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Luminescent Whole-Cell Cyanobacterial Bioreporter for Measuring Fe Availability in Diverse Marine Environments▽

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A *Synechococcus* sp. strain PCC 7002 Fe bioreporter was constructed containing the *isiAB* promoter fused to the *Vibrio harveyi luxAB* genes. Bioreporter luminescence was characterized with respect to the free ferric ion concentration in trace metal-buffered synthetic medium. The applicability of the Fe bioreporter to assess Fe availability in the natural environment was tested by using samples collected from the Baltic Sea and from the high-nutrient, low-chlorophyll subarctic Pacific Ocean. Parallel assessment of dissolved Fe and bioreporter response confirmed that direct chemical measurements of dissolved Fe should not be considered alone when assessing Fe availability to phytoplankton.

Despite recognition that Fe distribution and availability are important in terms of global aquatic production (10), the biogeochemistry of this trace element remains to be fully characterized. The complex chemical speciation of Fe in aquatic systems and the uncertainties associated with biological assimilation of Fe species (15) make it difficult to ascertain the fractions of chemically detectable Fe that are readily available to phytoplankton. Efforts to address this void have included the development of bioreporter constructs to assay Fe bioavailability. We previously described a freshwater cyanobacterial Fe bioreporter capable of yielding bioluminescence in response to Fe deficiency (11, 14, 25) and have demonstrated its applicability to measure Fe availability in the Laurentian Great Lakes (20, 26). Here we describe the development of a bioreporter suitable for studies of Fe availability in marine systems.

Plasmid pAM1414, a promoterless *luxAB* vector (1), was modified for integration into *Synechococcus* sp. strain PCC 7002 by adding recombination sites targeting *desB*, a gene encoding omega-3 acyl-lipid desaturase. Insertion of promoter:: *luxAB* sequences into *desB* will yield a phenotypically neutral construct at the assay temperature (25°C), yet the strain will be incapable of growth at temperatures of ≤15°C (30). Thus, promoter fusion constructs would be disabled for growth if the strain were inadvertently released into the environment.

Two consecutive 800-nucleotide fragments of *desB* were individually amplified by PCR with primers desB1_For and desB1_Rev, covering the 5′ half of the gene, and desB2_For and desB2_Rev, amplifying the 3′ end (Table 1). Ligation of *desB1* and *desB2* amplicons into unique NheI and SacI sites of pAM1414 yielded plasmid pMBB (2).

The promoter sequence of the Fe-responsive gene *isiAB* (19), including nucleotides from the transcription start site to nucleotide −317, was amplified by PCR with primers isiAB_For and isiAB_Rev, engineered with NotI and BamHI restriction sites, respectively (Table 1). The *isiAB* promoter fragment was ligated into the NotI and BamHI sites of pMBB, and the resulting construct, pMBR, was introduced into *Synechococcus* sp. strain PCC 7002 by genetic transformation (36).

Fe-dependent luminescence of strain BMB04 was assessed by incubating the bioreporter in trace metal-buffered medium A (35) containing 10 μg ml^{-1}spectinomycin and various additions of Fe. Chemical speciation of Fe in medium A was controlled by EDTA (0.1 mM) and was calculated by MINEQL+ ver. 4.5 software (Environmental Research Software, Hallowell, ME). Exponential-phase cells growing in medium A containing 100 nM Fe (pFe = 20.4) were collected by centrifugation at 4,000 × *g* for 8 min, washed twice in Fe-free medium, and resuspended in triplicate polycarbonate containers containing medium A with a defined free ferric ion content (pFe, 19.4 to 21.4). Cultures were incubated for 12 h at 25°C with constant bubbling and continuous illumination of 45 μmol quanta m^{-2} s^{-1} prior to bioluminescence measurement. Cellular bioluminescence was measured with a portable Femtomaster FB14 luminometer (Zylux Corp., Oak Ridge, TN) immediately following delivery of 2.7 mM *n*-decyl aldehyde (Sigma Chemical Co., St. Louis, MO) in 25% (vol/vol) methanol to the bioreporter cells by direct injection into 2 ml of cell culture.

