

PHYSIOLOGICAL CHARACTERIZATION OF A *SYNECHOCOCCUS* SP. (CYANOPHYCEAE) STRAIN PCC 7942 IRON-DEPENDENT BIOREPORTER FOR FRESHWATER ENVIRONMENTS¹

David Porta, George S. Bullerjahn, Kathryn A. Durham

Department of Biological Sciences, Bowling Green State University, Bowling Green, Ohio 43403, USA

Steven W. Wilhelm

Department of Microbiology, The University of Tennessee, Knoxville, Tennessee 37996, USA

Michael R. Twiss²

Department of Chemistry, Biology and Chemical Engineering, Ryerson Polytechnic University, 350 Victoria Street, Toronto, Ontario, M5B 2K3, Canada

and

R. Michael L. McKay³

Department of Biological Sciences, Bowling Green State University, Bowling Green, Ohio 43403, USA

The complex chemical speciation of Fe in aquatic systems and the uncertainties associated with biological assimilation of Fe species make it difficult to assess the bioavailability of Fe to phytoplankton in relation to total dissolved Fe concentrations in natural waters. We developed a cyanobacterial Fe-responsive bioreporter constructed in *Synechococcus* sp. strain PCC 7942 by fusing the Fe-responsive *isiAB* promoter to *Vibrio harveyi luxAB* reporter genes. A comprehensive physiological characterization of the bioreporter has been made in defined Fraquil medium at free ferric ion concentrations ranging from pFe 21.6 to pFe 19.5. Whereas growth and physiological parameters are largely constrained over this range of Fe bioavailability, the bioreporter elicits a luminescent signal that varies in response to Fe deficiency. A dose-response characterization of bioreporter luminescence made over this range of Fe³⁺ bioavailability demonstrates a sigmoidal response with a dynamic linear range extending between pFe 21.1 and pFe 20.6. The applicability of using this Fe bioreporter to assess Fe availability in the natural environment has been tested using water samples from Lake Huron (Laurentian Great Lakes). Parallel assessment of dissolved Fe and bioreporter response from these samples reinforces the idea that measures of dissolved Fe should not be considered alone when assessing Fe availability to phytoplankton communities.

Key index words: bioreporter; cyanobacteria; flavodoxin; iron; *isiAB*; Lake Huron; luminescent; *Synechococcus*

Abbreviations: decanal, *n*-decyl aldehyde; DFB, desferrioxamine B; F_m, 3'-(3,4-dichlorophenyl)-1',1'-dimethyl-urea-enhanced maximum fluorescence; F_v, variable fluorescence; P-I, photosynthesis versus irradiance; RA, rhodotorulic acid

Fe bioavailability in aquatic environments is largely controlled by chemical complexation and redox cycling, with the resulting chemical speciation of Fe exerting important controls on phytoplankton growth (Sunda 2000). Indeed, ecosystem scale fertilization experiments have now demonstrated that Fe bioavailability limits phytoplankton growth in areas of the open ocean characterized as "high nutrient, low chl" regions (Martin et al. 1991, Coale et al. 1996, Boyd et al. 2000). Other aquatic systems are also prone to Fe deficiency, including oligotrophic oceanic gyres (DiTullio et al. 1993, Greene et al. 1994, McKay et al. 2000), coastal marine waters (Hutchins and Bruland 1998, Hutchins et al. 1998), and even some freshwater systems (Schelske 1962, Wetzel 1966, Twiss et al. 2000, Durham et al. 2002).

Biochemical studies on Fe depletion in eukaryotic algae have demonstrated that Fe deficiency results in an exchange of the electron transfer catalyst ferredoxin for flavodoxin, an iron-free but functionally equivalent protein (La Roche et al. 1995, Doucette et al. 1996, McKay et al. 1997, 1999, Erdner et al. 1999). Moreover, it has been demonstrated that the accumulation of flavodoxin can serve as a cellular marker for Fe deficiency in natural phytoplankton assemblages (La Roche et al. 1996). Similarly, flavodoxin is demonstrated to replace ferredoxin in cyanobacteria under conditions of Fe deficiency (Straus 1994). In cyanophytes, flavodoxin is encoded by *isiB*, which together with *isiA* forms an operon under the control of a homologue of the Fe-dependent repressor Fur (Ghassemian and Straus 1996). The *isiAB* operon is widely

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²Present address: Department of Biology, Clarkson University, Potsdam, NY 13699-5805, USA.

³Author for correspondence: e-mail rmmckay@bgnet.bgsu.edu.

distributed among cyanophytes (Geiss et al. 2001), and with the exception of *Synechocystis* PCC 6803 where, in addition to Fe deficiency, salt stress is also reported to result in *isiAB* transcription (Vinnemeier et al. 1998), the operons known only to be responsive to Fe. Thus, its expression may serve as a diagnostic tool for Fe deficiency in natural populations of cyanobacteria.

Because the *isiAB* genes are readily recognized as a marker for Fe deficiency, we constructed a luminescent cyanobacterial bioreporter using the *Synechococcus* sp. strain PCC 7942 *isiAB* promoter fused to the *Vibrio harveyi luxAB* reporter genes (Durham et al. 2002). This tool provides us with a means of assessing Fe availability in freshwater environments, as perceived by the specific test organism. Here, we present a detailed physiological characterization of the bioreporter conducted during growth in defined trace metal-buffered medium. This characterization has enabled a quantitative assessment of the bioreporter luminescent response in relation to various physiological parameters and in relation to the free ferric ion content of the growth medium. In addition, we report on the use of the bioreporter to assess Fe bioavailability in natural water samples collected from Lake Huron, one of the Laurentian Great Lakes.

