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Tracking changes in bioavailable Fe within high-nitrate low-chlorophyll oceanic waters: A first estimate using a heterotrophic bacterial bioreporter

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[1] It is conventional knowledge that heterotrophic bacteria play a key role in the biogeochemical cycling of oceanic carbon. However, only recently has their role in marine iron (Fe) biogeochemical cycles been examined. Research during this past decade has demonstrated an inextricable link between Fe chemistry and the biota, as >99% of Fe in marine systems is complexed to organic chelates of unknown but obviously biotic origin. Here we present a novel approach to assess and compare Fe bioavailability in low Fe HNLC waters using a bioluminescent bacterial reporter that quantitatively responds to the concentration of bioavailable Fe by producing light. Originally tested in freshwater environments, this study presents the first characterization of this halotolerant reporter organism in a defined seawater medium and then subsequently in marine surface waters. Laboratory characterizations demonstrate that this reporter displays a dose-dependent response to Fe availability in our defined marine medium. Field tests were performed during the 10-day mesoscale FeCycle experiment (February 2003) in the Pacific sub-Antarctic high-nitrate low-chlorophyll region. Data from both biogeochemical measures and bioreporter assays are provided which describe how the bioreporter detected changes in Fe bioavailability that occurred during a natural shift in ambient dissolved Fe concentrations (~40 pM). Our data explore the use of heterotrophic bioluminescent reporters as a comparable tool for marine ecosystems and demonstrate the potential utility of this tool in elucidating the relationship between Fe bioavailability and Fe chemistry in complex marine systems.

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1. Introduction

[2] Iron (Fe) is an essential micronutrient for microorganisms since it serves as a cofactor for a wide variety of enzymes and redox catalysts [Raven, 1990; Geider and Laroche, 1994; Tortell *et al.*, 1999]. Although absolute requirements vary, it has been estimated from laboratory studies that cultured heterotrophic bacteria require a min-

imum Fe concentration of $\sim 10^{-8}$ M for growth [Braun and Hantke, 1997], yet such Fe concentrations are rarely measured in oceanic surface waters. Low inputs of Fe from aeolian and/or deep water upwelling sources, combined with rapid biological assimilation as well as abiotic removal due to scavenging by sedimenting particles, result in total dissolved Fe (DFe) concentrations ranging from 0.02 nM to 1 nM in many pelagic marine systems [Johnson *et al.*, 1997; Measures and Vink, 2001; Takata *et al.*, 2004]. Ecosystem-scale perturbation experiments have demonstrated that these low ambient Fe concentrations control phytoplankton growth, community composition and ecosystem function in large areas of the world's ocean, including the equatorial Pacific [Coale *et al.*, 1996], the subarctic Pacific [Tsuda *et al.*, 2003; Boyd *et al.*, 2004] and the Southern Ocean [Boyd *et al.*, 2000; Gervais *et al.*, 2002; Coale *et al.*, 2004]. In these regions, mesoscale "Fe enrichments" have resulted in phytoplankton blooms and a switch in the ecosystem from recycling- to export-dominated planktonic communities [Bishop *et al.*, 2004; Buesseler *et al.*, 2004].

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Table 1. Composition of BESAW Medium as Modified From *Berges et al.* [2001]^a

Substance	Final Concentration, M
<i>Anhydrous Salts Solution</i>	
NaCl	3.63×10^{-1}
Na ₂ SO ₄	2.50×10^{-2}
KCl	8.04×10^{-3}
NaHCO ₃	2.07×10^{-3}
KBr	7.25×10^{-4}
H ₃ BO ₃	3.72×10^{-4}
NaF	6.57×10^{-5}
<i>Hydrated Salts and Buffer Solution</i>	
MgCl ₂ •6H ₂ O	4.12×10^{-2}
CaCl ₂ •2H ₂ O	9.14×10^{-3}
SrCl ₂ •6H ₂ O	8.20×10^{-6}
Hepes Buffer	5.00×10^{-2}
<i>Nutrients</i>	
NaNO ₃	5.49×10^{-4}
NH ₄ SO ₄	2.00×10^{-2}
NaH ₂ PO ₄	2.10×10^{-5}
<i>Carbon Source</i>	
Glycerol	1.44×10^{-1}
<i>Trace Metals</i>	
ZnSO ₄ •7H ₂ O	2.54×10^{-7}
CoSO ₄ •7H ₂ O	5.69×10^{-9}
MnSO ₄ •4H ₂ O	2.42×10^{-6}
Na ₂ MoO ₄ •2H ₂ O	6.10×10^{-9}
Na ₂ SeO ₃	1.00×10^{-9}
NiCl ₂ •6H ₂ O	6.30×10^{-9}
<i>Iron</i>	
FeCl ₃ •6H ₂ O	$5.00 \times 10^{-9} - 5.00 \times 10^{-5}$
Na ₂ EDTA•2H ₂ O	1.00×10^{-4}
<i>Vitamins</i>	
Thiamine-HCl	2.97×10^{-7}
Biotin	4.09×10^{-9}
B12	1.47×10^{-9}

