas cyanobacteria commonly acquire iron using ferric-chelating siderophores (Wilhelm, 1995), eukaryotic algae generally use a cell surface ferric chelate reductase (Maldonado and Price, 2000; Weger *et al.*, 2002). Reflecting this difference, Hutchins *et al.* (Hutchins *et al.*, 1999) demonstrated distinct differences in the uptake efficiency of various molecules containing radiolabeled iron by cyanobacteria and diatoms.

In response, we adopted a separate immunochemical assay of iron deficiency in diatoms, an important component of the eukaryotic plankton in the Great Lakes (Munawar and Munawar, 2000). This assay evaluates the relative levels of the redox catalysts ferredoxin (Fd) and flavodoxin (Flvd) using polyclonal antisera raised against each protein (McKay *et al.*, 1999). When iron availability is low, Flvd replaces the iron-containing redox catalyst Fd to relieve the cellular iron burden in cyanobacteria (Straus, 1994) and numerous algae (Geider and La Roche, 1994). Thus, Flvd is a biochemical marker of iron deficiency in phytoplankton; its detection providing an *in situ* assessment of cellular iron nutrition (La Roche *et al.*, 1996). Biologically based assays of iron availability, as described here, are powerful tools to ascertain the physiological responses of living cells to a complex chemical milieu.

The primary objective of this study was to assess iron availability to prokaryotic picophytoplankton in Lake Superior over seasonal and spatial (nearshore *versus* offshore, depth resolved) scales using the iron-responsive cyanobacterial bioreporter. Parallel immunochemical assay of Flvd and Fd in diatoms conducted during May 2001 served as a complementary assessment of iron availability to the eukaryotic phytoplankton assemblage of the lake.

METHOD

Sample collection

Samples were collected from 13 hydrographic stations (Fig. 1) during three research cruises on Lake Superior, North America, during 2001–2002 on the R/V *Blue Heron*. The surveys included periods of vernal holomixis (May 2001) as well as summer stratification (July 2001, September 2002). At each station, sampling was preceded by a conductivity temperature depth (CTD) cast (Tables I–III). The CTD (SBE 911*plus*; Sea-Bird Electronics, Bellevue, WA, USA) was equipped with a 25-cm transmissometer and a chlorophyll (Chl) flourometer (WETStar; WET Laboratories, Philomath, OR, USA).

Water samples for analysis of metals and for the bioreporter assay were collected from discrete depths using a metal-clean *in situ* pumping system (Field and Sherrell, 2003). Water was collected from a laminar flow

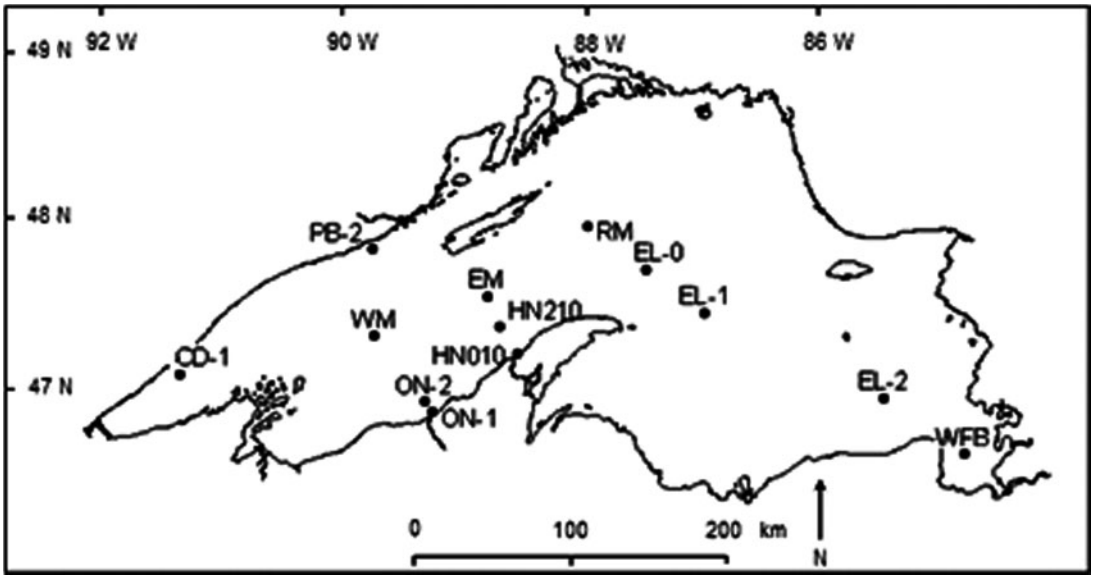

Fig. 1. Map showing location of stations occupied during 2001–2002 in Lake Superior, North America.