MATERIALS AND METHODS

Strains and culture conditions. Our studies used two cyanobacterial *Synechococcus* strain sp. PCC 7942 *psiAB::luxAB* constructs. Strain KAS100 carries a copy of the *isiAB::luxAB* promoter fusion integrated into the chromosome. Strain KAS101 is identical to KAS100 except that the aldehyde substrate synthesis genes *luxCDE*, under low level constitutive expression from the *psbAI* promoter (Liu et al. 1995), have been introduced into the chromosome at a second neutral site (Durham et al. 2002). Strain KAS101 is therefore capable of endogenous luminescence independent of added aldehyde substrate. Strains were grown in batch culture in trace metal-buffered Fraquil medium (Morel et al. 1975) at 24° C under continuous illumination of approximately 50 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Fraquil medium was prepared containing varying levels of total iron (FeCl_3) that corresponded to thermodynamically calculated free ferric ion concentrations (Twiss et al. 2001, $\text{pFe} = -\log[\text{Fe}^{3+} \text{ free ferric}]$): 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). Macronutrient stocks were treated with Chelex-100 resin before addition to the medium (Price et al. 1989). To further minimize trace metal contamination, all materials were soaked in 5%–10% HCl (trace metal grade, Fisher, Pittsburgh, PA, USA) for at least 24 h and rinsed thoroughly with Milli-Q water (Millipore Corp., Bedford, MA, USA) before use.

Experiments were carried out in semicontinuous batch cultures. Where appropriate, spectinomycin and kanamycin were added to 20 $\mu\text{g}\cdot\text{mL}^{-1}$ to maintain the integrity of the *luxAB/CDE* chromosomal constructs (Durham et al. 2002). Strains were cultured in 30 mL of medium contained in 50-mL polycarbonate tubes. Late exponential phase cells were diluted 1:30 into fresh medium. Growth curves were derived from daily measurements of *in vivo* chl *a* fluorescence using a TD-700 Fluorometer (Turner Designs, Sunnyvale, CA, USA). Regression analysis of logarithmically transformed data was used to determine exponential phase-specific growth rates. Estimation of the free ferric ion (pFe) content in metal-buffered medium was calculated using MINEQL+ (version 4.5) software (Twiss et al. 2001).

Photosynthetic measurements. PSII photochemical efficiency was assessed using the ratio F_v/F_m (Butler and Kitajima 1975),

measured according to McKay et al. (1997). Measures of *in vivo* fluorescence were made with a fluorometer on samples dark-adapted for 15 min before (F_0) and after (F_m) the addition of 30 μM 3'-(3,4-dichlorophenyl)-1',1'-dimethylurea. Chl *a* determination was conducted by fluorometry (Welschmeyer 1994) after overnight pigment extraction at 4° C in 90% (v/v) aqueous acetone. To measure photosynthetic rates, $\text{NaH}^{14}\text{CO}_3$ ([740 kBq; specific activity: 2.0 $\text{GBq}\cdot\text{mmol}^{-1}$] ICN Biomedicals, Irvine, CA, USA) was added to a 30-mL aliquot of exponential phase cells after a 30-min dark adaptation. The cell suspension was distributed into 7-mL glass scintillation vials that were incubated simultaneously under 24 different light intensities for 30 min using a temperature-controlled photosynthetron (CHPT Inc., Lewes, DE, USA) as described by McKay et al. (1997). Labile ^{14}C was volatilized by acidification of the samples with 50 μL of 6N HCl. Acid-stable ^{14}C assimilation was measured by liquid scintillation counting after the addition of 4.5 mL of scintillation cocktail (Ecolite(+); ICN) to each vial. Total activity of the added ^{14}C was determined by adding 20 μL of the sample at $t = 0$ to scintillation cocktail containing 200 μL β -phenylethylamine, whereas background activity was determined by dispensing a sample aliquot directly into formaldehyde before adding scintillation cocktail. Counting was conducted using a Searle Analytic (Des Plaines, IL, USA) Isocap-300 scintillation counter for which quench curves had been generated using ^{14}C standards. Disintegrations per minute were calculated for each sample from the ratio of counts per minute:counting efficiency, the latter of which was extrapolated from the quench curves. Photosynthesis-irradiance (P-I) curves were generated from which rates of photosynthesis were determined by using a nonlinear regression curve fitting function (SigmaPlot 4.0.1, SPSS Inc., Chicago, IL, USA).

Quantitative assessment of luminescence in Fraquil medium. The Fe-dependent luminescent response was measured in both KAS100 and KAS101 constructs under different Fe conditions. Late exponential to early stationary phase cells growing in Fraquil (pFe 20.6) were collected by centrifugation (10 min, 4000g) and rinsed twice in Fe-free Fraquil medium before being used to inoculate medium of defined free ferric ion content (pFe 19.5 to pFe 21.6). The cells were incubated for 12 h under standard culture conditions before luminometry. Luminescence was monitored in 2-mL aliquots using a portable luminometer (Femtomaster FB14, Zylux Corp., Maryville, TN, USA). The substrate for luciferase in the KAS100 constructs was provided by exposing the bioreporter to *n*-decyl aldehyde (decanal, Sigma Chemical Co., St. Louis, MO, USA), supplied either as vapors (aqueous solution diluted 1:9 with deionized water and provided in a sealed container) or by direct injection of 4 μL decanal (1% [v/v] solution in DMSO) into the culture aliquots. For KAS101, the aldehyde substrate was either provided from endogenous *luxCDE* expression or supplemented with exogenous decanal vapor exposure. Luminescence results (as relative luminescence units per second) were normalized to *in vivo* chl *a* fluorescence, which was adopted as a proxy for cell biomass.

Fe uptake assay and elemental analysis. For cellular Fe uptake measurements, a combination of $^{55}\text{FeCl}_3$ ([in 0.5 M HCl; specific activity: 1.0366 $\text{GBq}\cdot\text{mg}^{-1}$] New England Nuclear, Boston, MA, USA) and nonradioactive FeCl_3 was added to triplicate polycarbonate tubes each containing 30 mL of medium to generate total final iron concentrations yielding pFe 19.5 and pFe 21.6 (added ^{55}Fe was $\geq 10\%$ of the total Fe). Tubes were inoculated with the bioreporter and incubated at approximately 50 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and 25° C. At daily intervals, 10 mL of culture was filtered onto a 0.4- μm pore size polycarbonate membrane (Osmomics, Inc., Livermore, CA, USA), and cells were rinsed of extracellular-bound Fe using a Ti-citrate-EDTA reagent (Hudson and Morel 1989) modified for use with freshwater samples (NaCl content decreased to 0.3% final). Intracellular Fe was measured by liquid scintillation counting.

