



Characterization and field trials of a bioluminescent bacterial reporter of iron bioavailability

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Abstract

To better understand Fe cycling in marine and freshwater systems, we have developed a biomolecular tool to track the perceived bioavailability of Fe to heterotrophic bacteria. Bioluminescent reporters, constructed by fusing the *fepA*–*fes* promoter of *Escherichia coli* (an Enterobactin biosynthesis gene regulated by the ferric uptake regulatory [Fur] system) to a luxCDABE cassette, were integrated into the chromosome of a halotolerant *Pseudomonas putida*, which uses the Fur system to regulate high-affinity Fe uptake. The resultant *P. putida* bioreporter has been successfully tested both in lab and field studies. Laboratory cultures were maintained at a range of concentrations of total Fe (0–25 nM) or limited by the addition of concentrations of well-characterized siderophores (desferrioxamine B [DFB], ferrichrome, 2,2'-dipyridyl [DP] and Rhodotorulic acid [RA], 0–200 nM) and used to establish the dynamic range of this reporter system. Analysis of sample incubations after only 4 h suggest that both of the trihydroxamate-type siderophores DFB and ferrichrome efficiently reduced Fe availability, resulting respectively in a 1.77- and 1.88-fold increase in luminescence relative to Fe-replete conditions. In contrast, additions of the dihydroxamate-type siderophore RA and the synthetic chelator DP resulted in no response from the system, suggesting that cells could access Fe complexed to these compounds without activating high-affinity Fe transport systems.

Field studies were performed in the central basin of Lake Erie, which has previously been shown to undergo sporadic Fe limitation during summer stratification. DFB concentrations were titrated across a range of 0–50 nM into unfiltered water to manipulate Fe availabilities. Bioreporters expressed Fe stress (ca. a 2-fold increase in luminescence) at concentrations of DFB equivalent to the total (dissolved + particulate) Fe in the system (\approx 30 nM), indicative of the concentration of bioavailable Fe. In a similar experiment with 0.2- μ m pre-filtered water (2.25–5.24 nM Fe), a 6-fold increase in luminescence (relative to controls) was observed at the lowest (15 nM) concentration of chelators. The results of this study demonstrate the validity of bioreporters as a complimentary tool to measurements of total Fe. Moreover, these results suggest that a significant source of

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bioavailable Fe flux may be particulate in nature, and support the hypothesis that Fe recycling from microorganisms may play a key role in the regeneration of bioavailable forms of Fe.

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1. Introduction

Recent research has clearly demonstrated that Fe availability controls productivity and community structure in the High Nutrient, Low Chlorophyll (HNLC) marine areas (Martin and Gordon, 1988; Martin et al., 1994; Coale et al., 1996a,b; Boyd et al., 2000), some coastal marine systems (Hutchins and Bruland, 1998) and some large lakes (Wurtsbaugh and Horne, 1983; Twiss et al., 2000). Although Fe is the fourth most abundant element in the earth's crust, bioavailable Fe remains scarce in pelagic surface waters at near-neutral pH as a result of low input, biotic as well as abiotic adsorption, and rapid oxidization of the inorganic Fe(II) to the insoluble Fe(III) complexes (Millero and Sotolongo, 1989; Kuma et al., 1992).

As the term HNLC indicates, most investigations have focused on the impact of Fe depletion on the phytoplankton component (e.g., Hudson and Morel, 1990; Sunda et al., 1991; Sunda and Huntsman, 1995). However, recent improvements in the understanding of Fe chemistry have demonstrated that heterotrophic bacterioplankton in low Fe aquatic systems may also be directly or indirectly impacted. The application of electrochemical techniques has allowed for a reliable estimation of Fe-organic completion and redox speciation (Van den Berg et al., 1991; Wu and Luther, 1994, 1995; Rue and Bruland, 1995, 1997; Witter et al., 2000; Powell and Donat, 2001). The results of these novel techniques have led to the conclusion that more than 99% of the dissolved Fe in surface waters is bound to unknown organic ligands falling in two major classes (the stronger L1 and the weaker L2 ligand classes), which present stability constants similar to bacterial siderophores (Lewis et al., 1995; Rue and Bruland, 1995, 1997; Witter et al., 2000). This finding and further observations during the mesoscale fertilization experiments have supported the hypothesis that these ligands could have a biogenic origin: as microbial Fe chelates (sidero-

phores) produced by both photo- and heterotrophic prokaryotes to alleviate Fe stress (Wilhelm and Trick, 1994; Butler, 1998) or as the byproduct of viral- (Wilhelm and Suttle, 1999) and grazing-induced mortality (Hutchins and Bruland, 1994).

Traditional tools for the characterization of Fe bioavailability remain insufficient to determine whether the heterotrophic bacterial population is primarily limited by Fe availability or by dissolved organic carbon. While some experiments have shown that the bacterial community was responsive to Fe enrichment and that Fe requirements were high (Pakulski et al., 1996; Tortell et al., 1996), the response appears to be dependent on the chemical species of Fe that are provided (Granger and Price, 1999; Guan et al., 2001). Thus, the response to Fe enrichment cannot be separated from the effect of enhanced DOM production due to Fe stimulation of the primary producers (Kirchman et al., 2000). Since the heterotrophic bacterial component is suspected to contribute to the production of the Fe-complexing organic ligands in surface waters, a better understanding of the bioavailability of different organic Fe pools remains crucial for a resolution of the biogeochemical machinations of the "microbial ferrous wheel" (Kirchman, 1996).

To assess the response of heterotrophic bacteria to Fe bioavailability in aquatic systems, we have developed a mobile genetic system that can be used as a luminescent reporter for the characterization of Fe bioavailability to aquatic prokaryotes. The well characterized, ferric uptake regulator (Fur)-regulated bi-directional *fepA-fes* (ferric enterobactin uptake system-ferric Enterobactin esterase) promoter from *Escherichia coli* (Escobar et al., 1998) has been fused to the *Vibrio fischeri* luxCDABE reporter gene cassette on a transposable genetic element and harbored in *E. coli* SV-17. The resulting strain (denoted as *E. coli* pFeLux) can subsequently be mated with other bacteria to introduce the reporter stably into the

chromosome of the bacterium of interest. The structure and the regulation of the *fepA–fes* promoter system are well known. Through a single Fur-binding site (Escobar et al., 1998), it acts as a regulatory region for Enterobactin production and transport (Braun et al., 1998; Van der Helm, 1998). In the case of our reporter, de-repression of high-affinity Fe transport (displacement of the Fe–Fur protein complex from the promoter) results in light production (luminescence). As such, light production in these bacteria is directly linked to the Fe status of the cell. The present study reports on the characterization of the reporter after stable introduction into the chromosome of a halotolerant *Pseudomonas putida*. Laboratory work has been combined with a set of field experiments in an aquatic system (Lake Erie) where Fe availability is known to transiently limit primary production (Twiss et al., 2000). The results reported here demonstrate a differential availability of Fe in the presence of different chelators to the *P. putida* reporter strain. Moreover, the field studies suggest that much of the bioavailable Fe was located in the particulate (>0.2 µm) size class, indicative of the significant role that the recycling of Fe plays in aquatic environments.