Table I: Hydrographic stations and physicochemical parameters: May 2001

Station	z_m (m)	SD (m)	T (°C)	Chl *a* ($\mu g \cdot L^{-1}$)	Fe_d (nM)	pFe equivalency
CD-1	246	20	2.7	0.72	18.4	<20.6
PB-2	190	20	2.3	0.70	11.73 (40 m)	<20.6
WM	177	20	2.7	0.76	1.08	nd
ON-1	14	10	nd	1.62	nd	<20.6
ON-2	70	10	4.5	1.48	nd	<20.6
EM	179	20	2.7	0.76	1.92	20.8
RM	256	20	2.7	0.79	1.48 (50 m)	<20.6
EL-1	323	20	3.0	0.78	nd	>21.1

CD, Castle Danger; Chl *a*, chlorophyll *a*; EL, eastern lake; EM, eastern mid-lake; Fe_d, dissolved iron (<0.45 μm) measured at sampling depth except where indicated; nd, not determined; ON, Ontonagon; PB, Pigeon bay; pFe equivalency, concentration of free ferric ion in the sample; RM, 'real' mid-lake; SD, sampling depth; T, temperature at sampling depth; WM; western mid-lake; z_m, maximum depth.

hood containing an in-line capsule filter (0.45 μm Calyx Polypropylene capsule filter, GE Osmonics, GE Water Technologies, Trevose, PA, USA). In September, water was further subjected to manual ultrafiltration through acid-cleaned 0.02 μm Anotop syringe filters (Whatman International, Maidstone, UK).

A larger size fraction of plankton was collected during May only by vertical net tow (1 m diameter opening, 80 μm pore-size mesh). The net was deployed to 40 m depth at each station except for station ON-1, where it was deployed to 7 m due to the shallow depth of this site.

Analysis of Fe_d

Filtered water was collected into acid-cleaned, low density polyethylene bottles and frozen immediately. Analysis of metal content in the lake water was carried out by high-resolution inductively coupled plasma mass spectrometry, as described in Field and Sherrell (Field and Sherrell, 2003). Standardization was achieved by multielement standard additions to representative lake water samples, and process blanks associated with sample storage, acidification and analysis procedures were determined by treating Milli-Q deionized laboratory water as sample. Blanks were <2% of the lowest sample concentrations, and the instrumental detection limit (3 × SD of blank) was typically 20–50% of the blank. Measurement accuracy was quantified by analysis of SLRS-4 standard reference freshwater (National Research Council of Canada, Ottawa, Canada). Measurement precision, based on replicate analysis of a single sample, was <5% (1-sigma, $n = 10$).

Table II: Hydrographic stations and physicochemical parameters: July 2001 epi- and hypolimnion

Station	z_m (m)	SD (m)	T (°C)	Chl *a* (μg·L^{-1})	Fe_d (nM)	pFe equivalency
CD-1	246	3	15.1	0.93	75.8	<20.6
		101	3.9	0.37	5.5	20.96 ± 0.1
PB-2	190	5	10.8	0.60	5.51	20.6 ± 0.1
		87	3.9	0.48	7.22	20.72 ± 0.1
WM	177	5	10.1	0.46	3.42	nd
		103	3.9	0.49	5.76	nd
ON-1	14	5	18.1	0.52	11.67	20.6 ± 0.1
ON-2	70	5	17.2	0.45	7.42	20.77 ± 0.2
		50	4.4	0.60	5.66	20.63
EM	179	5	10.3	0.39	3.82	20.70 ± 0.1
		100	3.8	0.36	6.34	21.01
RM	256	5	7.4	0.37	2.43	<20.6
		61	3.9	0.55	3.45 (31 m)	<20.6
EL-1	323	5	9.4	0.26	1.27	21.03 ± 0.2
		100	3.8	0.37	1.73	21.93 ± 0.3
EL-0	157	5	11.0	0.47	2.05 (20 m)	21.04 ± 0.2
		84	4.3	0.39	2.65	20.73 ± 0.1
EL-2	200	5	11.9	0.53	1.63	20.93 ± 0.1
		100	4.1	0.31	1.82	21.03 ± 0.2
WFB	70	5	16.5	0.48	14.71	<20.6
		54	6.0	0.24	13.26	<20.6
HN010	10	5	8.2	0.81	23.70	nd
HN210	147	5	14.4	1.40	10.91	nd

HN, Hancock North; WFB, Whitefish Bay.
Abbreviations are as defined in Table I.

Table III: Hydrographic stations and physicochemical parameters: September 2002 epilimnion and subsurface chlorophyll (Chl) maximum

Station	SD (m)	T (°C)	Chl *a* (μg·L^{-1})	Fe_d (nM)[a]	pFe equivalency
WM	5	11.5	0.70	2.59 (0.34)	20.98 (21.04)[a]
	25	6.7	1.92	2.47 (0.83)	21.11 (21.08)
EM	5	12.7	0.75	2.40 (1.09)	nd
	25	7.3	1.87	1.41 (0.60)	nd

Abbreviations are as defined in Table I.
[a]Soluble (<0.02 μm) iron in parentheses.