Parallel tubes (to which no radioisotope was added) at the same pFe values were used to measure the carbon and nitrogen content of the bioreporter cells. At daily intervals, 15–25 mL of culture was filtered through a precombusted glass fiber filter

(GF/F) and dried overnight at 62° C. Elemental analysis was made using a Perkin-Elmer carbon, hydrogen and nitrogen (CHN) analyzer, Wellesley, MA, USA. Direct counts of autofluorescent *Synechococcus* cells were made on glutaraldehyde-preserved samples using an epifluorescent microscope (Model DMRXA, Leica, Leica Microsystems, Inc., Bannockburn, IL, USA) and Image Pro Plus Media Cybernetics, Carlsbad, CA, USA (version 4.1) software.

Effect of Fe chelators on luminescence. To determine how complexation to organic ligands influenced iron availability, we used commercially available metal chelators. Two trihydroxamate siderophores, desferrioxamine B (DFB) and ferrichrome, and the dihydroxamate siderophore, rhodotorulic acid (RA), were purchased from Sigma Chemical. The zinc (II) chelator, zinquin, was used as a control and was purchased from Toronto Research Chemical Company (Toronto, ON, Canada). Chelators were added to 1:1, 10:1, and 25:1 chelator:total Fe ratios in BG-11 medium (Allen 1968), containing 100 nM total Fe added as FeCl₃ (30-mL final volume). A culture of KAS101 growing in 100 nM Fe BG-11 medium was collected by centrifugation and used to inoculate treatments after resuspension twice in Fe-free medium. Test cultures were incubated for 24 h at 25° C and approximately 50 μmol photons·m⁻²·s⁻¹ before determination of bioreporter luminescence.

Assessing the bioreporter in natural waters. Water was assayed using the Fe bioreporter from six stations in Lake Huron and Georgian Bay (Table 1) collected during an August 1998 research cruise on board the *C.C.G.S. Limnos*. In each case, epilimnetic water from a depth of 10 m was sampled using a Teflon-coated Go-Flo bottle. Water temperature at this depth ranged between 19 and 21° C, and at most stations the epilimnion extended to approximately 20 m. Samples were processed through a 0.4-μm pore size polycarbonate filter and transferred to Teflon bottles before freezing. All water sampling materials were acid cleaned and rinsed with deionized (>18 MΩ·cm⁻¹) water before use, and all manipulations were conducted in a clean van on board the vessel. Dissolved Fe (<0.4 μm) was determined in lake water samples by graphite furnace atomic absorption spectroscopy (Perkin-Elmer AA-800). Analytical accuracy was confirmed by analysis of SLRS-4, a standard reference material for dissolved trace metals in fresh water (National Research Council of Canada).

The *Synechococcus* bioreporter strain KAS101 maintained in Fraquil medium was used to assess available Fe in each of the natural water samples. Before use of the bioreporter, cells were prepared in the same manner as for the laboratory characterization (see above). The bioreporter assay was carried out in replicate ($n = 4-5$) acid-rinsed polycarbonate tubes to which 20 mL of filtered lake water had been dispensed. Tubes were

amended with Fraquil phosphate and nitrate stocks and then inoculated with 1.5 mL of the bioreporter. Addition of 1000 nM Fe or, alternatively, 1000 nM DFB served as negative and positive controls, respectively. Luminescence was monitored after 12–13 h of incubation at 24° C under continuous light. Bioreporter growth was monitored by *in vivo* chl *a* fluorescence.

RESULTS

Fe-responsive growth and photosynthetic characterization of the bioreporter. Physiological characterization of the Fe bioreporter initially compared Fe-dependent growth rates and photochemical efficiency of KAS100 and wild-type *Synechococcus* sp. strain PCC 7942. Whereas no significant differences in growth rate were detected between strains, a slight difference was observed between treatments for the bioreporter strain KAS100 (one-way analysis of variance, $P < 0.05$) (Table 2). Specifically, cultures of the bioreporter grown at pFe 21.6 yielded a small (33%) but significant decrease in growth rate compared with pFe 20.6 and pFe 19.5 cultures (t -test, $P < 0.05$).

There were negligible differences in photochemical efficiency as assessed by F_v/F_m between Fe treatments for strain KAS100, although a slight reduction was measured for wild-type cells grown at pFe 21.6 compared with pFe 20.6 (t -test, $P < 0.05$) (Table 2). Otherwise, there were no discernible differences between the wild-type and KAS100 strains or between different treatments. These data show that the genetic modification of PCC 7942 with the *psiiA::luxAB* fusion did not yield any major physiological changes under laboratory culture conditions. As a result, subsequent experiments were conducted with the genetically modified strains only.

P-I curves were generated for KAS100 cells growing at pFe 21.6 and pFe 20.6. Photosynthetic efficiency as determined by the initial slope of the P-I curve was about two times higher in cells grown at pFe 20.6 compared with those growing at pFe 21.6 (0.3 ± 0.1 vs. 0.13 ± 0.01 g C·g chl⁻¹·h⁻¹/[μmol·m⁻²·s⁻¹], re-

TABLE 1. Construct KAS101 used to assess bioavailable Fe in water sampled from Lake Huron (mean ± SE, $n = 4-5$).