2. Methods and materials

2.1. Construction of *Fe* bioreporters

All bacterial strains and plasmids used in this study are listed in Table 1.

A 321-bp fragment containing the divergent promoters (*fepA–fes*) involved with high-affinity Fe uptake regulation in *E. coli* (Escobar et al., 1998) was amplified from *E. coli* K12 using the polymerase chain reaction and subsequently cloned into pCR2.1 according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). The 321-bp fragment was then inserted upstream of a promoterless *luxCDABE* gene cassette in a miniTn5:*luxTc^R* transposon to create a *fepA–fes::lux* fusion as previously described (Apple-gate et al., 1998; Hay et al., 2000). To facilitate the construction of the *lux* fusion, the *fepA–fes* plasmid DNA was linearized with *SpeI*, dephosphorylated, and subsequently ligated into the unique *XbaI* site of the transposon 5' to the *luxCDABE* gene cassette (Fig. 1). This enabled transformation into DH5α Library Efficiency competent *E. coli* (Life Technologies, Gaithersburg, MD) with selection on LB agar plates containing tetracycline (14 mg/l) and kanamycin (50 mg/l). Re-

Table 1
Bacteria and constructs used in this study

	Relevant genotype/characteristics	Reference
<i>Plasmids</i>		
pCR2.1	3.9-kb cloning vector for PCR products with 3' A overhangs; Ap ^R Km ^R	Invitrogen
pSWW1	pCR2.1 containing the 321-bp <i>fepA–fes</i> operator and divergent promoters	This study
pUTK83	Tn5 <i>luxCDABETc^R</i> Mini Tn5 <i>lux</i> transposon in pUT unique <i>NotI XbaI</i> cloning site for chromosomal insertion of <i>lux</i> reporters, <i>E. coli SV-17</i> , amp, Tc ^R on Tn	This study
pSWW2	pSWW1 linearized with <i>XbaI</i> and inserted into the <i>XbaI</i> site of pUTK83	This study
pSWW3	Tn5 <i>luxCDABETc^R</i> Mini Tn5 <i>lux</i> transposon containing <i>fepA–fes</i> 321-bp DNA fragment fused to <i>luxCDABE</i> in pUTK83	This study
<i>Bacterial strains</i>		
<i>E. coli</i> DH5αF'	Δ(<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> (rK ⁻ , mK ⁺) <i>phoA supE44 thi-1 gyrA96 relA1</i> (Tn5 Km ^R F' episome)	Invitrogen
<i>E. coli</i> INVF'	F'ϕ 80 <i>lacZM15Δ(lacZYA-argF)</i> U169 <i>deoR recA1 endA1 hsdR17</i> (rK, mK ⁺) <i>phoA supE44 thi-1 gyrA96 relA1</i>	Invitrogen
<i>E. coli</i> SV17	λ <i>pir recA thi pro hsdR M⁺</i> RP4:2-Tc:Mu:Km Tn7Tp ^R Sm ^R ; mobilizing strain for pUT/mini-Tn5 derivatives	de Lorenzo et al. (1993)
<i>E. coli</i> Lux	<i>E. coli</i> containing a chromosomally inserted Tn5:: <i>luxCDABETc^R</i> Mini Tn5, Km ^R	Hay et al. (2000)
<i>P. putida</i> FeLux	<i>P. putida</i> containing a chromosomally inserted Tn5 <i>fepA–fes::luxCDABETc^R</i> Mini Tn5, Tc ^R	This study
<i>E. coli</i> FeLux	<i>E. coli</i> DH5αF' containing a chromosomally inserted Tn5 <i>fepA–fes::luxCDABETc^R</i> Mini Tn5, Km ^R , Tc ^R	This study
<i>E. coli</i> pFeLux	<i>E. coli SV-17</i> containing pSWW3	This study

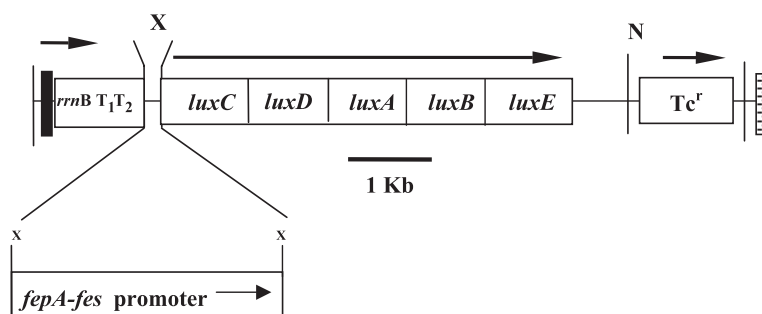


Fig. 1. Physical and genetic map of the bioreporter system. Details of the construction of the mini-Tn5-*luxCDABE* reporter/vectors have been published elsewhere (Applegate et al., 1998; Hay et al., 2000). X, *Xba*I site; N, *Not*I site; *rrmB* T₁T₂, transcriptional terminators from *E. coli rrmB*; Tc^r, tetracycline resistance gene.

sistant colonies were then isolated and genetically verified using restriction endonucleases. Once verified, the DNA was then digested with *Not*I, ligated and electroporated into *E. coli* SV-17 and plated on tetracycline. This step removed the pCR2.1 cloning vector and left the *fepA*–*fes* genetic element fused to the *lux* genes. The transformants were then patch-plated onto kanamycin to ensure sensitivity and removal of the cloning vector. Isolates were further screened by restriction mapping to orient the 321-bp insert relevant to the *lux* genes. Directionality was also verified using PCR with a *luxC* 3' primer and each of the primers used to amplify the *fepA*–*fes* promoter region. A clone containing a *fes*–*lux* fusion was chosen for further study. Once chosen, the clones were assayed for light production in the presence of Fe chelating agents using a Zylux FB-15 luminometer (Maryville, TN). Due to the high background level of bioluminescence from the reporter strain based on the multi-copy plasmid format, a single-copy chromosomal insertion was constructed.

Chromosomal-based Fe bioreporters were constructed by mating the *E. coli* SV-17 harboring the transposon with a halotolerant *P. putida* and *E. coli* DH5 with a chromosomal kanamycin marker as previously described (de Lorenzo et al., 1993). Matings were then plated on pseudomonas isolation agar amended with 14 µg/ml tetracycline or LB containing Km (50 mg/l) and Tc (14 mg/l). Colonies were patch-plated, evaluated for light production, and clones were chosen for further study. Further verification of the bioreporter was determined by a genetic fingerprint generated via PCR using chromo-

somal DNA and a conserved BOX primer (Rademaker and de Bruijn, 1997). This pattern was visualized on 1% agarose gel and compared to the banding pattern of the donor *E. coli* SV-17, *E. coli* DH5Km^R and wild-type *P. putida*.