Iron bioreporter assay

The development and characterization of the cyanobacterial iron bioreporter is described elsewhere (Durham *et al.*, 2002; Porta *et al.*, 2003). From each hydrographic station or sampling depth, filtered water was collected using the metal-clean sampling pump into acid-cleaned polycarbonate bottles and immediately frozen. During May, water was collected from a depth of 20 m in the isothermal water column, except for the shallow coastal station ON-1 and its offshore counterpart ON-2 where water was sampled from 10 m depth. During July and September, the water column was thermally stratified, and samples were collected at each station from locations in the epilimnion, hypolimnion and from the subsurface Chl fluorescence maximum located below the metalimnion. Replicate (n = 3) samples were

collected in 250 mL polycarbonate bottles at each site. In addition, the bioreporter was tested on the ultraltered (<0.02 μm) fractions collected from Station WM during September.

In our research laboratory, samples were thawed, and 20-mL aliquots were distributed into triplicate acid-cleaned polycarbonate tubes. Chelex-100 (Bio-Rad, Hercules, CA, USA)-treated Fraquil major nutrients (N, P) were added as was an iron-free Fraquil micronutrient stock. The formulation for Fraquil is available from the Cyanosite Website (http://www-cyanosite.bio.purdue.edu/). Tubes were inoculated with 1.5 mL of bioreporter cells prepared as described elsewhere (Porta *et al.*, 2003). Direct cell counts made as part of several assays indicated bioreporter cell density to be $\sim 6 \times 10^8$ cells·L^{-1}, a concentration comparable with the maximum reported autotrophic picoplankton cell density reported in the Laurentian Great Lakes (Caron *et al.*, 1985). Samples were incubated for 12 h at constant temperature (24°C), and light (50 μmol photons·m^{-2}·s^{-1}) following which 2-mL aliquots were removed and luminescence measured using a portable luminometer (Femtomaster FB14; Zylux Corp., Maryville, TN, USA). Luminescence was measured both prior to, and following, exposure to vapors of the exogenous substrate n-decyl aldehyde (Sigma, St. Louis, MO, USA) for 50 min. The exogenously supplied aldehyde was required to ensure saturation of luciferase with its substrate (Porta *et al.*, 2003). Positive and negative controls were provided by adding the iron chelator desferrioxamine mesylate (desferal; Sigma) and $FeCl_3$, respectively, to samples containing lake water and the bioreporter. Fraquil medium was prepared containing varying levels of total iron ($FeCl_3$) that correspond to thermodynamically calculated free ferric ion concentrations [Twiss *et al.*, 2001; pFe = –log(Fe^{3+} free ferric)]: 10 nM (pFe 21.6), 30 nM (pFe 21.1), 50 nM (pFe 20.9), 70 nM (pFe 20.8), 100 nM (pFe 20.6) and 1000 nM (pFe 19.5). Iron contained in synthetic culture medium is commonly expressed in terms of pFe to more readily facilitate comparisons between different studies where the synthetic medium formulation may differ. Similar to the pH scale, lower pFe values represent higher values of free [Fe^{+3}]. Coincident incubation of the bioreporter in Fraquil medium served as an internal calibration for the assay.

In conjunction with the assay of samples collected during September, we enumerated glutaraldehyde-preserved cells to normalize measures of luminescence. Chl *a* autofluorescence of *Synechococcus* cells was detected using a Leica DMRXA Microscope with epifluorescence attachment (Leica Microsystems, Buffalo, NY, USA) and Image Pro Plus software (version 4.1; Media Cybernetics, Silver Spring, MD, USA). For all other assays, bioreporter luminescence was normalized against *in vivo* Chl *a* fluorescence measured using a TD-700 fluorometer (Turner Designs, Sunnyvale, CA, USA).

Assay of iron-responsive redox proteins Flvd and Fd in net plankton

Plankton concentrated by net tow were fractionated immediately following collection using 210-μm polypropylene mesh (Spectra/Mesh; Spectrum Laboratories, Rancho Dominguez, CA, USA) in order to minimize contribution of zooplankton. A portion of this 80–210 μm fraction was preserved in Lugol's iodine for microscopic analysis, whereas the remainder was collected onto replicate 20 μm pore-size polycarbonate filters, transferred to cryotubes and frozen in liquid nitrogen within 30–60 min following collection of the net plankton.

Total protein was extracted from net plankton samples by sonication in lysis buffer containing 4% (w/v) sodium dodecyl sulphate (SDS) and 68 mM sodium carbonate. Following centrifugation (5 min at 3000 × *g*), a portion of the supernatant was reserved for protein assay using bicinchoninic acid (Pierce, Rockford, IL, USA) and bovine serum albumin as a standard. To the remaining supernatant were added 1 volume of 0.2 M dithiothreitol and 2 volumes of electrophoresis sample buffer [8% (w/v) SDS, 15% (v/v) glycerol and 0.05% (w/v) bromophenol blue]. Proteins were resolved by discontinuous gel electrophoresis (Laemmli, 1970) prior to electrophoretic transfer to nitrocellulose membranes (Nitrobind, 0.45 μm; GE Osmonics), according to Towbin *et al.* (Towbin *et al.*, 1979).