	Latitude N Longitude W	Dissolved Fe (nM)	Bioreporter luminescence ^a	pFe Equivalent ^b (linear regression)	+DFB ^c	+Fe ^d
Lake Huron						
121	43°31'01" 82°10'00"	9.5 ± 1.1	13.2 ± 2.8	20.54	340.1 ± 11	5.9 ± 1.2
123	44°40'02" 82°49'56"	16.7 ± 0.8	17.6 ± 2.0	20.58	609.5 ± 48	7.1 ± 1.2
124	44°55'00" 81°50'00"	7.1 ± 0.4	8.4 ± 2.5	20.50	130.4 ± 15	4.4 ± 1.1
126	45°44'59" 83°55'01"	39.7 ± 1.4	7.9 ± 2.3	20.50	344.8 ± 3	7.1 ± 0.7
Georgian Bay						
128	45°07'58" 80°47'05"	15.9 ± 0.7	9.0 ± 2.7	20.51	174.1 ± 10	4.2 ± 1.0
129	45°27'00" 81°19'58"	22.3 ± 1.0	13.8 ± 3.1	20.55	889.7 ± 40	7.6 ± 2.2

^aLuminescence normalized to *in vivo* chl *a* fluorescence.

^bCalculated from linear regression of luminescent response after exposure for 20 min to decanal vapors (Durham et al. 2002).

^cPositive control; addition of 1000 nM desferrioxamine B (DFB).

^dNegative control; addition of 1000 nM Fe.

TABLE 2. Comparison of growth rates and photochemical efficiency of wild-type *Synechococcus* sp. strain PCC 7942 and *pisiA::luxAB* construct KAS100.

	KAS100			PCC 7942		
	21.6	20.6	19.5	21.6	20.6	19.5
pFe	21.6	20.6	19.5	21.6	20.6	19.5
μ (d ⁻¹)	0.53 ± 0.20	0.80 ± 0.16	0.80 ± 0.21	0.69 ± 0.14	0.78 ± 0.16	0.88 ± 0.23
F _v /F _m	0.41 ± 0.04	0.39 ± 0.11	0.53 ± 0.06	0.46 ± 0.03	0.55 ± 0.03	0.49 ± 0.02

Values are means ± SE. μ , growth rate.

spectively) (Fig. 1). Likewise, the rate of light-saturated photosynthesis measured for cells grown at pFe 20.6 (54.40 ± 3.0 g C·g chl⁻¹·h⁻¹) was greater than twice that recorded for cells growing at pFe 21.6 (24.52 ± 0.9 g C·g chl⁻¹·h⁻¹). These data are consistent with previous studies on Fe-responsive photosynthetic parameters conducted with cyanobacteria (Öquist 1971, Reuter and Unsworth 1991).

Effect of mode of delivery of aldehyde substrate on bioreporter luminescence. In most whole-cell bioreporters that incorporate *luxAB* promoter fusions, the aldehyde substrate required by luciferase is injected into the medium to elicit a luminescent response (Blouin et al. 1996, Sticher et al. 1997, Gillor et al. 2002). Yet there is some concern over potential cytotoxic effects of direct injection, and some investigators advocate delivery by exposure to aldehyde vapors instead (Kondo and Ishiura 1994, Andersson et al. 2000). To test both the intensity and reproducibility of the luminescent response under low Fe conditions, we tested the efficacy of providing the decanal substrate to KAS100 by both approaches.

In the case of aldehyde injection directly into the bioreporter cell suspension, a strong luminescent response was observed within 10 min (Fig. 2). After this time, however, light emission began to decrease, decaying to a stable faint signal within 3 h. When the aldehyde substrate was provided by exposure to vapors, the luminescent response increased rapidly during the first 30 min, followed by a more steady increase,

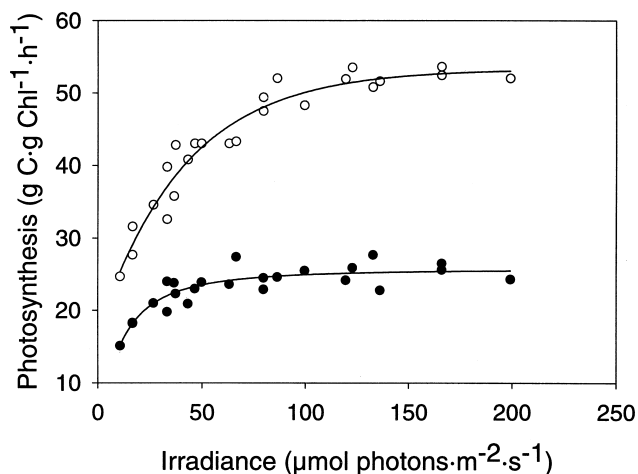


FIG. 1. P-I curves of KAS100 cultured at pFe 20.6 and 21.6. ●, pFe 21.6; ○, pFe 20.6.

reaching its maximum output at 3 h (Fig. 2). As a result, we chose substrate delivery by vapors for subsequent work when exogenously supplied substrate was required.

Non-steady state response of the bioreporter to varying free [Fe³⁺]. Figure 3 compares endogenous luminescence of KAS101 (Fig. 3A) with the luminescent signal elicited from cells provided decanal vapors for 50 min (Fig. 3B) after an incubation of the bioreporter for 12 h in Fraquil medium at each defined pFe. Discernible changes were measured in the luminescent response of cells to varying [Fe³⁺] (Fig. 3). Whereas luminescence was quenched in cells incubated in medium containing pFe 19.5, a small but genuine response was measured for cells incubated at pFe 20.6. In addition, cells exposed to the lowest Fe regimen (pFe 21.6) exhibited approximately six to seven times greater luminescence compared with cells incubated at pFe 20.6.