Growth rates assessed by measuring in vivo chlorophyll *a* fluorescence (25) or by direct enumeration of glutaraldehyde-preserved cells (26) did not differ between bioreporter strain BMB04 and wild-type *Synechococcus* sp. strain PCC 7002 when they were cultured in Fe-sufficient medium A (two-tailed *t* test; $P = 0.29$; df = 2). Wild-type cells possessed a growth rate of 0.15 ± 0.03 h^{-1} compared to 0.12 ± 0.02 h^{-1} for the biore-

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TABLE 1. Primers used in construction of the Fe bioreporter

Primer name	Sequence (5′→3′)	Sequence amplified
desB1_For	GGCCCGCTAGCCCCTTCACCCTCAAGGATGTGAAAGCAG	800 bp of *desB1*
desB1_Rev	CCGGGGCTAGCCCAATATTATGGTGAATTTCGTTGAAAA	
desB2_For	GGCCCGAGCTCCACCCATGTCGCCCACCACATTTTCCATA	800 bp of *desB2*
desB2_Rev	CCGGGGAGCTCCTTCGGCAGCGGCAGCATCCTCTGGCTAA	
isiAB_For	GGCCCGCGGCCGCGACTTAGTTAATTTAGCGTAGTTTGCG	327 bp of *isiAB* promoter
isiAB_Rev	CCGGGGGATCCGGATTGGCTTTATCCTACAATTATTCTCA	
luxAB_For	GGCGCGGTTTACAAGCATAAAGCTCTAGAG	400-bp fragment
luxAB_Rev	GGCGGGTGAGTTGTTCAAAATCAGGCTCGA	

porter strain. Accordingly, subsequent experiments were conducted only with the genetically modified strain.

Non-steady-state growth rates determined between 6 and 12 h following inoculation into new medium were highest when the bioreporter was inoculated into Fe-sufficient medium (pFe, 19.4; $0.14 \pm 0.03\ h^{-1}$) but declined by ca. 40% when cells were cultured at a pFe of 20.4 ($0.08 \pm 0.01\ h^{-1}$) and by nearly 70% for cells cultured at a pFe of 21.4 ($0.04 \pm 0.03\ h^{-1}$). These results differ from the steady-state response of wild-type *Synechococcus* sp. strain PCC 7002 in response to Fe, whose growth rates decreased with concomitant decreases in available Fe through pFe 20 yet recovered to a near-maximal value when cultures were grown at a pFe of 21 (41). Growth recovery was attributed to induction of a higher-affinity Fe acquisition system involving the production of siderophores of which *Synechococcus* sp. strain PCC 7002 produces both catechol and hydroxamate types in response to Fe deficiency (40). Using the CASAD assay (32), we failed to observe the production of siderophores over the time course (12 h) of our assays (data not shown).

A dose-response curve was generated relating bioreporter luminescence to the free ferric ion content of medium A between pFe 19.4 and 22.4 (Fig. 1). Through this variable [Fe^{3+}]

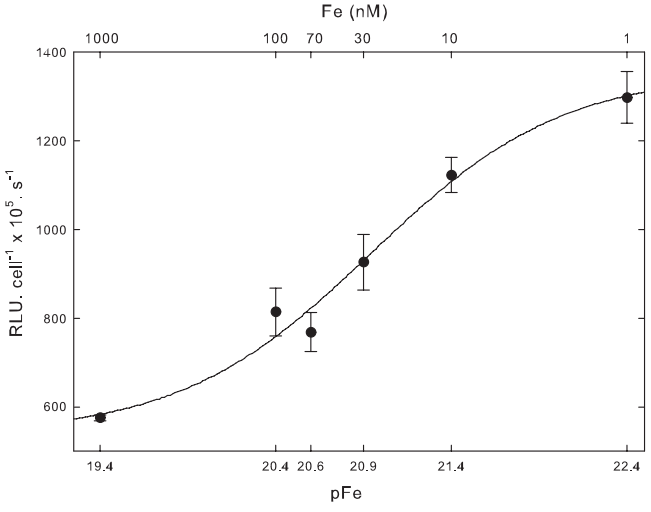

FIG. 1. Standard dose-response curve of bioreporter luminescence normalized to cell number and measured after 12 h of incubation in synthetic medium A with various free ferric ion contents. The total Fe concentration (nanomolar) in the medium is shown at the top. Luminescence is described according to a three-parameter sigmoidal curve (see equation 1) ($R^2 = 0.957$). pFe = $-\log$ [Fe^{+3}].