The presence of Fd and Flvd in net plankton samples was assessed by western blot analysis using polyclonal antiserum raised against *Phaeodactylum tricornutum* Flvd (La Roche *et al.*, 1995) and *Thalassiosira weissflogii* Fd (McKay *et al.*, 1999). Replicate blots (n = 2–3) were processed using standard immunoblotting procedure (Towbin *et al.*, 1979) and subsequently incubated with an affinity-purified goat anti-rabbit IgG-alkaline phosphatase conjugate (Amersham Biosciences, Piscataway, NJ, USA). Immunoreactive proteins were visualized by chemifluorescence using the ECF™ substrate (Amersham Biosciences) followed by detection using a Storm 860 imaging system and analysed using ImageQuant software (version 5.2; Amersham Biosciences).

Net plankton preserved in Lugol's iodine were identified and enumerated by light microscopy using a Palmer-Maloney chamber. For each station, a minimum of 400 cells was counted.

RESULTS

During May, the water column was isothermal (2–3°C) except at shallow stations ON-1 and ON-2, where there was modest thermal structure. Chl *a* ranged between 0.7 and 0.85 μg·L^{-1} (Table I) and varied by no >15% throughout the top 100 m of the water column at any single

station. Size-fractionated Chl demonstrated an abundant picoplankton (0.2–2 μm) population through the entire western basin of the lake comprising 22–37% (median: 27%) of total Chl *a*. Microplankton (>20 μm) comprised no >4% of total Chl *a*, except at stations ON-1 and ON-2 where this size fraction accounted for ~15% of total Chl.

During July and September, thermal stratification was evident at all stations (Tables II and III). In July, surface water temperatures ranged between 7 and 12°C (median: 11°C) at offshore sites and increased to 17–18°C at nearshore stations ON-1, ON-2 and WFB. Total epilimnetic Chl *a* was low, ranging from 0.26 to 1.4 $\mu g \cdot L^{-1}$ (median: 0.48). As in May, the picoplankton size fraction was abundant, comprising 22–50% (median: 36%) of total Chl in the epilimnion. As documented in previous studies of Lake Superior (Barbiero and Tuchman, 2001), a distinct subsurface Chl maximum existed at most stations, extending from the lower reaches of the metalimnion and into the hypolimnion and with its peak generally positioned between 30 and 40 m depth in July (not shown) and ~25 m in September (Table III).

Spatial–temporal variation of Fe_d in Lake Superior

Spatial variability in the concentration of Fe_d (<0.45 μm) in surface waters was observed between nearshore and offshore locations during both spring and summer surveys (Tables I and II). For the purpose of our analysis, we define a nearshore station as one situated within 3 km from shore (CD-1, PB-2, ON-1, WFB and HN-010). Surface waters of stations CD-1 and PB-2, both located along the north shore of Lake Superior, contained 8–12 times higher levels of Fe_d compared with mean levels measured at offshore stations sampled during the spring survey (WM, EM and RM; mean: 1.5 nM Fe). By July, surface water Fe_d had diminished at Station PB-2 by >50%, whereas Fe_d measured in the epilimnion of Station CD-1 increased by a factor of 2 with an integrated value (2 depths) of 51.5 nM. Elevated surface water Fe_d was also measured in Whitefish Bay (WFB), located at the eastern outflow of the lake and along transects ON-1/ON-2 and HN010/HN210, both of which are situated perpendicular to the Keweenaw Peninsula. By comparison, mid-lake stations surveyed in July maintained low surface water Fe_d with levels ~4 nM in the western lake and levels ranging between 1.3 and 4 nM in the eastern lake. In summary, surface water Fe_d was maintained at low nanomolar levels at deep, offshore stations and was elevated at stations located closer to shore.

Examining vertical profiles at each station, there was little variability in levels of Fe_d measured through the isothermal water column during the spring survey (R.M. Sherrell, Rutgers University, unpublished data). By contrast, in July, open lake stations commonly showed slight to moderate elevation (10–40%) of Fe_d in the hypolimnion compared with surface waters (Table II).

Iron availability assessed using a cyanobacterial iron-dependent bioreporter

Bioreporter luminescence was negatively correlated with the amount of Fe_d measured in each sample (Fig. 2). An association between luminescence and log transformed Fe_d was tested using a Spearman rank correlation, a test free of assumptions about normality or constancy of error variance. The two variables were significantly correlated ($r_s = 0.71$, $P < 0.001$).

The relationship between bioreporter response and the available iron content was developed as part of a previous characterization of the *Synechococcus* KAS101 iron bioreporter (Durham *et al.*, 2002; Porta *et al.*, 2003). In the present analysis, a series of standards run concurrently with each assay provided a dose-response relationship for the Lake Superior samples. The standards demonstrated that the luminescent response was linear through the range of pFe 20.6 to pFe 21.1. A regression drawn through this range was used to calculate an equivalent pFe concentration for each Lake Superior sample based on the luminescence measured from each of the lake samples (Tables I and III).