Despite sizeable differences in absolute luminescence values between the two assays, in each case luminescence plotted as a function of pFe was described according to a four-parameter sigmoidal curve (Fig. 3). Equation 1 describes the relationship between luminescence and pFe depicted in Figure 3A with the bioreporter using endogenous substrate yielded by

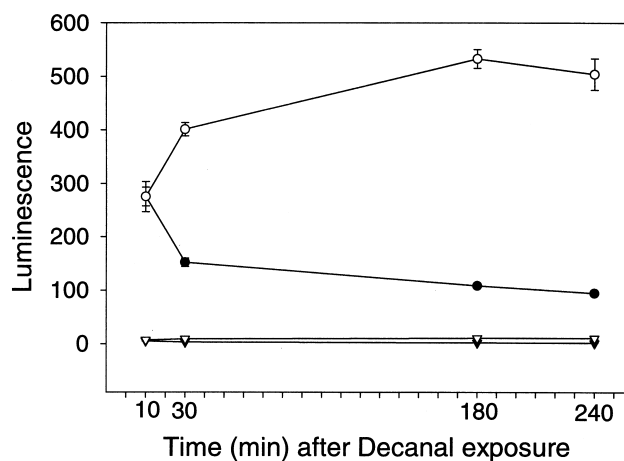


FIG. 2. Comparison of the luminescent response of KAS100 after the addition of exogenous decanal substrate by injection or by vapor exposure. Bioreporter cells were incubated in medium of the appropriate pFe for 12 h, after which luminescence was measured after exposure to vapors of decanal or direct injection of decanal (means ± SE). ○, pFe 21.6, vapor exposure; ●, pFe 21.6, direct injection; ▽, pFe 19.5, vapor exposure; ▼, pFe 19.5, direct injection.

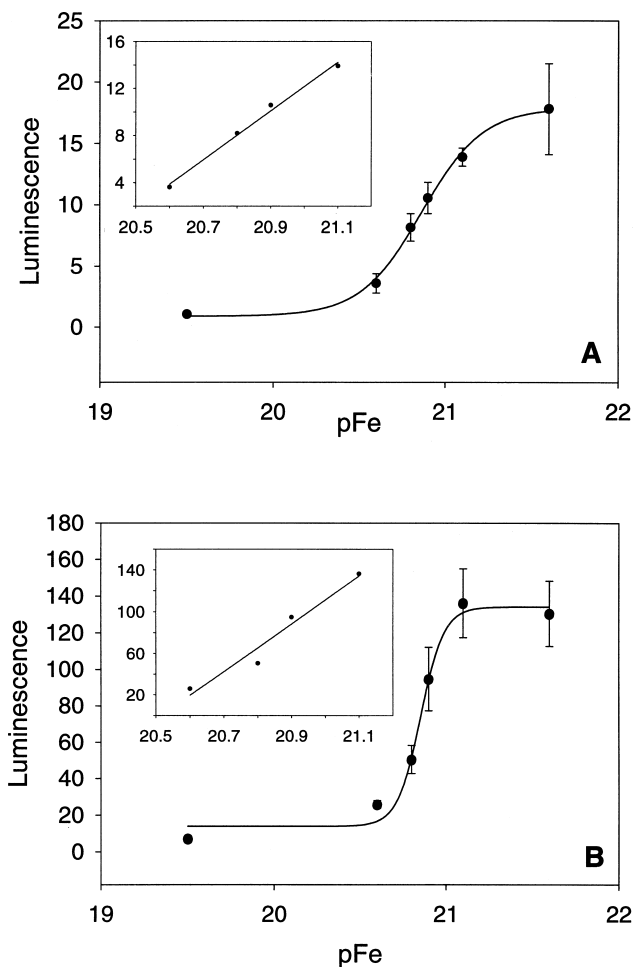


FIG. 3. Dose-response characterization of luminescence as a function of the free ferric ion content of Fraquil medium. (A) Luminescent response of strain KAS101 after 12 h of incubation at pFe values indicated. No exogenous aldehyde substrate was supplied. (B) The same cell samples as used in A, except that the decanal substrate was supplied by exposure to vapors for 50 min (means \pm SE). Insets: regression analysis of linear region of curves.

luxCDE function. Equation 2 describes the response elicited after 50 min of exposure to vapors of decanal:

$$y = 0.889 + \frac{17.013}{1 + e^{-\left(\frac{x-20.864}{0.178}\right)}} \quad (R^2 = 0.997) \quad (1)$$

$$y = 14.543 + \frac{120.265}{1 + e^{-\left(\frac{x-20.853}{0.068}\right)}} \quad (R^2 = 0.989) \quad (2)$$

where y is the normalized luminescence and x is pFe.

Alternatively, the dose-response relationship between luminescence and pFe could be described as a simpler linear relationship due to the inherent characteristics of the central region of the sigmoidal calibration curves (Fig. 3, insets). This region, extending

between pFe 21.1 and pFe 20.6, highlights the detection window of the dynamic range of this Fe bioreporter. A linear regression drawn through this region in Figure 3A provided the following equation:

$$y = -422.640 + 20.705x \quad (R^2 = 0.992) \quad (3)$$

Similarly, a linear regression fitted to this region in Figure 3B was represented by the following equation:

$$y = -4708.249 + 229.501x \quad (R^2 = 0.957) \quad (4)$$

Relationship between bioreporter elemental composition and luminescent response. The luminescent response was similarly measured in relation to cellular Fe quotas determined using ^{55}Fe (Table 3). The Fe:C ratios determined for the *Synechococcus* PCC 7942 bioreporter reflect the Fe composition of the medium with a low Fe:C ratio (3.1 $\mu\text{mol Fe}:\text{mol C}$) resolved for cells grown at a low $[\text{Fe}^{3+}]$ (pFe 21.6) and a substantially higher ratio (22.6 $\mu\text{mol Fe}:\text{mol C}$) resolved for Fe-replete cells grown at pFe 19.5.

The bioreporter luminescent response was negatively correlated to intracellular Fe:C (Table 3). Although the luminescent response was effectively quenched in Fe-replete cells (pFe 19.5) having a relatively high Fe:C ratio, a strong luminescent signal was elicited under low Fe conditions. It appears that the maximum luminescent response coincided with a depletion of intracellular Fe to a level of approximately 0.7 $\text{amol Fe}\cdot\text{cell}^{-1}$. Unlike the cellular Fe quota, bioreporter luminescence was not well correlated to variation in the cellular C:N ratio that ranged from 4.3 to 5.7 (Table 3). These values were indicative of N-replete cells.