^aNutrients and salts were prepared as two-time concentrated stocks solutions, Glycerol was added to the salt solution as 30 mL of a separately Chelex-100 treated stock solution (35% v/v, in BESAW salts), and trace metals and vitamins were added from $\times 1000$ and $\times 10,000$ stock solutions, respectively.

[3] Although Fe-deficient surface seawaters are generally dominated by microbial food web communities containing a high proportion of picoplankton (0.2–2 μm), both laboratory and field experiments suggest that autotrophic [Wilhelm, 1995; Achilles *et al.*, 2003] and heterotrophic [Pakulski *et al.*, 1996; Hutchins *et al.*, 1999a, 2001a; Weaver *et al.*, 2003; Arrieta *et al.*, 2004] bacterioplankton components may be Fe-limited as well. Despite larger cell surface area to volume ratios that should give bacterioplankton an advantage [Eldridge *et al.*, 2004, and references therein], empirical models suggest that bacteria are disadvantaged compared to phytoplankton due to slower uptake kinetics and their higher Fe:C requirements [Maldonado and Price, 1999]. Therefore the predominance of bacterioplankton in most HNLC regions is quite puzzling if only total DFe concentrations are considered. Indeed, it has been suggested that changes in the chemical speciation and size-class partitioning of Fe exert more influence than the total concentration of DFe on the planktonic

group(s) that dominate the specific communities [Hutchins *et al.*, 1999b, 2001a; Poorvin *et al.*, 2004; Wells and Trick, 2004]. Unfortunately, while physiological proxies such as flavodoxin and ferredoxin have been developed to correlate algal Fe stress to water column Fe concentrations [McKay *et al.*, 2005], there are no analytical tools currently available which can be employed to compare the fractions of Fe in seawater that are biologically available to either phytoplankton or bacteria [Gerringa *et al.*, 2000; Wells and Trick, 2004].

[4] Here we describe a new approach to assess Fe bioavailability in seawater: a heterotrophic bacterial reporter that responds specifically to Fe stress by producing a quantifiable bioluminescent signal. In the current study we have used this new tool to determine whether changes in bioavailable Fe occurred within a surface seawater patch labeled with sulfur hexafluoride (SF₆). The halotolerant isolate *Pseudomonas putida* was genetically engineered to produce light (luminescence) when the native high-affinity Fe transport system(s) of the host cell are derepressed [Mioni *et al.*, 2003], resulting in the bioreporter strain *P. putida* FeLux. This enables the detection of variations in bioavailable Fe in situ. In the present study, we describe the initial laboratory characterization of this *P. putida* Fe-dependent bioreporter in a chemically defined and modeled synthetic seawater medium (BESAW) that can be used as a standard for comparison between systems. We also present a set of field measurements performed in late austral summer (February 2003) in the sub-Antarctic Pacific region during the 10-day “FeCycle” mesoscale study. To the best of our knowledge, this is the first portable tool available to directly assess the bioavailability of Fe to a heterotrophic prokaryote in marine systems.

2. Material and Methods

2.1. Strain and Laboratory Calibration

[5] *Pseudomonas putida* FeLux [Mioni *et al.*, 2003] stock cultures were maintained on Pseudomonas Isolation Agar (Remel) supplemented with 50 $\mu\text{g mL}^{-1}$ of tetracycline (Tc) at 30°C. Prior to the experiment, several colonies were screened. The colony with the largest dynamic range of light production relative to the control (no addition) after an addition of Fe (1.5 nM) or desferrioxamine B (DFB, 1.5 nM) was selected for the field experiments. Cultures from the selected colony were maintained in sterile Marine Broth 2216 (Difco) supplemented with 50 $\mu\text{g mL}^{-1}$ of Tc at 25°C in sterile 50-mL polycarbonate tubes on an orbital shaker. Pre-acclimation of the bioreporter cells prior to experiments was performed in BESAW, a completely defined medium modified from the Enriched Seawater/Artificial water recipe (ESAW [Berges *et al.*, 2001]) and optimized for heterotrophic bacteria growth (Table 1). Final free ferric ion concentrations (expressed as both pFe, the $-\log [\text{Fe}^{3+}]$, and Fe(III)', the sum of the inorganic Fe³⁺ species) as well as the concentration of other free trace metals were inferred using the chemical equilibrium software MINEQL+ ver.4.06 (Table 2). To reduce residual Fe, all nutrient stocks and water were treated with Chelex-100 resin [Price *et al.*, 1988/1989]. All culture materials were soaked in dilute HCl