We previously demonstrated that steady-state growth rates of the *Synechococcus* bioreporter were suppressed by ~30% when grown at pFe 21.6 compared with growth at pFe 20.6, a level that supported iron sufficient growth (Porta *et al.*, 2003). Extending this understanding of iron-responsive growth of the bioreporter to an examination

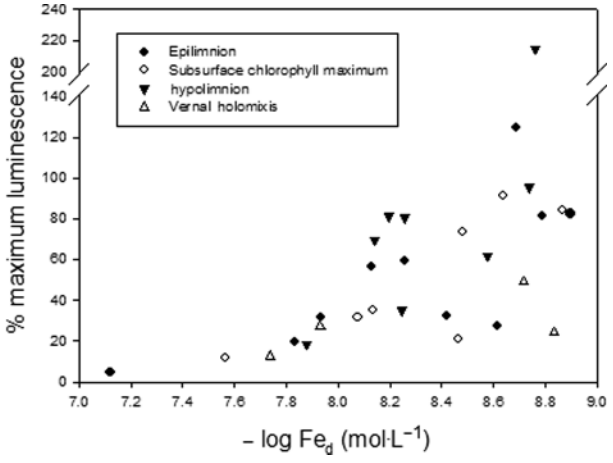

Fig. 2. Bioreporter luminescence expressed against the –log function of total dissolved iron (Fe_d) for samples collected during May (vernal holomixis) and July (thermal stratification) 2001. Bioreporter luminescence elicited from water collected from various depths is reported as a percentage of maximum luminescence elicited by a series of accompanying calibration standards.

of seasonal trends in iron bioavailability in Lake Superior, we demonstrated that during May, bioreporter luminescence was generally low (Table I), suggesting high availability of iron to the picocyanobacteria of which the bioreporter is representative. Water from most sites sampled in May generated a pFe equivalency <20.6, suggestive of an iron sufficient environment for the bioreporter.

Distance from shore was a better predictor of iron bioavailability than was season (Tables I and II). Water collected from two offshore stations sampled during May (EM and EL-1) elicited higher luminescence than did the nearshore sites ($P < 0.01$; one-way analysis of variance followed by Tukey honestly significance difference test). For Station EM, a station where we have accompanying measures of Fe_d, this coincided with lower levels of Fe_d in May compared with nearshore sites (Table I). Conversely, bioreporter luminescence was reduced at two additional offshore stations sampled in May (ON-2 and RM) suggesting higher bioavailability of iron at these sites. For Station RM at least, such a finding was inconsistent with the low levels of Fe_d measured at this station in May (1.5 nM).

A nearshore–offshore comparison for two locations sampled in July demonstrated that iron availability was higher at the nearshore sites as might be predicted based solely on analysis of Fe_d (Table II). Fe_d measured at Station WFB was nine times greater than at Station EL-2, the nearest offshore station on this cruise track. Consistent with this, bioreporter luminescence was low at the nearshore site (19% of maximum; pFe < 20.6), whereas it attained near maximal response (81%; pFe = 20.9) at the offshore site (unpaired two-tailed *t* test, $P < 0.05$). Of similar magnitude was the difference in response measured along the Hancock North transect extending from the Keweenaw Waterway, a navigation channel that bisects the Keweenaw Peninsula, to a site located 21 km offshore. Surface water from Station HN010 (1 km offshore) contained >2 times more iron than did water collected from the epilimnion at Station HN210, the offshore site. This difference was reflected in the response of the *Synechococcus* bioreporter which yielded a luminescent signal one order of magnitude higher with the offshore water sample compared with the nearshore site (unpaired two-tailed *t* test, $P < 0.0001$) (McKay *et al.*, 2004). Overall, with the exception of Station PB-2, nearshore sites sampled during July exhibited luminescent values of $\leq$25% of the maximum response achieved with the accompanying calibration standards. In each instance, Fe_d at these sites was >11 nM (Table II). A third transect, extending from a site located 1 km offshore of the Keweenaw Peninsula (ON-1) to a site 10 km offshore (ON-2), failed to demonstrate a nearshore–offshore trend in iron availability (unpaired two-tailed *t* test, $P > 0.05$, df = 4), despite parallel chemical analysis of Fe_d that showed surface waters at Station ON-1 to contain ~40% more iron compared with Station ON-2 (Table II).

Considering only offshore locations sampled in July, bioavailability of iron was highly variable (21–100% of the maximum response, one-way analysis of variance, $P < 0.05$). However, with the exception of Station ON-2, Fe_d was uniformly low in surface waters at these same sites, ranging between 1.3 and 3.8 nM (Table II).

Despite elevated levels of Fe_d present in the hypolimnion at most mid-lake stations, for the most part, iron availability did not increase with depth (Fig. 2). Only at one station (EL-0) was the bioreporter luminescent response low in the hypolimnion compared with surface waters, consistent with the higher concentration of Fe_d measured in these samples.