Response of the bioreporter to exogenous ligands. The Fe specificity of the luminescent response was supported by results yielded by three of the model ligands: DFB, RA, and ferrichrome. In each case, addition of the Fe chelator yielded a robust luminescent response from the bioreporter (Table 4). By comparison, addition of the synthetic quinoline Zn chelator zinquin (Kimura and Koike 1998) yielded no luminescent response (Table 4), which substantiates the Fe-specific nature of the bioreporter. Considering each chelator alone, it was apparent that luminescence was not affected by varying the ratio of chelator:total Fe (one-way analysis of variance). Although each chelator was effective at yielding a strong luminescent response from the bioreporter, differences were evident between ligands. Notably, there was a distinction between the two trihydroxamate siderophores, with DFB seemingly more effective at sequestering Fe from *Synechococcus* compared with

TABLE 3. Bioreporter luminescence and elemental composition of construct KAS100 after 36 h of incubation (mean \pm SE, $n = 3$).

pFe	Bioreporter luminescence ^a	Fe:C ($\mu\text{mol}:\text{mol}$)	C:N (mol:mol)
21.6	297.5 \pm 73.4	3.1 \pm 0.3	5.7 \pm 0.7
19.5	0.7 \pm 0.1	22.6 \pm 0.9	4.3 \pm 0.1

^aLuminescence normalized to *in vivo* chl *a* fluorescence.

TABLE 4. Luminescence of KAS101 in response to the iron chelators DFB, RA, and ferrichrome and the zinc chelator zinquin. No exogenous aldehyde was provided.

	Ligand ratio		
	1:1	10:1	25:1
DFB	919.2 ± 33.4	1113.2 ± 27.6	880.2 ± 107.6
RA	588.4 ± 423.7	1184.9 ± 609.2	811.9 ± 50.4
Ferrichrome	343.2 ± 69.4	536.7 ± 21.6	270.9 ± 46.7
Zinquin	4.1 ± 1.0	3.0 ± 0.8	3.0 ± 0.0

Data were obtained following 24 h of incubation. (mean ± SE, $n = 3$).

ferrichrome. At each ligand:Fe ratio tested, addition of DFB yielded a two to three times greater luminescent response over that of ferrichrome (t -test, $P < 0.05$). The dihydroxamate RA, when added at a 25:1 ratio, was also more effective at withholding Fe from the bioreporter compared with ferrichrome (t -test, $P < 0.05$). In contrast, responses elicited from the bioreporter by DFB and RA did not vary significantly (t -test), suggesting they were equally effective as Fe complexing agents in the presence of *Synechococcus* PCC 7942.

Assessing bioavailable Fe in Lake Huron. The bioreporter was used to assess Fe availability in field samples collected from the epilimnion of pelagic Lake Huron. Modest variability in dissolved Fe was evident in the samples analyzed from the lake (Table 1). Hydrographic profiles conducted at each station indicated the waters to be thermally stratified during the time of sampling (M. Charlton, Environment Canada, personal communication). With the exception of station 126, where 39.7 ± 1.4 nM Fe was recorded, total dissolved Fe did not exceed 17 nM in the Lake Huron stations sampled. The samples acquired from stations 128 and 129 in Georgian Bay, a large inlet of Lake Huron, contained 15.9 ± 0.7 nM and 22.3 ± 1.0 nM of dissolved Fe, respectively. Although three stations sampled in Lake Huron (121, 124, and 126) were considered "near-shore" sites, it should be noted that dissolved Fe measured at two of these sites (121 and 124), both located along the eastern shore of the lake, was markedly lower than the single mid-lake site sampled (123).

Relating our chemical assessment of dissolved Fe to the luminescent response of the bioreporter, only water sampled from the near-shore site, station 126, located along the northwestern shoreline of the Bruce Peninsula, elicited a seemingly straightforward response from the Fe bioreporter. Here, high dissolved Fe measured in the sample (39.7 ± 1.4 nM) corresponded with a quenched bioreporter response (Table 1). In fact, the luminescent response elicited by this sample did not vary (t -test) from that of its corresponding negative control, which was amended with 1000 nM Fe. This indicated that dissolved Fe was not only present at a high level in this sample, but that it was also readily bioavailable. A quenched luminescent response similarly indicated high bioavailable Fe at

station 124 despite the fact that chemically measured dissolved Fe did not exceed 7 nM. The luminescent response elicited by water sampled from this station was not significantly different from that reported at station 126 (t -test) despite there being more than five times higher Fe levels measured at the latter site. A similar case was demonstrated by comparison between samples collected from station 121 and station 124. Although a higher dissolved Fe was measured at the former station (t -test, $P < 0.05$), this station also elicited a higher luminescent response, indicating relative lower Fe availability. Similarly, comparison between the two sites sampled in Georgian Bay indicated that Fe was less available to the Fe bioreporter at station 129 (50% higher luminescent response; t -test, $P < 0.05$) despite there being a nearly 50% higher concentration of dissolved Fe measured at this station compared with station 128 (t -test, $P < 0.01$).

DISCUSSION

It is becoming increasingly clear that in aquatic environments, Fe availability is a key factor controlling phytoplankton production, diversity, and standing crop biomass. However, because most Fe is present either in particulate form or is complexed to uncharacterized organic ligands and perhaps not directly available to phytoplankton, there is growing consensus against accepting chemical measures of dissolved and particulate Fe as sole proxies for identifying Fe-deficient aquatic environments. In response to the need for another means of measuring dissolved Fe besides strict chemical analysis, we developed a cyanobacterial bioreporter to assess the bioavailable fraction of dissolved Fe in freshwater systems. Constructed using the Fe-responsive *isiAB* promoter from the cyanobacterium *Synechococcus* PCC 7942, the bioreporter emits variable luminescence depending on Fe availability with Fe deficit manifested through an elevated luminescent response. We demonstrated the general efficacy of the bioreporter through its ability to detect Fe deficiency in natural water samples collected from Lake Erie (Durham et al. 2002). Here we report a complete characterization of the bioreporter in defined trace metal-buffered medium. Considering that Fe is an essential nutrient and that both intracellular Fe reserves and external Fe levels must be considered when interpreting the Fe-dependent luminescent response, a detailed physiological characterization of the *Synechococcus* PCC 7942 bioreporter was warranted before its quantitative use in the field.