range, discernible changes were measured in the luminescent response of cells, with luminescence being more than twofold higher associated with cells growing under low-Fe conditions (pFe, 22.4) compared to Fe-sufficient conditions (pFe, 19.4). Luminescence plotted as a function of pFe could be described according to a three-parameter sigmoidal curve (equation 1), a response similar to that documented for a suite of freshwater Fe bioreporters (11, 14, 25).

$$y = \frac{1{,}940.2}{1 + e^{-\left(\frac{x - 21.06}{1.82}\right)}} \quad (1)$$

To characterize strain BMB04 and assess its use to measure iron availability under environmentally relevant conditions, a set of physiological, physical, and chemical parameters were varied. These included time of incubation, growth temperature and irradiance, the biomass of bioreporter used to seed test media, and salinity. Whereas cellular luminescence was indistinguishable between parallel cultures grown in medium A containing 10, 100, and 1,000 nM Fe following 6 h of incubation, differences in luminescence were resolved by 12 h and continued through 24 h of incubation (not shown). Accordingly, we chose a 12-h incubation time for subsequent bioreporter assays. The response time reported here represents a dramatic improvement over other currently available cell culture methods that attempt to assess Fe availability (e.g., see reference 37).

The initial cell density used in assays was varied from 10^4 to $10^6\ ml^{-1}$, a range selected to represent the picocyanobacterial concentrations found in the marine systems tested in this study (4, 17). In media containing 10 to 1,000 nM Fe, the initial biomass had no influence on cellular luminescence following 12 h of incubation (Fig. 2). Unlike the initial cell biomass, temperature and light had dramatic effects on bioreporter growth and luminescence. At temperatures of ≤15°C, the bioreporter was incapable of growth (Fig. 2) as a result of the integration of the promoter fusion vector pMBB into *desB* (30). Consistent with previous studies with the wild-type strain (29), growth was most rapid when cells were grown at 37°C ($0.2 \pm 0.05\ h^{-1}$) whereas growth at 25°C proceeded at <50% of this rate (Fig. 2). By contrast, cellular bioluminescence was highest at 25°C whereas cells grown at 37°C exhibited only ca. 30% of the maximum luminescence (Fig. 2), an observation consistent with the reported thermal instability of bacterial luciferase (7).

Cell culture under 80 μmol quanta $m^{-2}\ s^{-1}$ supported the highest rates of growth, whereas growth rates declined by ca. 40% under each of the other light fluence rates tested (Fig. 2;

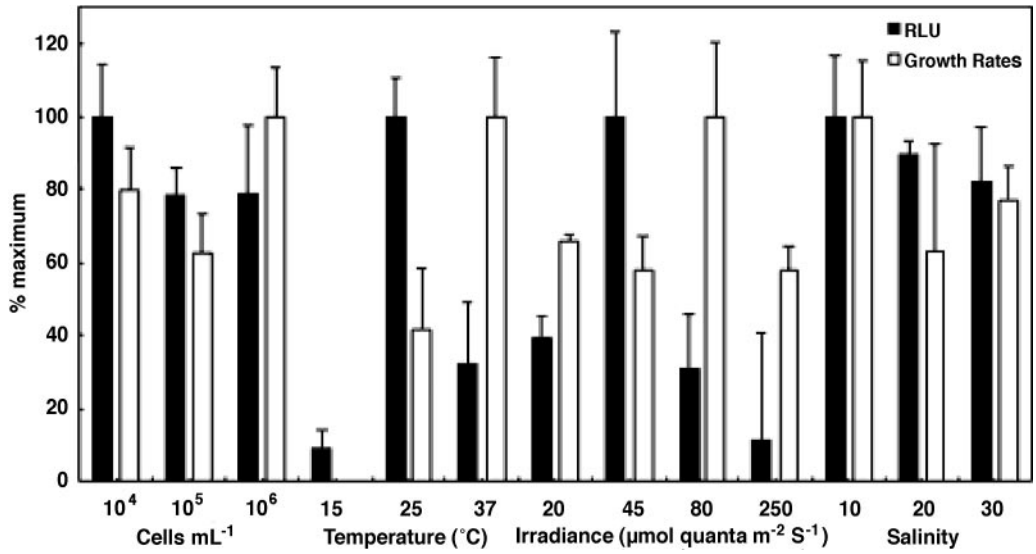

FIG. 2. Influence of biotic and abiotic factors on Fe bioreporter luminescence following 12 h of incubation. Relative luminescence units (RLU) and growth rates are reported as percentages of the maximum luminescence and growth achieved under each parameter assessed, respectively (mean ± standard error, $n = 3$).