Water collected at depths of 5 m (epilimnion) and 25 m (subsurface Chl fluorescence maximum) at Station WM during September was filtered (0.45 μm) and further resolved into a soluble (<0.02 μm) fraction by ultraltration. Analysis of Fe_d in these fractions demonstrated that at each depth, most of the iron was present in a presumptive colloidal phase (0.02–0.45 μm; Table III). At 5 m depth, only 13% of the dissolved (<0.45 μm) iron was characterized as soluble (<0.02 μm), whereas this increased to 33% at 25 m depth. Use of the *Synechococcus* bioreporter revealed no difference in bioavailability of iron between filtered and ultrafiltered samples at these depths (Fig. 3; unpaired two-tailed *t* test, $P > 0.05$, df = 6) suggesting that over the 12-h course of the assay, the bioreporter responded solely to iron contained in the operationally defined soluble phase of these samples.

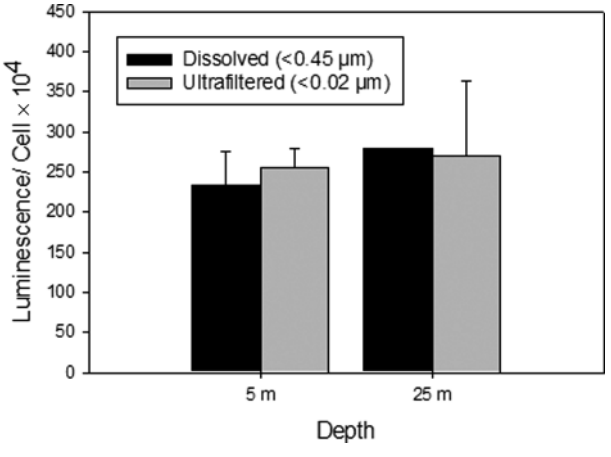

Fig. 3. Effect of ultrafiltration of dissolved iron (Fe_d) on bioreporter response. An operationally defined soluble iron fraction (0.02 μm) was obtained from Station WM in September at two depths representing the epilimnion (5 m) and the subsurface chlorophyll (Chl) maximum (25 m). The bioreporter was used to compare iron availability between soluble and dissolved (0.45 μm) fractions.

Abundance of Flvd and Fd in Lake Superior net plankton during spring

Diatoms accounted for >90% of the total phytoplankton cells enumerated in the 80–210 μm fraction of net plankton in May with *Asterionella* being the most numerous genus (Table IV). Of the eight stations occupied during May, *Asterionella* spp. accounted for 50% or greater of the total net plankton in this size class at five stations.

Western blot analysis using polyclonal antisera raised against diatom Flvd and Fd demonstrated the presence of Fd in net plankton sampled from each site during May. However, Fd staining was not consistent for all stations with an intense signal evident at only three of the eight stations sampled (PB-2, CD-1 and WM). Blots were used to quantify staining intensity and to derive a Fd index [Fd/(Fd + Flvd)] (Table IV). A Fd index of 1 (i.e. no Flvd was detected) was recorded at five of the stations sampled. Of the three remaining stations, the Fd index did not decline below 0.74.

DISCUSSION

Overview of bioreporter response

The bioreporter was used to infer patterns of iron availability to picocyanobacteria in Lake Superior during cruises conducted in May and July 2001 and in September 2002. When considering all samples from all sites, dates and depths, the response of the bioreporter was negatively correlated to the amount of Fe_d measured in the lake samples. Measures of Fe_d thus do provide a first-order indication of iron availability.

Table IV: Floristic analysis of net plankton and ferredoxin (Fd) index: May 2001 percent composition

Station	*Asterionella* spp.	*Fragilaria* spp.	*Aulacoseira* spp.	*Tabellaria* spp.	Fd index
CD-1	69	16	11	3	0.78 ± 0
PB-2	75	21	3	–	0.74 ± 0.1
WM	66	27	3	2	0.78 ± 0
ON-2	49	30	10	4	1.0
ON-1	20	49	15	11	1.0
EM	44	44	6	3	1.0
RM	34	57	2	5	1.0
EL-1	71	14	1	10	1.0

Abbreviations are as defined in Table I.
More than four hundred cells from at least four fields (10×) were counted.

From each of the nearshore stations sampled in May, we derived a pFe of <20.6, implying that the bioreporter was iron replete in these samples as based on our prior physiological characterization (Porta *et al.*, 2003). However, also included among this group of iron-replete sites were two offshore stations, ON-2 and RM. Station ON-2 is located 10 km offshore, situated on a broad, shallow coastal shelf extending from the Keweenaw Peninsula, and thus in many ways is more characteristic of a nearshore station. During the spring, this station can be influenced by a strong thermal bar that constrains offshore transport of terrigenous materials (Chen *et al.*, 2001). Station RM, however, is a true pelagic site, located ~60 km offshore and having a depth of 256 m. Notwithstanding the low absolute amount of Fe_d measured at Station RM (1.5 nM), the cyanobacterial bioreporter perceived an iron sufficient environment here. In contrast, other open lake sites having similar low levels of Fe_d evoked an iron-deficient response from the bioreporter. This specific result indicates that the bioavailability of iron does not always and reliably relate to the Fe_d concentration, perhaps because of differences in the type (Hutchins *et al.*, 1999) and the recalcitrancy (Maranger and Pullin, 2003) of organic iron-complexing ligands. The existence of varied iron chelators might also explain why on several occasions, field samples elicited >100% of the maximum luminescence obtained from accompanying calibration standards using synthetic Fraquil medium where EDTA served as the sole iron chelating agent.