Characterization of Fe-responsive growth demonstrated that the bioreporter growth rates and photochemical efficiency (F_v/F_m) were essentially unchanged while growing under the steady state Fe conditions assessed in this study, the exception being a minor, albeit significant, reduction in growth rate for bioreporter cells cultured at pFe 21.6. This is consistent with previous work with *Synechococcus* PCC 7942, where it was shown that cells grown in low Fe medium (pFe 21) maintained similar (>90%) rates of growth compared

with Fe-replete (pFe 17) cells (Falk et al. 1995). By contrast, studies documenting the Fe-responsive growth of marine *Synechococcus* show markedly different results. For example, there was no difference in growth recorded for *Synechococcus* DC2 cultured at either pFe 17.6 or pFe 18.6, yet a significant decline in growth was evident for cells grown at pFe 19.6 (Kudo and Harrison 1997). In another study, Wilhelm et al. (1996) documented declining growth rates in the marine *Synechococcus* PCC 7002 that extended between pFe 17 and pFe 20, with a dramatic recovery in growth exhibited by cells grown at pFe 21 that was attributed to the onset of siderophore production at this $[\text{Fe}^{3+}]$.

Despite the lack of variation in Fe-responsive growth observed in the present study, the bioreporter yielded markedly different luminescent response under the various steady-state growth conditions. Luminescence from cells cultured in medium containing pFe 19.5 was at baseline, whereas cells cultured at pFe 20.6 responded with a luminescent signal approximately two times greater than the “background” signal elicited at pFe 19.5. Accordingly, cells maintained at the lowest Fe regimen (pFe 21.6) exhibited approximately six times greater luminescence compared with cells incubated at pFe 20.6. These results are consistent with a previous study of marine diatoms cultured under varying $[\text{Fe}^{3+}]$ in which distinct changes in the accumulation of flavodoxin (*isiB* gene product in cyanobacteria) were evident despite only subtle changes recorded in growth rate and F_v/F_m (McKay et al. 1997). Likewise, our results are consistent with the finding that the expression of flavodoxin is best characterized as an early stress response event in cyanobacteria and that accumulation of flavodoxin does not need to be tied to a depression in physiological function of the cell (Sandmann and Malkin 1983, Sandmann et al. 1990).

The higher magnitude of the luminescent response observed in Figure 3B compared with Figure 3A is attributed to the addition of exogenous decanal, which was apparently required to enhance aldehyde substrate levels around the luciferase reporter enzyme beyond that provided by endogenous *luxCDE* expression. In each case, luminescence plotted as a function of pFe was described according to a four-parameter sigmoidal curve, similar to that observed in a preliminary characterization of this bioreporter in which cells were provided decanal vapors for 20 min (Durham et al. 2002). Although a sigmoidal response curve has been described for other whole-cell bioreporters (Schreier et al. 2001), it should be noted that, in some cases, the performance of *luxAB*-dependent whole-cell bioreporters has been alternately described by an exponential rise to maximum (see Fig. 6 in Sticher et al. 1997), a linear relationship (see Fig. 3 in Hay et al. 2000), or by single exponential decay curves (see Fig. 3 in Gillor et al. 2002). In any event, the response observed in the present study highlights an important feature of the Fe bioreporter, that being the limited dynamic range (pFe 21.1 to pFe 20.6) over which it may serve as a quantitative environmental sensor.

The luminescent response of the bioreporter was also characterized in relation to cellular Fe quotas. The Fe:C ratios recorded during the present study (3.1–22.6 $\mu\text{mol}:\text{mol}$) fall within the lower end range of those reported for other cyanobacteria (Kudo and Harrison 1997, Berman-Frank et al. 2001). Kudo and Harrison (1997) reported ratios of 14–143 $\mu\text{mol Fe}:\text{mol C}$ for the marine *Synechococcus* sp. strain DC2; however, this ratio represented total cell-associated Fe (intracellular plus extracellular) and thus the values were likely elevated. Indeed, a recent analysis demonstrated that intracellular Fe quotas are up to 60%–70% lower (Berman-Frank et al. 2001). In that study, intracellular Fe:C ratios of cultured *Trichodesmium* sp. IMS101, a marine diazotrophic cyanobacterium, were between 3.1 and 69.5 $\mu\text{mol Fe}:\text{mol C}$ (Berman-Frank et al. 2001).

The somewhat lower range of Fe:C values reported here were likely due to several factors. The higher range values reported by Kudo and Harrison (1997) reflect cells cultured under light conditions that were roughly half of those used in the present study. Higher Fe requirements are expected under low light conditions given the greater Fe allocation to thylakoid membrane components (Raven 1990). Considering high-light cells only, a lower range of Fe:C ratios (14–38.5 $\mu\text{mol Fe}:\text{mol C}$) was present in Fe-deplete and Fe-replete cells, respectively (Kudo and Harrison 1997). In contrast to N_2 -fixing cyanobacteria, for example, *Trichodesmium* (Berman-Frank et al. 2001), our results show relatively low Fe:C ratios associated with *Synechococcus* PCC 7942, which supports the argument that nitrogen fixation carries a high Fe requirement (Raven 1988, McKay et al. 2001). Presumably the higher Fe quotas reported for *Trichodesmium* were reflective of its diazotrophic capacity.

If the luminescent response is considered as a function of the cellular Fe quota, it is clear that bioreporter luminescence was negatively correlated to intracellular Fe:C. Although the luminescent response was effectively suppressed in cells maintained at pFe 19.5 having a relatively high Fe:C ratio (22.6 $\mu\text{mol}:\text{mol}$), a strong luminescent signal was elicited under low Fe (pFe 21.6) conditions.

Unlike the cellular Fe quota, bioreporter luminescence was not well correlated to variation in the cellular C:N ratio, which indicated N-replete cells. This observation is consistent with previous studies that demonstrate that C:N:P stoichiometry is not a useful indicator of Fe deficiency in phytoplankton (Greene et al. 1991, Berman-Frank et al. 2001).