2.2. Laboratory characterization of the Fe-dependent bioreporters

2.2.1. Study organisms

Three bioluminescent reporters were examined. The Fe-dependant bioreporters *P. putida* FeLux (chromosomally integrated construct) and *E. coli* pFeLux (plasmid construct) and *E. coli* FeLux (chromosomally integrated construct) were used to evaluate the effect of heterologous siderophores on Fe availability by monitoring endogenous bioluminescence. The recombinant *E. coli* Lux (described as *E. coli* #82; Hay et al., 2000), containing the *luxCDABE* cassette integrated into the chromosome at a neutral site and lacking the *fepA*–*fes* promoter, was used as control to demonstrate that the response of the *luxCDABE* system itself was independent of bioavailable Fe. No control strain is available for *P. putida* at this time.

2.2.2. Culture conditions

The first set of laboratory experiments were performed by growing the bioreporters overnight (~ 8 to 12 h) with shaking (1000 rpm) at 37 °C in 250-ml, acid-washed polycarbonate Erlenmeyer flasks filled with an Fe-replete medium: a modified recipe of the Luria Bertani medium. For 1 l of medium, 10 g of

tryptone, 5 g of yeast extract and 10 g of NaCl were dissolved in Milli-Q water, and the pH was adjusted to 7.5 with NaOH. After microwave sterilization (Keller et al., 1988), tetracycline was added to 50 µg/ml. No attempts were made to deferrate the medium since variations in the concentration of Fe contaminant found in 8-hydroxyquinoline can result in variations in residual Fe concentrations and thus, variations between assays (Schwyn and Neilands, 1987; Hersman et al., 2000). When an optical density at 600 nm (OD₆₀₀) of 0.1 (1 cm pathlength) was reached, 40-ml aliquots of the initial culture were transferred in 50-ml sterile polycarbonate tubes. The 40 ml of culture samples were either amended, to final concentrations ranging of from 0 nM (unamended control) to 200 nM of the appropriate chelator, or up to 25 nM of inorganic Fe (FeCl₃). Sub-samples (10 ml) were collected in triplicate in 15-ml sterile polycarbonate tubes and incubated at 25 °C in a temperature-controlled shaker during the experimental period (6 h). Fe availability, estimated by changes in the luminescence of the bioreporters, was evaluated at 0, 2, 4 and 6 h by measuring the light produced by 1 ml of culture. Growth was followed in the cultures during the incubation period by measuring the optical densities (OD₆₀₀) of each sample (at 0, 2, 4 and 6 h). Light production (relative light units, Rlu) was normalized per OD₆₀₀ for each triplicate at each time-point. Reported values are the means of three replicate cultures for the time-point for which the signal was the strongest ($t=4$ h for *P. putida* or $t=6$ h for *E. coli*).

2.2.3. Fe chelators tested

Four well characterized hydroxamate-type Fe(III) chelates were tested, as described above: two trihydroxamates, the actinomycete siderophore desferrioxamine B (desferrioxamine mesylate, Sigma) and the basidiomycete siderophore ferrichrome (Sigma); one linear dihydroxamate-containing siderophore produced by *Rhodotorula pilimanae*, Rhodotorulic acid (Sigma) [RA]; and one non-metabolizable synthetic chelator 2,2'-dipyridyl (Acros Organic) (Table 2). Stock solutions of each chelator (50 µM) were prepared in acid-washed polycarbonate flasks. Chelators were dissolved in 1 ml of 50% ethanol and adjusted to final volume with Chelex-100-treated Milli-Q water (Price et al., 1988/89).

Table 2

Relative stability constants of Fe chelates used in this study

	Ligand	log $K_{Fe^{3+}+L}$	Reference
Trihydroxamates	DFB	21.6	Witter et al. (2000)
	Ferrichrome	21.6	Witter et al. (2000)
Dihydroxamate	Rhodotorulic acid	19.39	Nguyen van Duong et al. (2001)
Natural ligands	L1 class	18.6–22.9	Witter et al. (2000)

The range provided for natural ligands represents the extremes from multiple literature values.

2.3. Field testing

2.3.1. Stations and sample collection

Water was collected from the central basin of Lake Erie (Environment Canada Station 84, 81.65°W, 41.93°N) during a research cruise on the CCGS *Linmos* in July 2001. Data from the ship's CTD were used to collect temperature profiles to model the water column structure. Surface water (5–10 m) was collected with a metal-clean surface pumping system comprising a Teflon double diaphragm pneumatic pump (Husky 307™, McMaster-Carr™) and PFA Teflon™ tubing deployed off of the port side of the ship. Water was pumped directly into an on-deck Class-100 clean room facility. The system was allowed to flush for 30–60 min prior to each water collection. Water collected for manipulation was pre-filtered in-line through acid-cleaned 210-µm screening (Nytex™) to remove large zooplankton, etc. Measurements of nutrient concentration were made at the National Laboratory for Environmental Testing (Environment Canada) using standardized techniques (NLET, 1994). Sample pre-processing (filtration) was completed on the ship. Samples were stored frozen prior to analysis. Total chlorophyll-*a* content of seston sampled from depth and collected onto 0.2-µm pore-size filters was quantified (Turner designs TD-700 fluorometer) using the non-acidification protocol (Welschmeyer, 1994).

To test the *P. putida* FeLux reporter in the field, 1-ml aliquots from overnight cultures of the bioreporter were inoculated into 40 ml water samples. Two sets of field trials were carried out (July 13, 2001 and July 17, 2001). In the first trial, triplicate acid-cleaned polycarbonate tubes were filled with lake water and DFB was added to give final concentrations of 0, 10, 20, 30, 40, and 50 nM. Samples were incubated at ambient temperatures in reduced light conditions.

Sub-samples (1.5 ml) were taken every 2 h throughout a 24-h period to measure light production. In the second experiment, the same process was repeated with the addition of a filtered (0.2 μm) treatment. Due to a limitation in the available number of clean sample tubes, concentrations of DFB were added at 0, 15, 30 and 45 nM in the filtered water treatment.

2.3.2. Measurements of dissolved (<0.2 μm) and total Fe

Samples for dissolved Fe analysis were collected as described above. Lake water was filtered through acid-cleaned 0.2- μm pore-size polycarbonate filters (Millipore) in a Teflon filtration ring (Savillex, Minnetonka, MN), and frozen in Teflon bottles until analysis. Water was acidified with 0.1 ml of concentrated ultraclean nitric acid (Baseline/Seastar, Sidney, BC) per 1 l of lake water and Fe content was determined by direct injection using Zeeman-corrected transversely heated graphite furnace atomic absorption spectrophotometry (Perkin Elmer AAnalyst-800) using pyrolytically-coated L'Vov platform graphite tubes and magnesium nitrate as a matrix modifier. A certified freshwater reference material (SLRS-4; National Research Council of Canada) was diluted to the expected range of Fe concentrations in the sample and analyzed with the samples. Particulate Fe (>0.2 μm) content in the lake water was determined by collecting seston from 0.3 l of lake water onto 0.2- μm pore-size polycarbonate filters (Millipore). Filters were digested in 1 ml of concentrated nitric acid in 8-ml Teflon jars (Savillex). Fe content in filter digests was determined as above.