In July, distinct nearshore–offshore trends in iron availability were evident along two transects (HN010 *versus* HN210 and WFB *versus* EL-2), both showing differences in the level of Fe_d measured at the transect end points. For the HN transect, we previously reported biochemical evidence (see *Discussion* on Flvd) consistent with enhanced iron-deficient conditions existing for diatoms at the offshore location (McKay *et al.*, 2004). Flvd, the biochemical marker for iron deficiency used in the study, was greatly decreased in samples collected from Station HN010, the nearshore site (McKay *et al.*, 2004). This pattern of Flvd staining is consistent with a higher terrigenous particle load constrained close to shore by the Keweenaw current. This phenomenon may also explain the lack of a distinct nearshore–offshore difference in iron availability between stations ON-1 and ON-2 located along the Ontonagon transect where both stations are situated within the influence of the current.

Soluble and colloidal iron in Lake Superior

The so-called 'dissolved' fractions of iron or other substances are operationally defined as those fractions which pass through filters of defined pore size of 0.2 or 0.45 μm. Iron contained in this 'dissolved' fraction is not, however,

truly 'dissolved'; much of it in fact is colloidal (1 kDa–0.2 μm). In estuarine and coastal regions, the colloidal fraction frequently exceeds 80% of total Fe_d (Powell *et al.*, 1996; Wen *et al.*, 1999; Wells *et al.*, 2000). Similar findings have been reported for rivers (Whitehouse, 1990; Ross and Sherrell, 1999). In the open ocean, there is considerable variability in the fraction of colloidal iron contained in the dissolved phase ranging from 80 to 90% in subtropical oligotrophic gyres (Wu *et al.*, 2001) to 5 to 50% in high nutrient, low chlorophyll (HNLC) waters of the equatorial Pacific (Wells, 2003) and the subarctic Pacific (Nishioka *et al.*, 2001). In Lake Superior, the colloidal phase (0.02–0.45 μm) was variable, accounting for >85% of Fe_d in a sample collected from the epilimnion at the offshore Station WM but only 55–60% of Fe_d in surface waters at Station EM, another offshore site.

We used the cyanobacterial iron bioreporter to compare the availability of iron contained in the soluble (<0.02 μm) fraction with iron contained in the dissolved (<0.45 μm; soluble + colloidal iron) phase for samples collected at Station WM. The bioreporter responded only to iron contained in the soluble fraction in this sample as shown by the fact that the luminescent response did not vary when the bioreporter was incubated with water from either fraction. This pattern of response is consistent with reports that iron uptake by phytoplankton is restricted to soluble iron species (Rich and Morel, 1990; Wells *et al.*, 1995). We acknowledge, however, that several recent reports demonstrate that some phytoplankton, diatoms in particular, can acquire iron complexed to colloids (Nishioka and Takeda, 2000; Chen and Wang, 2001; Chen *et al.*, 2003).

The apparent preference of the cyanobacterial bioreporter for soluble iron might also serve to inform our understanding of iron availability through different strata of the water column. In the present study, there were few discernible patterns relating iron availability with depth, despite the observation that levels of Fe_d were modestly enhanced in the hypolimnion at a majority of pelagic stations occupied in July. We note that at Station WM during September, soluble iron species accounted for >30% of Fe_d at a depth of 25 m compared with only 10% at the surface (5 m). We also note that total Fe_d was essentially unchanged at these two depths (~2.5 nM). It follows then that iron availability should be higher at 25 m compared with the 5 m sampling depth. However, this was not so. In assessing the dissolved fraction (<0.45 μm), iron availability was not significantly enhanced with depth, with each sampling depth yielding a pFe of ~21. This apparent discrepancy may be related to differences in the iron-complexing ligands that exist at different depths as has been suggested for marine systems (Rue and Bruland, 1995). It is likely that iron contained in the soluble fraction is highly complexed to low molecular weight organic ligands. Further, the cyanobacterial bioreporter exhibits differential affinity to ferric-binding ligands (Porta *et al.*, 2003), an observation that may underlie the present results. Given this speculation, future studies that address the nature and spectrum of iron-complexing ligands in freshwater systems are warranted.

Contrary to our experience in Lake Superior, we documented discernable patterns in depth-resolved iron availability as part of a recent investigation in Lake Erie, demonstrating high bioavailability of iron in the hypolimnion at pelagic stations located in both the eastern and central basins of the lake (Porta *et al.*, in press). In each instance, lower bioreporter response recorded from the hypolimnion was consistent with higher levels of Fe_d measured in this stratum. Remineralization of detritus in concert with organic complexation of the remineralized iron contributes to the elevated levels of Fe_d typically reported from waters below the mixed layer. In the case of a Lake Erie central basin station that we analysed, entrainment of sediment into the shallow hypolimnion (<5 m) likely also contributed to elevated Fe_d.