$P < 0.05$). Bioluminescence was maximal under 45 μmol quanta m^{-2} s^{-1} following 12 h of incubation (Fig. 2). The diminished luminescence associated with growth under high light (250 μmol photon · m^{-2} · s^{-1}) was consistent with the deleterious effects of light on reduced flavin mononucleotide, the flavin cofactor required of bacterial luciferase (Y. Hihara, Saitama University, Saitama, Japan, personal communication).

Acknowledging the salinity gradient present in samples from the Baltic (Table 2), as well as in other brackish systems, we tested the bioreporter in medium A, whose salinity was varied over a range of 10 to 28 (as measured by the practical salinity scale), the latter being the ambient salinity of medium A. Over this range, there was no change in the growth rate of the bioreporter ($P = 0.087$; df = 2) nor was bioluminescence altered ($P = 0.85$; df = 2) (Fig. 2). The cyanobacterium *Synechocystis* sp. strain PCC 6803 responds to increasing salinity by up-regulating transcription of the Fe-responsive gene *isiAB*, a response to increased oxidative stress associated with a shift in salinity (38). That the bioluminescence of Fe bioreporter BMB04 was unresponsive to changes in salinity further defines the Fe specificity of this bioreporter.

Bioreporter assays of Baltic Sea samples. Fe bioavailability was measured with the bioreporter in samples collected from three stations in the Baltic Sea during July 2005 (Table 2). Station IOW 271, located in the Gotland Basin, was stratified and featured distinct thermal, salinity, and redox gradients (Table 2) typical for the Baltic (21, 24). To assess Fe bioavailability, bioreporter cells growing in trace metal-buffered medium A containing 100 nM Fe were collected at early exponential phase by centrifugation for 8 min at 4,000 × *g* and rinsed twice in Fe-free medium A. Triplicate acid-rinsed polycarbonate bottles containing 50-ml seawater samples were inoculated with bioreporter cells to provide a cell density of 10^5 ml^{-1}. Because surface macronutrient concentrations were low at each of the stations, we tested the bioreporter response in medium A with or without amendment with NO_3^- and PO_4-P_i. Compared to growth in regular medium A, growth of strain BMB04 was only marginally reduced (12%) when 8.3 μmol $liter^{-1}$ NO_3^- and 2.3 μmol $liter^{-1}$ PO_4-P_i (paired two-tailed *t* test, $P < 0.05$) were provided yet was strongly reduced (ca. 88% lower) in cultures not amended with NO_3^- and PO_4-P_i. In these same cultures where macronutrients were not

TABLE 2. Physiochemical characteristics of Baltic Sea hydrographic stations[a]

Station	Date (July 2005)	Depth (m)	Temp (°C)	Salinity	$[O_2]$ (μM)	$[NO_3^-]$ (μM)	$[PO_4$-$P_i]$ (μM)	[DFe] (nM)
IOW 213	6	10	16.4	7.55	Saturated	0.02	0.38	27.5
IOW 271	8	5	17.0	7.13	352.37	0.05	0	12.6
IOW 271	8	20	6.3	7.14	324.23	0.06	0.22	13.7
IOW 271	8	50	2.7	7.46	353.26	0.45	0.76	16.5
IOW 271	8	150	6.0	12.35	0.45	1.33	2.59	15.3
IOW 271	8	225	6.0	12.73	0	0	4.99	601
Bocknis-Eck	28	5	18	14.14	285.1	0.04	0.02	49

[a] Station coordinates: IOW 213, 55°14.95′N, 15°58.14′E; IOW 271, 57°19.20′N, 20°03.00′E; Bocknis-Eck, 54°31.2′N, 10°2.5′E. Salinity corresponds to the practical salinity scale. Sampling was done as described elsewhere (13, 24).