Characterization of bioreporter response was also made in the presence of known metal chelators provided at different ratios of chelator:total Fe. These results confirm the Fe-specific nature of the bioreporter because each of the characterized Fe-specific ligands was successful at withholding Fe from the bioreporter, albeit with varying degrees of success. Iron was most effectively withheld by DFB, whereas ferrichrome treatment consistently elicited the lowest luminescent

response. To some extent, the bioreporter was expected to access at least some of the complexed Fe from the ligands. *Synechococcus* PCC7942 itself produces hydroxamate siderophores under conditions of Fe deficiency (Kerry et al. 1988), a trait common to most cyanobacteria (Wilhelm and Trick 1994). Although not substantiated for this particular cyanophyte, many prokaryotes that produce siderophores are also capable of exploiting heterologous siderophores through a phenomenon known as siderophore piracy (Hutchins 1995), thereby providing a mechanism to access organically complexed Fe. This was recently demonstrated by Hutchins et al. (1999), who showed that marine cyanobacteria were capable of acquiring Fe from a range of heterologous siderophores, albeit with varying efficiency. In contrast to results presented here, marine prokaryotes, including the cyanobacteria assessed by Hutchins and colleagues, were more successful at acquiring Fe from DFB compared with ferrichrome. These differences speak to the wide variability that might be expected among phytoplankton in accessing Fe from different ligands and points to a need for the development of additional Fe-responsive bioreporters using taxa demonstrating contrasting modes of Fe acquisition.

Field studies in the Great Lakes (S. W. Wilhelm, R. M. L. McKay, C. G. Trick, and M. R. Twiss, unpublished data) have demonstrated that the addition of these chelators alters Fe availability to the natural plankton community. In our field studies, as well as those conducted in marine systems (Wells et al. 1994), the addition of trihydroxamate siderophores (DFB, ferrichrome) typically reduces Fe availability. Addition of the dihydroxamate RA also decreases Fe availability; however, RA additions at low concentrations (1–10 nM) at times have been shown to stimulate community Fe assimilation rates (S. W. Wilhelm and C. G. Trick, unpublished data). Although the details of this “Fe-shuttling” effect by RA have yet to be elucidated, development of this and other Fe-bioreporter systems provide a useful tool for investigating these events.

Perhaps the most striking observation from the chelator studies was the high absolute luminescent response elicited by each chelator assessed. These values ranged from 15 to 55 times higher than those elicited by “low Fe” water samples and point to the existence of another component of the dose-response curve that was not effectively elucidated using Fraquil medium of defined free ferric ion content. Why heterologous chelators elicited such an elevated luminescent response from the bioreporter is unknown. We consistently observe this response in positive controls receiving DFB addition processed alongside our field samples (Durham et al. 2002). Yet, despite this exaggerated response, field samples routinely elicit a luminescent signal close to the dynamic range of the bioreporter.

The ultimate goal of this project was to develop a luminescent bioreporter to be used as a quantitative tool to assess Fe availability in fresh waters. To this end, we have had several opportunities to assess sam-

ples of water from the North American Great Lakes collected using metal-clean approaches and for which conventional total dissolved Fe analysis has been conducted. Despite its status as the second largest of the Great Lakes and the fifth largest lake in the world, Lake Huron receives relatively little study. Reflecting this, our measures of dissolved Fe are among the few measures of trace metals made for this lake. In fact, to our knowledge, only one additional published study contains measurements of dissolved Fe for Lake Huron for which specific attention was afforded to trace metal contamination during sample acquisition and processing (Rossmann and Barres 1988). In that study, the authors present median dissolved element concentrations for each of the Great Lakes with dissolved Fe for Lake Huron surface waters ($n = 19$ stations) averaging 14.3 nM during a 1980 survey.

What is perhaps most striking about the results obtained with the samples from Lake Huron are the relative low luminescent responses compared with that elicited by water sampled from Lake Erie (Durham et al. 2002). In fact, the luminescent response elicited by water from nearly every station assessed in Lake Huron lies at or below the lower threshold of the pFe-specific dynamic range of the bioreporter, thereby precluding its use as a quantitative tool in this instance. Despite the fact that most of the Lake Huron stations possessed comparable levels of total dissolved Fe with those measured at an open lake station in Lake Erie, the bioreporter exhibited markedly higher luminescence when incubated with water collected from the Lake Erie station (Durham et al. 2002). This speaks directly to the issue of bioavailability and demonstrates why consideration of only chemically determined levels of dissolved Fe are inappropriate. Obviously, additional factors may contribute to rendering dissolved Fe relatively less bioavailable to photoautotrophs in Lake Erie compared with Lake Huron. Most likely this issue relates to differences in the types of organic Fe-complexing ligands present in each system. Studies from marine systems demonstrate that upward of 99.99% of dissolved Fe is organically complexed to allochthonously derived organic acids or to autochthonous Fe-complexing ligands such as siderophores and tetrapyrroles (Wu and Luther 1995, Rue and Bruland 1997). The watershed of Lake Huron is predominantly covered by forest, whereas that of Lake Erie is dominated by drainage through agricultural and urban landscapes. Such a difference in land use is likely to be reflected in the types of organic matter contributed to each lake. Based on a survey of five hydrographic stations surveyed in July 2000, dissolved organic carbon in pelagic Lake Erie was 2.0 ± 0.4 mg·L⁻¹. Hydrophobic content (similar to fulvic acid) ranged from 40% to 60% (R. Bourbonniere, Environment Canada, personal communication). Indeed, a recent study comparing a predominantly forest-covered watershed to one dominated by urban development in coastal South Carolina suggested that Fe solubilized by ligands derived from the former was more

readily available to photoautotrophs than Fe derived from the urban landscape (Kawaguchi et al. 1997). In our case, the bioreporter response may reflect such differences in Fe bound to organic ligands. Accordingly, it is important that future use of the Fe bioreporter is coordinated with assessment of not only total dissolved Fe but also Fe speciation to more fully understand the factors that influence Fe bioavailability in aquatic systems.

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