Fd index

By relying solely on a prokaryotic bioreporter, we cannot comment with certainty on iron availability to eukaryotic cells in Lake Superior. Considering the dichotomy in iron acquisition strategies between prokaryotes and eukaryotes, development of a suitable eukaryotic iron bioreporter is warranted. *In lieu* of an appropriate freshwater diatom bioreporter, we applied a biochemical approach to assess iron deficiency among members of the mixed diatom assemblage of Lake Superior in May. This approach measures the cellular concentration of Flvd and Fd, two proteins that serve, among other roles, as redox catalysts in photosynthetic electron transport. The inducible nature of Flvd, in particular, confers a diagnostic capacity to this protein as an indicator of iron deficiency, an attribute that has been exploited to demonstrate iron deficiency in marine environments (La Roche *et al.*, 1996; Maldonado *et al.*, 2001). Notably, *isiB*, part of the dicistronic operon from which the iron-responsive promoter for our cyanobacterial bioreporter is derived, encodes for Flvd in cyanophytes. This provides additional relevance to our choice in measuring Flvd as an indicator of iron deficiency among diatoms in Lake Superior.

We used antisera directed against marine diatom Flvd and Fd to detect these proteins in net plankton. Prior characterization has shown that the anti-Flvd serum cross-reacts only with diatoms (La Roche *et al.*, 1995) including freshwater species (McKay *et al.*, 2004),

whereas the anti-Fd serum shows broad reactivity with diatoms and with several non-diatom taxa (McKay *et al.*, 1999). Fd was detected in net plankton at all of the sites sampled during the spring cruise, whereas Flvd was detected at only three sites. We interpret the lack of a detectable Flvd signal at five stations sampled as an indication that the diatom net plankton at these sites were iron replete during the period of vernal mixing. Using the analysis of Erdner *et al.* (Erdner *et al.*, 1999) as a guide, the slight decrease in the Fd index reported for three stations in the present study was likely not accompanied by concomitant decreases in growth and photosynthesis among the diatoms sampled at these locations.

Despite detecting Fd in diatoms from all stations, there were large differences observed in the staining intensity of Fd between stations with particularly strong immunoreactions associated with the protein profiles resolved from samples collected at nearshore stations PB-2 and CD-1 and also from pelagic Station WM, where the strong Fd signal was inconsistent with the low measured Fe_d of 1.1 nM. Considering solely measured concentrations of Fe_d, it is likely that Station WM would be classified as a candidate site for iron deficiency. This designation is supported in part by the observation of Flvd accumulation associated with at least a component of the diatom assemblage endemic to this site. That Fd persisted in diatoms sampled from Station WM provides further support to our contention that Fe_d does not serve as an absolute proxy for the iron status of the endemic phytoplankton community.

Thus, during May, prokaryotes and eukaryotes provided a mostly unified response in terms of iron availability. The eukaryotic response was indicative of iron replete conditions at five of eight stations where the Fd Index measured 1. The slightly decreased Fd index of ~0.75 measured at the three remaining stations was not expected to be manifested as physiological iron deficiency (Erdner *et al.*, 1999). Likewise, the prokaryotic response, represented by the cyanobacterial iron bioreporter, was indicative of iron-sufficient conditions at most sites.

However, several exceptions to this general analysis served to highlight important differences between the two approaches and also perhaps to reinforce the underlying dichotomy between eukaryotes and prokaryotes in terms of iron acquisition. Whereas the bioreporter indicated iron-sufficient conditions existing at all nearshore stations in May, some Flvd accumulation was recorded among diatoms sampled at stations PB-2 and CD-1 despite conditions of elevated Fe_d (>11 nM). Thus, at least a component of the diatom assemblage at these nearshore stations had perceived reduced iron availability resulting in the induction of Flvd expression. This represents one advantage of the immunochemical approach, especially when combined with immunofluorescence microscopy (Boyd *et al.*, 2000) as it can identify variability in the response to iron availability within the community. By contrast, the bioreporter serves solely as a representative member of a specific community, in this instance, the picocyanobacteria.

At the other end of the spectrum, the cyanobacterial bioreporter showed signs of iron deficit at two open-lake stations sampled during May, EL-1 and EM. Corresponding immunochemical analysis of the diatom assemblage existing at these stations, however, showed no signs of iron deficit as Flvd was not detected in immunoblots. Although we offer no direct evidence, the difference in perceived iron availability between diatoms and the cyanobacterial iron bioreporter may be related to the quality of iron complexing ligands present rendering iron in a form readily available to diatoms but not so to cyanobacteria. Although not a straightforward task, it is clear that interpretation of future work will benefit from analysis of iron speciation concomitant with the use of the iron bioreporter.

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