added, cellular bioluminescence was inhibited, likely because of impaired amino acid and nucleic acid synthesis. By contrast, bioluminescence was highest (paired two-tailed *t* test, $P < 0.05$) when the bioreporter was grown in medium A containing reduced NO_3^- (8.3 μmol liter^{-1}) and PO_4-P_i (2.3 μmol liter^{-1}). As a result, assessment of Fe availability was conducted on Baltic Sea samples amended with 8.3 μM NO_3^- and 2.3 μM PO_4-P_i, macronutrient concentrations similar to those measured in the nutricline at station IOW 271 (Table 2). Cultures were incubated for 12 h under the growth conditions described above prior to bioluminescence measurement.

Measurement of dissolved Fe (DFe; <0.4 μM) at stations IOW 213 and IOW 271 was done by graphite furnace atomic absorption spectroscopy with Zeeman correction as described previously (24). For station Bocknis-Eck, labile and total dissolvable Fe levels (<0.2 μM) were measured by voltammetry by established methods (8). Previous studies have demonstrated distinct spatial gradients in DFe between nearshore-offshore environments in the Baltic Sea (18, 22). Consistent with this, surface water DFe was highest at the nearshore station Bocknis-Eck (49 nM) and lowest in surface waters of the Gotland Basin (12.6 nM; Table 2). Whereas surface water from the Gotland Basin yielded the highest bioluminescent response from the Fe bioreporter (Fig. 3), bioreporter luminescence was not always correlated with the DFe content of samples. DFe measured in samples from station Bocknis-Eck was nearly twofold higher than that measured in samples from a 10-m depth in the Bornholm Basin (station IOW 213; Table 2), yet luminescence from the bioreporter was higher associated with the Bocknis-Eck site (pFe equivalent = 22.4 ± 0.3 versus 21.5 ± 0.2 at station IOW 213). This was not expected given the higher DFe but is likely related to Fe speciation. As a nearshore, shallow (ca. 30 m) station subject to transient sediment resuspension, it is likely that most of the DFe at Bocknis-Eck was colloidal and not readily exchangeable. Vertical gradients in DFe are also evident in the Baltic and are accentuated in the presence of anoxic bottom waters (18, 22, 23). Consistent with this, in the present study the bioreporter response at 20-m and lower depths was quenched and suggestive of higher Fe availability relative to that at the surface (Fig. 3). Likewise, below the redoxcline, the bioreporter perceived Fe-sufficient conditions consistent with a 40-fold increase in DFe at a 200-m depth compared to that at the surface (Fig. 3 and Table 2).

Results obtained with the cyanobacterial bioreporter provide support for previous studies demonstrating Fe deficiency in surface waters of the Baltic (12, 28, 34, 39). Characterization of the Fe bioreporter demonstrated that non-steady-state growth rates were suppressed by 40% and 70% when the bioreporter was grown at a pFe of 20.4 and 21.4, respectively, compared to Fe-sufficient conditions. With the exception of the Fe-sufficient subredoxcline sample collected from station IOW 271, all other samples elicited enhanced luminescence consistent with suppressed non-steady-state growth rates. Consistent with a relatively low DFe level (12.6 nM), the highest luminescence was measured in surface water from station IOW 271. By July, the time during which water was sampled at this station, a large surface bloom of diazotrophic filamentous cyanobacteria (*Aphanizomenon* sp. and *Nodularia spumigena*; M. Nausch, Institut für Ostseeforschung Warnemünde [IOW], Warnemünde, Germany, personal communication) had formed and it is tempting to speculate that the Fe-deficient condition was attributed to biological Fe drawdown. It is also possible that Fe availability was constrained because of complexation with refractory organic compounds such as humic substances, as demonstrated previously for cyanobacteria (16). Spatial and seasonal gradients of surface dissolved organic carbon (DOC) exist in the Baltic (31), as does a vertical gradient in both total organic carbon (33) and DOC (K. Nagel, IOW, personal communication) at the Gotland Basin site. During July, surface DOC concentrations are highest compared to other seasons of the year, with mean DOC concentrations acquired between 1995 and 2005 ranging between ca. 300 and 400 μM C (K. Nagel, personal communication). In the deep waters of the Gotland Basin, lower DOC concentrations were measured (mean, 267 ± 20 μM C; 1995 to 2005), mainly owing to the origin of this water from the North Sea. Thus, it appears that in the surface and deep waters of the Gotland Basin there are two separate systems for DOC and this likely influences Fe bioavailability. Given this consideration, it is clear that future data derived from the bioreporter should include parallel measurements of Fe speciation.