2.3.3. Effect of chelators on Fe assimilation in freshwaters

To determine the effect of chelators on Fe uptake by the planktonic community, ethylenediaminetetraacetic acid (EDTA) and DFB were titrated into water samples and ^{55}Fe assimilation rates determined. Water was collected during a July 2000 cruise using an acid-clean Teflon-coated Go-Flo bottle suspended on a Kevlar line and triggered by a Teflon messenger. For uptake experiments, 0.1 μCi of [^{55}Fe]- FeCl_3 was added to triplicate polycarbonate bottles (500 ml) for each concentration of the two chelates (50, 100 and 200 nM) as well as to a set of control bottles. Samples were incubated in an on deck incubator with solar influence reduced to ca. 37% by neutral density screening.

After a 48-h incubation, 100 ml were removed from each bottle and filtered onto a 0.2- μm pore-size polycarbonate filter (diameter, 47 mm). Filters were rinsed with a Ti/citrate/EDTA rinse (Hudson and Morel, 1989) modified for use in freshwater by omitting the NaCl (C.G. Trick, personal communication). Radioactivity collected on filters was measured by liquid scintillation with background radioactivity measured and subtracted from all sample counts.

3. Results

3.1. Laboratory characterization

3.1.1. Fe-independent expression of the lux gene

To confirm that the lux gene itself was independently expressed from Fe bioavailability, bioluminescence was measured after addition of the siderophore DFB and Fe in the *E. coli* Lux recombinant, containing the lux gene cassette but not the *fepA-fes* promoter (Fig. 2A). No significant variation (*t*-test, $p > 0.05$ [150 nM DFB–10 nM Fe], $p > 0.05$ [150 nM DFB–25 nM Fe]) of the light production normalized per OD₆₀₀ was observed over the experimental range of relative Fe concentrations. The ratio of luminescence between the Fe-limited medium (150 nM DFB) and the Fe-replete medium (25 nM Fe) was 0.933. A decline of about 20% (from 3.84 ± 0.45 to $3.14 \pm 0.55 \text{ d}^{-1}$) in growth rate was observed for *E. coli* Lux when shifted to Fe-deficient media (150 nM DFB). However, no significant decrease of the growth rate was observed between the unamended control medium and the deficient medium (*t*-test, $p > 0.05$). The constitutive and stable expression of luminescence under the range of relative Fe concentrations suggests that the lux genes themselves are not regulated by the level of bioavailable Fe in the culture medium.

3.1.2. Effects of Fe chelates on *fepA-fes* promoter activity in *E. coli*

Regulation of the promoter *fepA-fes* activity (demonstrated by lux gene expression) by bioavailable Fe was confirmed by repeating a similar experiment with the *E. coli* FeLux recombinant (Fig. 2B). By contrast to the strain *E. coli* Lux, a significant variation of the activity of the bioreporter by the strain *E. coli* FeLux was observed (*t*-test [5 nM Fe–

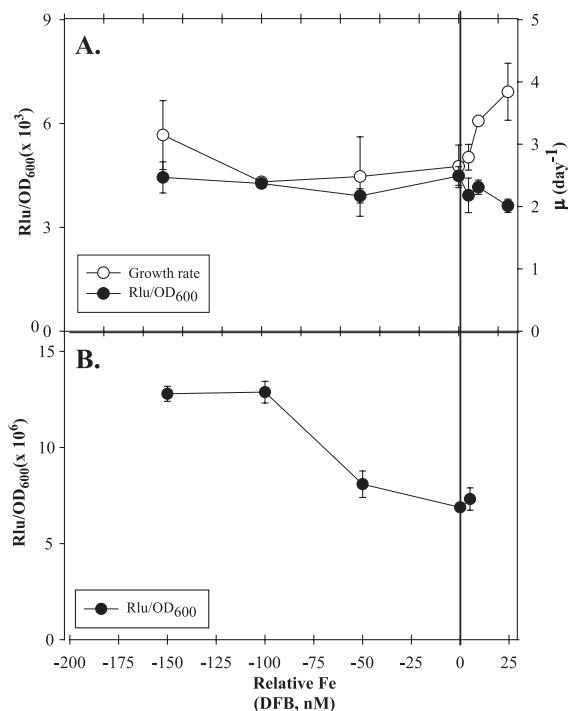


Fig. 2. (A) Fe-independent expression of the *luxCDABE*. Cultures of *E. coli* Lux, containing the *lux* cassette, but not the *fepA–fes* promoter, were grown on LB medium supplemented with increasing amounts of DFB (50–150 nM, represented as “-relative Fe”) or FeCl₃ (5–25 nM). The control medium (0 nM) was neither amended with DFB nor with Fe. The OD₆₀₀ and bioluminescence (measured in RLU) were determined every 2 h over a 6-h experimental period. Growth rates (day⁻¹; ○) were calculated from linear regressions based on the OD₆₀₀ data. The bioluminescence value correspond to the mean of light production normalized per OD₆₀₀ at *t*=6 h (●) (corresponding to the optimal activity). Error bars represent standard deviation of the means (*n*=3). (B) Induction of bioluminescence in the *E. coli* FeLux bioreporter upon DFB addition. The bacterial culture was grown as indicated previously described for the bioreporter strain *E. coli* Lux. Bioluminescence values are means for the light production normalized per OD₆₀₀. Error bars represent standard deviation of the means (*n*=3).

150 nM DFB], *p*=0.00087)). The bioluminescence expressed by *E. coli* FeLux was inversely related to Fe availability. Light production relative to the control increased dramatically with the concentration of DFB added (up to 1.87-fold between the control and 100 nM DFB), indicating that DFB chelated Fe in a form that was not available to *E. coli*. These data demonstrate that Fe availability regulates bioreporter expression.