Table 2. Total Concentrations of Trace Metals Added to BESAW Medium (pH 7.8) With an EDTA Concentration of 100 μ M and Correspondence to Free Metal Ion Concentrations for Each Treatment as Computed Using the Chemical Equilibrium Program MINEQL

[Fe], M	EDTA, M	[Fe ³⁺], M	Fe(III)'	pFe	pZn	pCo	pMn	pNi
5×10^{-9}	10^{-4}	$10^{-21.15}$	0.9	21.2	9.38	11.2	6.12	13.1
5×10^{-8}	10^{-4}	$10^{-20.15}$	9.3	20.2	9.38	11.2	6.19	13.1
2×10^{-7}	10^{-4}	$10^{-19.54}$	37	19.5	9.38	11.2	6.18	13.1
5×10^{-7}	10^{-4}	$10^{-19.14}$	93	19.1	9.38	11.2	6.18	13.1
7.5×10^{-7}	10^{-4}	$10^{-18.97}$	140	19.0	9.38	11.2	6.18	13.1
5×10^{-6}	10^{-4}	$10^{-18.12}$	980	18.1	9.36	11.2	6.17	13.1
5×10^{-5}	10^{-4}	$10^{-16.84}$	1.8×10^4 ^a	16.8	9.09	10.9	6.02	12.8

^aFe concentrations at this level will lead to Fe precipitation within the medium.

(5%) and rinsed with Chelex-100 treated Milli-Q water and culture vessels were microwave-sterilized prior to use. All manipulations were performed under a class-100 laminar flow hood using aseptic and trace-metal clean techniques.

2.2. Laboratory Characterization of *P. putida* FeLux as Fe-Dependent Bioreporter

[6] Reagents and medium for the characterization of the bioreporter were prepared prior to the day of the experiment. Sterile aliquots of Fe-free BESAW (18 mL) were dispensed into polycarbonate Oakridge tubes (Nalgene) and supplemented with Fe from an Fe:EDTA concentrated stock. The concentration of FeCl₃•6H₂O was altered in each premix to create an increasing range of bioavailable Fe. The final concentration of EDTA was maintained constant (100 μ M) for all treatments. For pre-acclimation, 5 mL of culture grown in Marine Broth 2216 (Difco) was transferred to a 500-mL polycarbonate Erlenmeyer flask containing 95 mL of sterile, Fe-deficient BESAW (pFe 21.2, Table 2) and incubated overnight in the dark at 25°C with shaking. The experiment was initiated by transferring 2 mL this culture to each replicate tube ($n = 3$ for each treatment). The optical density at 600 nm (OD₆₀₀) and light production were measured at 2-hour intervals over an experimental period of 12 hours using a spectrophotometer (Biomate 5, ThermoSpectronic corp.) and luminometer (FB-15, Zylux Corp.). Samples in the luminometer were measured after a 1-s delay period with a 5-s integration time and blank subtraction. The sensitivity of the FB-15 PMT is 1000 luciferase molecules per reaction (manufacturer's specifications). For each time point, light production was normalized to OD₆₀₀. The results of the experiment are expressed as light levels at different pFe (or Fe(III)') equivalents. This allows for the characterization of Fe in different chemically modified media and comparison (using the calibrated BESAW response as a standard) between samples and systems.