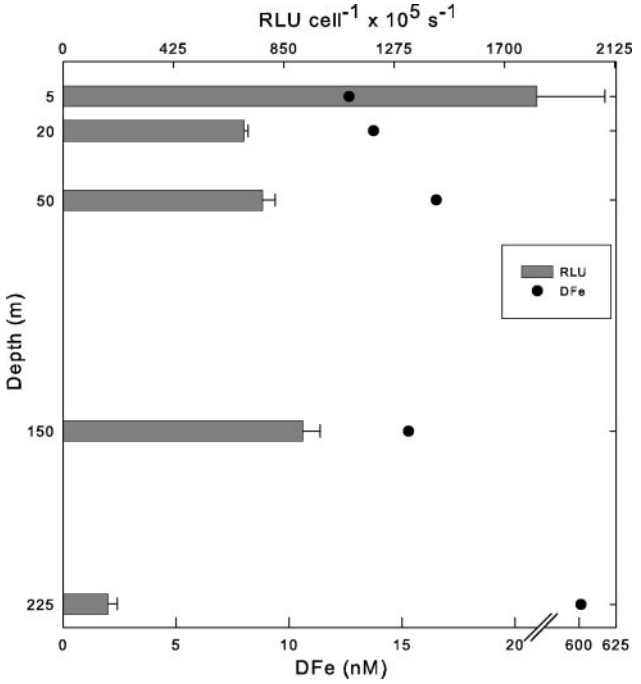

FIG. 3. Depth profile of [DFe] and bioavailable Fe as measured with the cyanobacterial bioreporter at station IOW 271 (Gotland Deep) in the Baltic Sea. Note the axis break corresponding to [DFe]. The subredoxcline sample (225 m) was degassed immediately upon thawing by bubbling for 10 min with Ar prior to inoculation with bioreporter cells. This treatment had no apparent deleterious effect on the bioreporter cells, as there was no difference in the luminescent response elicited from controls (medium A containing 7.5 and 250 nM Fe) compared to those that had not been bubbled with Ar gas (data not shown).

Bioreporter assays of subarctic Pacific samples. Fe bioavailability as determined with the bioreporter was also measured on samples collected during the SERIES Fe fertilization experiment in the subarctic Pacific 50 km northeast of Ocean

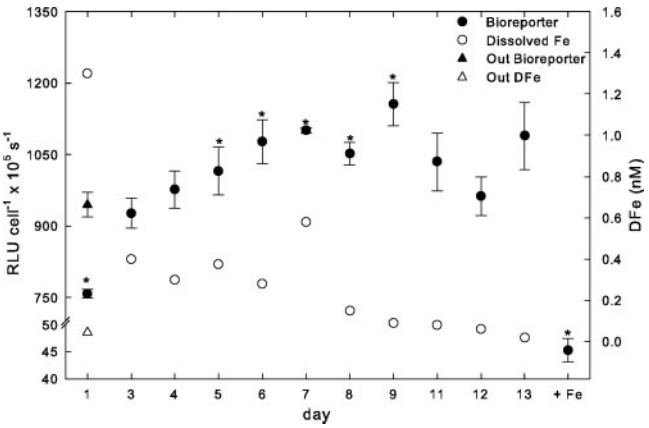

FIG. 4. Bioreporter responses in relation to measured concentrations of DFe during SERIES. Black circles represent the bioluminescence generated by the bioreporter, whereas open circles represent [DFe]. A sample collected from outside the patch on 11 July 2002 (day 1) is plotted with triangles (▲, RLU; △, [DFe]). Asterisks demonstrate bioreporter responses significantly ($P < 0.005$) different from that elicited by the sample from outside the patch ($n = 5$). A negative control to which 1,000 nM Fe was added is designated +Fe. Note the axis break corresponding to the luminescent response of the bioreporter.