3.1.3. Effects of Fe chelates on *fepA–fes* promoter activity in *P. putida*

A similar set of experiments was repeated with the *P. putida* Fe sensor that we constructed for use in natural aquatic systems: *P. putida* FeLux. As with the *E. coli* FeLux, the lux expression of *P. putida* FeLux responded to shift in Fe availability in the culture medium (Fig. 3). A significant variation in the activity of the bioreporter by the strain *P. putida* FeLux was observed (*t*-test [15 nM Fe–100 nM DFB], *p*=0.014). Light production relative to the control increased markedly with the concentration of DFB added (up to 1.77-fold between the control and 100 nM DFB). Conversely, it decreased when Fe was added to the medium (~ -20% between control and 25 nM Fe). The increase of bioluminescence as a function of Fe availability follows a dose-dependent pattern as shown by the linear relationship with the relative Fe concentration between 100 nM DFB and 10 nM Fe ($y = -9270x + 9.53 \times 10^5$, $r^2 = 0.93$). The slight decline in luminescence observed between 100 nM DFB and 150 nM DFB may be a consequence of a physiological decline of the bacteria under Fe starvation leading to a slowdown of the transcriptional activity of the promoter. Such a decline was not observed with the strain *E. coli* FeLux at a similar concentration of DFB. The growth rate for the bio-

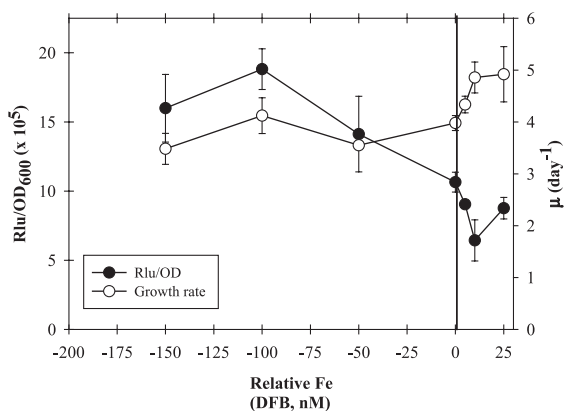


Fig. 3. Induction of bioluminescence in the *P. putida* FeLux bioreporter upon DFB addition. The bacterial culture was grown as indicated previously described for the *E. coli* bioreporter strains. Bioluminescence values are means for the light production normalized per OD₆₀₀ at *t*=4 h, (corresponding to the optimal activity). Error bars represent standard deviation to the means (*n*=3).

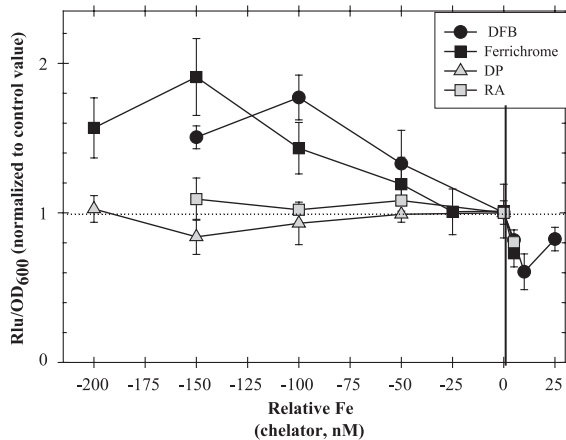


Fig. 4. Bioluminescence induction in *P. putida* FeLux grown in LB medium supplemented with DFB (●), ferrichrome (■), 2,2'-dipyridyl (△) and RA (□). OD₆₀₀ and bioluminescence were determined for each triplicate over a 6-h experimental period as indicated previously. The data are plotted as the ratio of the light production at *t*=4 h normalized per the correspondent OD₆₀₀ (RLU/OD₆₀₀) between the treatment amended with 100 nM of chelator and the control (non-amended). Error bars represent standard deviation of the means (*n*=3).

reporter in Fe-replete conditions (control, $3.98 \pm 0.14 \text{ day}^{-1}$) is similar to previously reported growth rate for Fe-replete *Pseudomonas* sp. ($3.59 \pm 0.32 \text{ day}^{-1}$; Granger and Price, 1999). No significant change relative to the unamended control was observed in the growth rate upon titration of Fe by DFB (-7% ; *t*-test, $p>0.05$) or upon enrichment with Fe ($+25\%$, *t*-test, $p>0.05$).

3.1.4. Influences of different chelators on Fe availability to the *P. putida* FeLux bioreporter

To assess the differential effects of Fe-binding ligands on the bioavailability of Fe, we challenged the bioreporter *P. putida* FeLux with various well-defined chelators (Fig. 4). The addition of different ligands resulted in a variety of responses. The strongest bioluminescence was observed upon addition of DFB and ferrichrome. Relative light production increases linearly ($r^2=0.95$) between 5 nM Fe and 150 nM of ferrichrome, with a similar slope to that calculated upon addition of DFB ($r^2=0.92$). The decline of light production at concentrations of ferrichrome higher than 150 nM is mostly likely due to a decrease in the metabolic activity of the cells upon Fe

starvation. However, this decline is observed at higher concentrations (but similar Rlu) of ferrichrome (150 nM) relative to DFB (100 nM). Therefore, even though they possess identical Fe-affinity constants (Table 2), ferrichrome does not appear to be as efficient as DFB in sequestering the initially bioavailable Fe from this strain.

Unlike the DFB and ferrichrome treatments, the DP or RA treatments did not exhibit an increase of luminescence (Fig. 4). Compared to the unamended control, the addition of DP or RA did not significantly influence light production (*t*-tests, $p>0.05$). Within the range of our experimental concentrations, the synthetic chelator DP and the siderophore RA appear less efficient than DFB and ferrichrome at sequestering Fe from *P. putida* FeLux culture medium.

3.1.5. Transcriptional regulation of the FeLux reporter in different microbial hosts

Because the results described above suggested that heterologous chelators alter the availability of Fe to *P. putida*, further experiments were done to evaluate the impact of the same ligands on Fe nutrition of *E. coli* strains. Two *E. coli* bioreporters were used: *E. coli* pFeLux (parental strain), where the *fepA-fes:lucC*-

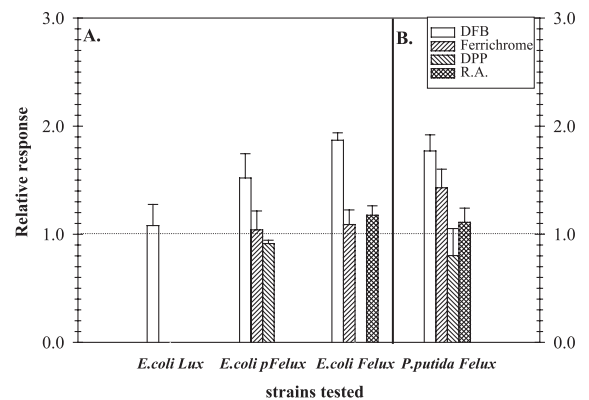


Fig. 5. Intercomparison of the induction of bioluminescence among strains after the addition of 100 nM DFB, ferrichrome, DPP or RA. Data are expressed as ratio of bioluminescence normalized per OD₆₀₀ between the treatment amended with 100 nM of chelator and the unamended control ($\text{Light}/\text{OD}_{600}[100 \text{ nM chelator}] \div \text{Light}/\text{OD}_{600}[\text{control}]$). Errors bars represent standard deviation of the means (*n*=6) for each points. (A) Responses of *E. coli* control (*E. coli* Lux), *E. coli* parental strain (*E. coli* pFeLux) and *E. coli* bioreporter (*E. coli* FeLux) at *t*=6 h. (B) Responses of *P. putida* bioreporter (*P. putida* FeLux) at *t*=4 h.