[7] In a separate experiment, Fe-replete bioreporter cultures were used to calibrate the OD₆₀₀ value to cell concentration. Cells were preconditioned as described above and transferred to BESAW medium of pFe 18.1. At 2-hour intervals, cells were sampled to measure the luminescence and optical density at 600 nm (Figure 1a). In parallel, 1 mL of culture was fixed with glutaraldehyde (2.5% v/v) and enumerated by epifluorescence microscopy after treatment with acridine orange [Hobbie *et al.*, 1977]. Since the OD₆₀₀ values correlated strongly ($R^2 = 0.988$) with the density of bioreporter cells (Figure 1b), light production normalized to OD₆₀₀ was converted to light

emitted per reporter cell using the empirically determined linear function,

$$y = 3.61 \times 10^8 x - 3.46 \times 10^6, \quad (1)$$

where y is the concentration of bioreporter cells mL⁻¹ and x is the corresponding OD₆₀₀ value. Reported values are the

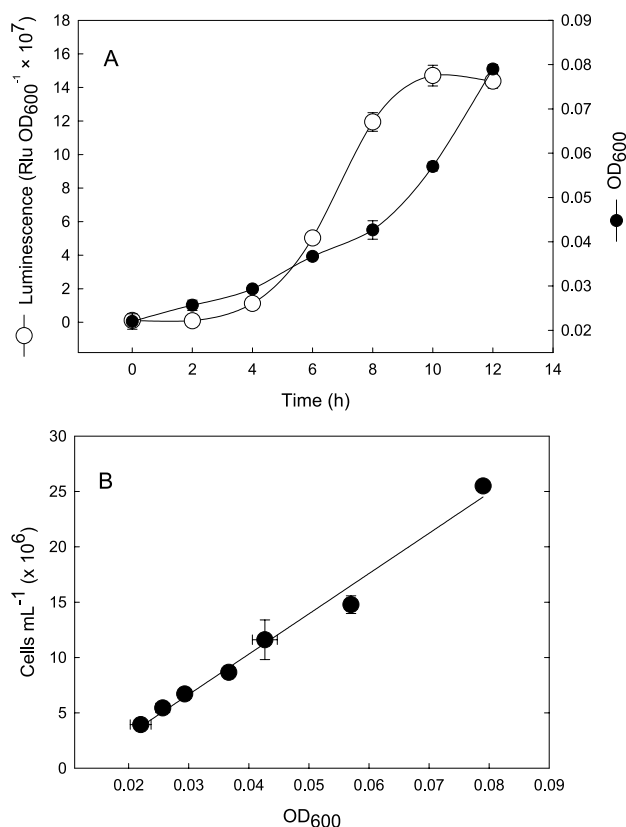


Figure 1. Laboratory characterization of the *P. putida* FeLux bioreporter in the trace metal buffered BESAW medium. (a) Typical growth curve and light production (Rlu: relative luminescence units) of *P. putida* FeLux in BESAW medium (pFe 18.1). Error bars represent the standard deviation between triplicates when larger than the symbol. (b) Typical calibration curve of OD₆₀₀ and cell concentration in Fe-replete BESAW medium with resulting linearization ($R^2 = 0.988$; slope = 3.61×10^8 , y intercept = -3.46×10^6). Error bars represent the standard deviations of triplicates when larger than the symbol size.

means of triplicate cultures (\pm S.D.) from the time-point for which the signal was the strongest ($t = 12$ hours). Owing to the high reproducibility of the data, calibration data will be presented here as the mean of the data obtained from three experiments performed in identical conditions ($n = 9$ per treatment).

2.3. Site Selection and Survey

[8] The FeCycle mesoscale experiment was designed to develop an unperturbed Fe budget by following a patch of surface water in a HNLC region labeled with SF₆ as a tracer [see *Boyd et al.*, 2005]. The study site was previously characterized by subpolar high nutrient–low silicic acid–low chlorophyll [HNLSiLC; *Dugdale and Wilkerson*, 1998] waters that are representative of the majority of the Southern Ocean surface waters ($\sim 65\%$ [Coale *et al.*, 2004]). During austral summer in this region, surface waters are typically depleted ($< 1 \mu\text{M}$) of silicic acid (Si(OH)₄) as well as Fe; indeed it has been suggested that diatom stocks remain low due to a Si(OH)₄-Fe co-limitation [Boyd *et al.*, 2001; *Hutchins et al.*, 2001b]. The austral summer provides optimal conditions to study possible mechanisms that control HNLC assemblages as during this period of the year the HNLSiLC community is dominated by prokaryotic and picoeukaryotic organisms [Boyd *et al.*, 2001, 2005; *Hutchins et al.*, 2001b].