Station Papa. Details of SERIES have been published elsewhere (3, 4). On 10 July 2002, designated day 0 of the study, the site was fertilized with an acidic solution of ferrous sulfate concomitant with addition of the tracer sulfur hexafluoride to provide mixed-layer concentrations of ca. 1 nM and ca. 400 fM, respectively, over a patch of 4.75 by 4.75 nautical miles. A second Fe infusion was made on day 6 to provide an additional 0.6 nM DFe in an expanding rectangle of 7.3 by 3.7 nautical miles. The patch was monitored for 25 days from 10 July until 4 August. The parameters measured included temperature, salinity, in vivo chlorophyll fluorescence (including variable fluorescence/maximum fluorescence), and nutrient concentrations.

Filtered (<0.2 μm) samples for DFe and bioreporter analysis were collected in acid-rinsed Teflon bottles from day 1 until day 11 of the study. "Out-patch" samples from a location ca. 16 km away from the fertilized patch were collected for experimental controls. DFe measured during SERIES showed a transient increase to >1 nM on day 1 (11 July) following Fe fertilization, with levels decreasing thereafter through day 6 (17 July), when additional Fe was infused into the patch (Fig. 4). Within 1 to 2 days of Fe addition, ecosystem level community responses, manifested as increases in both chlorophyll *a* and photochemical efficiency (variable fluorescence/maximum fluorescence) were observed concomitant with an initial increase in the abundance of endemic marine *Synechococcus* spp. (4). Consistent with this, the Fe bioreporter perceived increased Fe availability (20% lower luminescence) associated with a sample collected 1 day after Fe fertilization (11 July) compared to a control sample collected from outside of the patch (two-tailed *t* test; $P < 0.0005$; Fig. 4). When the "out patch" sample was spiked with 1,000 nM Fe as a negative control, bioreporter luminescence was quenched almost completely, confirming that the luminescent response was Fe dependent (Fig. 4).

The low luminescent response of the Fe bioreporter evident 1 day following the initial Fe infusion was followed by a >30% increase (two-tailed *t* test; $P < 0.0005$) in bioluminescence through day 6, indicating lower Fe availability as the study progressed. Associated with this response was a taxonomic shift in phytoplankton in the patch characterized by a fourfold increase in autotrophic nanoflagellates (4). *Synechococcus* sp. abundances returned to ambient levels by day 4, as did nanoflagellate abundances by day 14. Diatom stocks showed few changes between days 1 and 5. Thereafter, from day 6, exponential increases in the abundance of diatoms induced a bloom condition (3, 4).

The lack of response by the bioreporter to the second Fe infusion was unexpected, although by this time the patch may have already contained high levels of Fe-binding ligands as a result of grazing and viral lysis (15). We submit that the Fe bound to these dissolved organic ligands was not readily accessible to the bioreporter. In previous mesoscale Fe fertilization experiments, it has been demonstrated that Fe-binding ligands of various sizes and affinities for Fe accumulate in the mixed layer within days of Fe infusion (5, 9, 27). Despite the elevated concentrations of these ligands, diatom blooms have occurred during each fertilization study. This is consistent with the specific mechanism of Fe acquisition by diatoms, which rely on cell surface ferrireductase activity compared to the use of siderophores by cyanobacteria (15).

The *Synechococcus* Fe bioreporter is presented as a tool to assess the bioavailability of Fe in seawater since it provides a measurement of Fe availability from the perspective of a living organism. The bioreporter assay can be viewed as a method complementary to chemical approaches, which provide a first-order indication of nutrient status but are less flexible in discriminating between biologically available and more refractory forms of Fe. Traditional approaches to bioavailability assessment such as nutrient amendment studies can introduce artifacts that may confound the interpretation of results (6). Implementing a living system such as a bioreporter organism will assist in gaining a better understanding of the availability of Fe from the perspective of a living cell. Further, whereas we recognize that a bioreporter constructed in *Synechococcus* sp. strain PCC 7002 does not adequately represent the diversity of picocyanobacteria of marine systems, the BMB04 strain can be viewed as a prototype in which detailed characterization can be carried out prior to expansion of the technology to other ecologically relevant phototrophs.

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