Table 3
Fe concentrations in Lake Erie, July 2001

Date, time	Location (depth)	Dissolved Fe (nM)	Particulate Fe (nM)
July 13, 10:00*	Station 84, 10 m	3.98 (± 0.14)	ND
July 13, 16:00	Station 84, 10 m	3.26 (± 0.86)	ND
July 13, 16:30	Station 84, 10 m	6.77 (± 0.20)	ND
July 17, 10:00*	Station 84, 10 m	3.21 (± 0.37)	ND
July 18, 10:00	Station 84, 10 m	2.62 (± 0.65)	28.6 (± 3.2)
July 14, 10:00	Put-In-Bay, OH, surface	23.8 (± 0.49)	ND
SLRS-4 Standard (certified as 1844 ± 89)		1735	1941

Dissolved ($<0.2 \mu\text{m}$) Fe concentrations (\pm S.D., $n=3$) in Lake Erie were taken for bioreporter work (*) as well as for other times during the cruise to demonstrate the consistency of Fe concentrations. Samples from a coastal harbor (Put-In-Bay, OH) are presented to contrast the neritic and pelagic environments. Particulate Fe samples were collected after the effects of filtration on bioavailability were noted (\pm S.D., $n=5$). ND=no data.

DABE construct is maintained as an extra-chromosomal element (plasmid), and *E. coli* FeLux with the bioreporter chromosomally inserted. Data obtained for these two bioreporters (as well as the for *P. putida* strains) are compiled in Fig. 5. The addition of the heterologous siderophores yielded different responses dependent upon both the chelator tested and the bioreporter host strain. DFB addition resulted in the strongest increase in luminescence for each strain. In addition, the response of the three bioreporters to DFB addition compared to their respective control was about the same magnitude (~ 1.7 – 1.8). The expression of the bioreporter construct as a plasmid may explain the large variations observed for *E. coli* pFeLux relative to the strains with the construct in their chromosome, as excess multiple copies of the promoter region within a cell may not be regulated by a limited abundance of Fe–FUR repressor. However, these data demonstrate that DFB-complexed Fe is not directly available to either *P. putida* or *E. coli*.

As with *P. putida* FeLux, neither the addition of DP nor the addition of RA to *E. coli* pFeLux resulted in a significant variation in luminescence (relative to the unamended control). However, in contrast to *P. putida*, both *E. coli* strains did not exhibit any significant difference of luminescence between the treatment amended with 100 nM of ferrichrome and

the unamended control (*t*-test, $p>0.05$ for *E. coli* pFeLux, $p>0.05$ for *E. coli* FeLux). These data suggest that ferrichrome can be used as a source of Fe by *E. coli*.

3.2. Field data

3.2.1. Station description

As predicted for our July samplings, significant stratification of the water column (a thermocline at ca. 18 m, where $Z_{\text{max}}=26$ m) established conditions where Fe concentrations would be expected to be drawn down in the upper-mixed layer (Twiss et al., 2000). Total dissolved phosphorus, 4.3 $\mu\text{g/l}$ (0.14 μM), and total dissolved nitrate, 202 $\mu\text{g/l}$ (3.26 μM), in the upper mixed layer concentrations were accompanied by concentrations of chlorophyll-*a* indicative of oligotrophic/mesotrophic conditions. During our experiments, the in situ chlorophyll-*a* ranged from a high of 1.95 (± 0.11) $\mu\text{g/l}$ on July 12, down to 1.55 (± 0.04) $\mu\text{g/l}$ on July 15, 2001.

Dissolved Fe concentrations demonstrated a small range of variability over the study period in the surface waters (10 m) of station 84 (Table 3). The dissolved Fe ranged between 2.62 nM (July 18) and 6.77 nM (July 13) during the survey. The dissolved Fe concentrations did not exceed 7 nM during the assessment of the luminescent responses

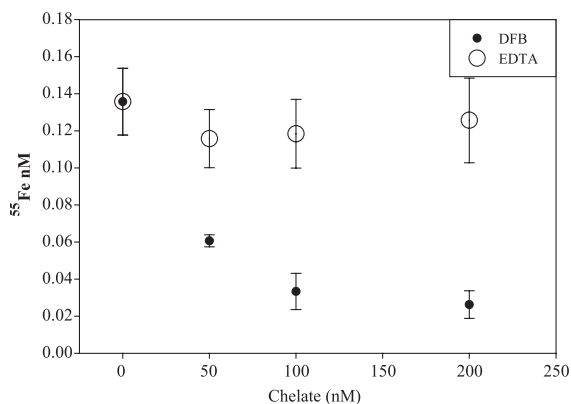


Fig. 6. Effect of chelating agents on ^{55}Fe assimilation in freshwaters. Fe uptake into particles was estimated using the Ti/citrate/EDTA method as altered for use in freshwaters (see Methods and materials). Results indicate that increasing concentrations of DFB inhibited Fe uptake by the biological community while the synthetic chelator EDTA had no effect.

of the bioreporter on July 13 (3.98 ± 0.14 nM) and July 17, 2001 (3.21 ± 0.37 nM). Particulate Fe concentrations evaluated on July 18 (28.6 nM) were nearly an order of magnitude larger than the dissolved Fe concentrations.

3.2.2. Effects of DFB on Fe uptake in by the Lake Erie microbial community

To demonstrate the validity of using DFB as an Fe sequestering agent in this natural system, we have included information from a July 2000 cruise in Lake

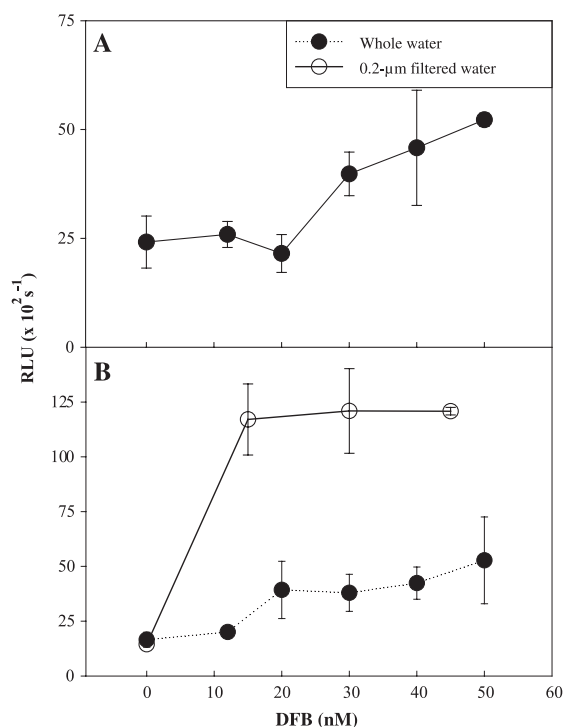


Fig. 7. Response of the *P. putida* #4 bioreporter to increasing concentrations of DFB in water from the central basin of Lake Erie. (A) Luminescence (RLU s^{-1}) is shown versus the concentration of DFB added to whole water (\bullet : $<210 \mu m$) samples collected July 13, 2001 and amended with DFB and incubated at $t=6$ h. Increased light production at concentrations >20 nM suggests that Fe stress occurred at DFB concentrations greater than this. (B) Luminescence (RLU s^{-1}) is shown versus the concentration of DFB added to whole water (\bullet : $<210 \mu m$) and filtered water (\circ : $<0.2 \mu m$) samples collected on July 17, 2001 and amended with DFB and at $t=6$ h. The results clearly demonstrate that DFB additions to filtered water samples induce Fe deficiency faster than in whole lake water, implying a significant component of the bioavailable pool was removed by 0.2- μm filtration.

Erie where DFB as well as the synthetic chelator EDTA were added and Fe assimilation measured with the radiotracer ^{55}Fe . Addition of increasing concentrations of DFB resulted in a decrease of the Fe uptake by the ambient community, indicating that DFB sequestered Fe in a form that was not available for uptake by biota in the sampled lake water (Fig. 6). In contrast, EDTA, which has a lower affinity (and specificity) for Fe relative to DFB, did not affect Fe uptake by the community. These data support the laboratory observations of a differential efficiency among cationic chelators to sequester bioavailable Fe and demonstrate that Fe–DFB is not recognized as an Fe source by the biota sampled.

3.2.3. Assessment of Fe availability in pelagic Lake Erie

Addition of the fungal siderophore DFB into whole water ($<210 \mu m$) from central basin of the Lake Erie induced a significant luminescent response at concentrations of DFB higher than 30 nM ($p>0.05$, Fig. 7A). Bulk light production increased ca. 2-fold (exact number) between 20 and 50 nM of added DFB. These data suggest that DFB efficiently decreases the bioavailability of Fe when added at concentration above 20 nM in whole water, subsequently leading to Fe stress in the microbial community. When a similar experiment was performed both in whole water and in filtered water ($<0.2 \mu m$), the bulk increase in luminescence upon DFB addition was 2-fold higher in the filtered water than in whole water (Fig. 7B). The onset of luminescence also occurred at lower concentrations of DFB in the filtered treatment, which suggests that a substantial fraction of the bioavailable Fe was removed by filtration. These results and those of the laboratory studies are considered below.

4. Discussion

Two significant conclusions are drawn from this study. The first is that genetically engineered microbial strains (bioreporters) can be used to rapidly (4–6 h) characterize the bioavailability of Fe under different conditions in both laboratory and field conditions, providing a necessary compliment to the analytical techniques that are currently in place for the characterization of Fe concentration and chemical specia-

tion. This novel tool may allow for the determination of which factor (Fe or DOM) is the primary limiting resource of heterotrophic production in HNLC environments. Moreover, it may also provide insight on the influence of the chemical speciation of Fe on microbial community structure in a platform that is simpler to apply (the *lux* reporter system) than most other reporters (e.g., Loper and Lindow, 1994). The second conclusion from this study is that Fe bioavailability (at least, in the central basin of Lake Erie) is strongly dependent on the presence of Fe in the particulate (>0.2 μm) pool. Although others have suggested that the biota themselves may represent one of the most rapidly cycled pools of bioavailable Fe (e.g., Wells et al., 1995), we believe this represents some of the first data that truly addresses this hypothesis. These results are discussed below in addition to other perceived advantages (and limitations) of these bioreporters.

4.1. Characterization of Fe-L availability using bioreporters

Several studies in recent years have used xeno-siderophores to alter Fe assimilation by marine phytoplankton and bacterioplankton. As a result of these studies, contrasting information concerning the use of these chelates (particularly DFB) has arisen. DFB has been demonstrated to effectively reduce the available Fe to most members of the phytoplankton community (Wells et al., 1994; Wells, 1999; Hutchins et al., 1999a; Timmermans et al., 2001). And while some evidence suggests that phytoplankton communities in the sub-Arctic Pacific can access at least small amounts of DFB-bound Fe (Maldonado and Price, 1999), results from other studies suggest the flux of DFB-complexed Fe to the cell surface is probably not sufficient to satisfy cellular requirements (Hutchins et al., 1999b). Recently, Martinez et al. (2001) demonstrated the production of desferrioxamine G (a structural relative to DFB) by a marine *Vibrio* sp. isolated from a marine invertebrate larva. As such, they concluded that the production of this compound inferred its utility as a bioavailable Fe-chelate complex to the marine community. In the current study we have shown DFB reduces Fe availability to our reporter strains under both laboratory and field conditions. In both cases, the high-affinity Fe transport

system (as denoted by the activity of the *fepA*–*fes* promoter) was derepressed upon nanomolar addition of the fungal siderophore DFB. In addition, DFB additions also reduced uptake of ^{55}Fe by the microbial community in pelagic Lake Erie. Thus, DFB sequesters a substantial amount of available Fe originally present in the environment. As a result, we are able to titrate the available Fe to a point where it is no longer bioavailable to our reporter strains.

As part of this work we have demonstrated the functionality of this mobile genetic construct in strains of both *E. coli* and *P. putida*, and that it can be used to characterize the bioavailability of various chelates to some microorganisms. A dose-dependent response of the bioreporter *P. putida* FeLux was observed during the laboratory assays upon enrichment in Fe and restriction of Fe by addition of the siderophores DFB and Ferrichrome. This dose-dependent response was observed while the addition of chelators had no significant impact on the growth rate of the bioreporters. The minimal impact of Fe stress on the growth rate of *P. putida* in this study is similar to data previously reported for the same species (Granger and Price, 1999). Therefore, the bioluminescence production by FeLux bioreporter may be sensitive to alterations in Fe bioavailability that do not immediately affect cellular growth rate over the experiment period but may affect heterotrophic metabolism. Additionally, the linearity of this dose-dependent response underlines the interest of using this type of bioreporter as a quantitative tool in aquatic systems as previously reported (Durham et al., 2002). The decline of the light, observed for the highest concentrations of siderophores tested on *P. putida* FeLux, suggests the effective range of use for this biological tool in characterizing Fe availability. Any physiological stress, such as deficiency in respiratory electron transport system ETS, can lead to a reduction in the metabolism of the bioreporters and subsequently reduced transcription (and therefore expression) of the *lux* reporter gene. Indeed, it has been estimated that heterotrophic metabolism, dependent on the maintenance of respiratory ETS, requires 60% more Fe per mole of cytochrome-*c* than does the photosynthetic ETS (Raven, 1988); subsequent studies have shown that respiratory ETS is a site primarily affected by Fe limitation (Tortell et al., 1996). However, the results of the field assays seem to indicate that the range of

linear dose-dependant response is wide enough to use the *P. putida* FeLux in environmental samples.

The ability to cross-feed on exogenous siderophores is an important survival strategy for eubacteria in their natural environment. Previous studies have suggested that different strains of *P. putida* were able to use siderophores from other species (Loper and Henkels, 1999, Jurkevitch et al., 1992a,b). In this study, the absence of luminescence increase following the addition of nanomolar concentrations of the weak chelator DP is consistent with the literature data: when used in a culture medium to titrate available Fe, DP is generally added at millimolar concentrations (Khang et al., 1997; Loper and Henkels, 1999). The experimental range of DP concentrations was probably too low to alter significantly the Fe availability to *P. putida* and, therefore to derepress the high-affinity transport systems. The absence of a response to the addition of RA in this study may also indicate that this siderophore was not efficient at sequestering Fe away from *P. putida* FeLux. This may be due to a lower specificity and lower affinity of RA for Fe relative to DFB, or due to the ability of this strain to assimilate this xenosiderophore. Conversely, the dose response observed after addition of ferrichrome suggests that this fungal siderophore is almost as efficient as DFB at sequestering Fe away from *P. putida* FeLux, leading to the derepression of the high-affinity transport systems. This finding seems surprising in consideration of previous work, demonstrating that a terrestrial *P. putida* was able to utilize Fe-bound ferrichrome via a Fe^{3+} -ferrichrome uptake system (Jurkevitch et al., 1992a,b). However, the same authors observed that the rate of uptake of ^{55}Fe complexed to ferrichrome by *P. putida* St3 was very low compared to other siderophores such as pseudobactin (Jurkevitch et al., 1992a). It is therefore possible that the ability to use Fe-ferrichrome complexes varies among strains of the same species.

In contrast to for the response of *P. putida* FeLux, no significant light was detected for the both *E. coli* bioreporters after addition of ferrichrome. Like many bacteria, although *E. coli* does not produce ferrichrome, it does produce Fhu A receptor, which will transport the ferrichrome (Fecker and Braun, 1983). Moreover, while the expression of Fhu A is also thought to be regulated by the FUR protein, leakiness in this regulation can lead to the expression of up to a hundred copies of the receptor on the surface of cells

maintained in Fe-replete conditions (Hantke and Braun, 1998). This strain-dependent response to Fe-bioavailability highlights the importance of constructing genetically mobile reporters so that the response of a variety of cells to Fe speciation can be examined. In the case of our construct, we specifically chose a FUR-regulated promoter as the *fur* gene is well distributed throughout many prokaryotes and displays significant homology between several strains (Hantke and Braun, 1998; Hantke, 2001). As such it is predicted that this construct can be moved into other cultivable marine prokaryotes such as *Vibrios* and other *Pseudomonads*, thereby providing a broader selection of bioreporters to employ in attempting to model the bioavailability of Fe to native bacterial community.

4.2. Fe availability from the particulate size class

Previous studies using DFB to reduce Fe assimilation have typically involved the addition of DFB (100 nM, Wells, 1999; 50 nM, Hutchins et al., 1999a; 100 nM, Hutchins et al., 1999b) well in excess (ca. 5- to 100-fold) of the total dissolved Fe concentrations. While our observations confirmed the ability of DFB to sequester Fe away from both our reporter strains and the natural community (cf. ^{55}Fe uptake data) in Lake Erie, we also noted that filtration removed a significant proportion of the bioavailable Fe. Assuming that dissolved Fe is not lost onto the filters (our protocols were essentially the same as those used in other Fe-clean studies) then this suggests that a significant proportion of the bioavailable Fe was associated with the particulate size class. Assuming that the availability to the reporter strains is an indication of the availability of Fe to the greater community and that there is a 1:1 relationship between the chelate/Fe complex and availability, then in the case of the whole water from the central basin of Lake Erie, somewhere between 20 and 30 nM Fe is biologically available to the planktonic community. The 1:1 relationship has been well established with respect to the Fe-binding kinetics of DFB, and this has further been shown to induce Fe stress in a recently developed reporter for Fe bioavailability to freshwater cyanobacteria (Porta et al., 2003). This concentration of available Fe seems to closely match our estimates of total Fe (dissolved plus particulate) from our samples on July 18, 2001 (ca. 30 nM). As such, it

supports previous suggestions that much of the Fe that is bioavailable in the system (either directly or indirectly) is associated with particulate (and most likely biological) material (Wells et al., 1995). Moreover, it stresses the need to examine the total Fe pool and not just the dissolved Fe pool as has been carried out in some studies of Fe bioavailability (e.g., Christian et al., 2002). As stressed elsewhere (Hutchins and Bruland, 1994; Wilhelm and Suttle, 2000), recycling of Fe by grazing and viral activity continuously supplies Fe back to the microbial community. Protozoan grazing in pelagic surface waters can actively recycle bioactive metals between particulate and dissolved phases (Twiss et al., 1996) and viruses in this lake have been shown to be responsible for ca. 12–23% of the microbial mortality (Wilhelm and Smith, 2000). As such, the removal of biological particles from samples thereby reduces a significant source of persistently remobilized Fe. Moreover, it has been demonstrated that organisms within the planktonic community possess mechanisms for accessing Fe associated with abiotic particles (e.g., Maranger et al., 1998). It is becoming increasingly clear from this and other studies (Hutchins et al., 1999b; Wells, 1999; Maldonado and Price, 1999) that the temporal component (i.e., the kinetics of interactions with biota) of a compound's bioavailability requires consideration equal to that given to its chemical structure.

5. Conclusions

As researchers and politicians continue to debate how Fe fertilization may allow for the use of the oceans as sinks for anthropogenically produced CO₂ (Chisholm et al., 2001, 2002; Neufeld et al., 2002; Johnson and Karl, 2002), it is surprising that we still have no clear concept of what represents the bioavailable component of the aquatic Fe pool. Obviously, any understanding of the physiology and nutritional status of the different members of the planktonic community (including the heterotrophic bacterioplankton) remains hampered by this lack of knowledge. As our knowledge of the identity of the “bioavailable pool” grows, we can hypothesize that this store of Fe represents a heterogeneous mixture of Fe-containing compounds and that the availability of these compounds is often on

time scales outside (either too short or too long) our experiments. The tool described in the current text, when combined with the current arsenal of the modern-day aquatic biogeochemistry, should allow for a better characterization and temporal scaling of the bioavailable Fe pools in different aquatic regions and lead to a better understanding of the true regulatory role Fe plays in these environments. Future studies, using different hosts as well as multiple ligands under different competitive regimes, should help us provide some clarity in these areas.

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