[9] The FeCycle mesoscale experiment was conducted during late austral summer (30 January 2003 to 12 February 2003) in the vicinity of the NIWA's Southern mooring site ($\sim 46^{\circ}30$ S, $178^{\circ}30$ E) on board of the RV *Tangaroa*. The study site location was selected based on a pre-release oceanographic survey (circa XTB section, CTD and underway seawater sampling) and local remote sensing (SeaWiFS, SSH, SST, mooring data) as described by *Boyd et al.* [2005]. Once the study site was selected, surface waters were labeled over a 47-km² area using the chemically inert tracer sulfur hexafluoride (SF₆) in a manner similar to that used during the SOIREE voyage [Boyd *et al.*, 2000; *Bowie et al.*, 2001]. However, unlike the SOIREE experiment, the labeled patch was not infused with Fe. SF₆ concentrations were analyzed during daily underway mapping of the patch as described elsewhere in this special section [Boyd *et al.*, 2005].

2.4. Field Sample Collection

[10] All samples for Fe bioreporter analysis were collected at the center of the SF₆-labeled patch using a trace-metal clean pumping system (Almatec) as described in a related paper [McKay *et al.*, 2005]. Waters were homogenized in an acid-cleaned container and subsequently filtered (0.2 μm), collected in an acid-cleaned polycarbonate flasks and stored frozen (-20°C) until use. Samples were collected prior to sunrise ($\sim 5:00$ local time) from a depth of approximately 5–7 m.

[11] Seawater samples for Fe chemistry were also concurrently collected using the trace metal clean pumping system. Total dissolved Fe concentrations (DFe, $< 0.2 \mu\text{m}$) were determined on samples using a combination of the solvent extraction techniques described by *Danielsson et al.* [1978] and *Bruland et al.* [1979]. Briefly, 250 g of seawater was buffered to a pH of 4.5 with purified ammonium acetate

buffer in a 250-mL Teflon separation funnel. Purified ammonium pyrrolidine-dithiocarbamate (APDC)/sodium diethyl-dithiocarbamate (DDC) solution was then added followed by 5 mL of cleaned chloroform. The sample was extracted by shaking for 3 min. After allowing the phases to separate, chloroform was drained and the sample extracted a second time with 5 mL of chloroform. Following extraction, chloroform primary and secondary extracts were pooled, acidified with 75 mL of Teflon-distilled HNO₃ (t-HNO₃), shaken, and left to react for 1 hour. Metals back-extracted from the chloroform phase were diluted to 1 mL with Nanopure water, shaken, and left overnight before analysis by graphite furnace atomic adsorption spectrometry using a Perkin-Elmer 4100 ZL with Zeeman background correction.

[12] As a proxy for phytoplankton biomass in the water column total chlorophyll *a* (chl *a*) was determined from duplicate 50-mL samples collected on 0.2- μm pore size polycarbonate filters (Osmonics) after extraction (~ 24 hours) in 90% acetone. Chl *a* was quantified with a Turner Designs TD-700 fluorometer using the non-acidification protocol of *Welschmeyer* [1994].

2.5. Determination of in Situ Fe Bioavailability to the Bacterial Bioreporter

[13] Preacclimated *P. putida* FeLux bioreporter cells (BESAW medium, pFe 21.2) were used to estimate bioavailable Fe in the seawater samples obtained during FeCycle. The following Chelex-100 treated (except vitamins) and filter-sterilized nutrient stocks were added to filtered (0.2- μm) seawater samples to ensure that the level of all nutrients except Fe were sufficient to support the growth of the bioreporters: 20 μM NH₄NO₃, 10 μM NaH₂PO₄, 1 μM glycerol, and 100 μL vitamin mix (ESAW vitamins recipe [Berges *et al.*, 2001]). Enriched seawater (18 mL) was dispensed into Oak-ridge tubes. Triplicate samples were either unaltered (control treatment) or supplemented with FeCl₃ (1.5 nM final concentration). Amendment of the seawater samples was performed at least 24 hours before the addition of preconditioned bioreporter cells. At time zero, 2 mL of pre-acclimated *P. putida* FeLux bioreporter cells were added to each replicate. OD₆₀₀ and light production were measured at 2-hour intervals over a period of 12 hours using a spectrophotometer (Biomate 5, ThermoSpectronic corp.) and a luminometer (FB-15, Zylux corp.). At each time point, light production was normalized to OD₆₀₀ and then converted to light production per reporter cell. Bioavailable Fe levels (estimated as Fe(III)', which equals the sum of the inorganic Fe species) were predicted from estimated cellular luminescence using the calibration curve established from BESAW medium treatments with known pFe. Calibration treatments were run in parallel to seawater treatments for each experiment.

2.6. Statistical Analyses

[14] Statistical analyses for all data were performed using SPSS (version 12) software. Analyses of variance and independent *t*-tests (two-tailed) were performed assuming equal variance on mean values. The homogeneity of variance was tested in each analysis using the Levene test. For

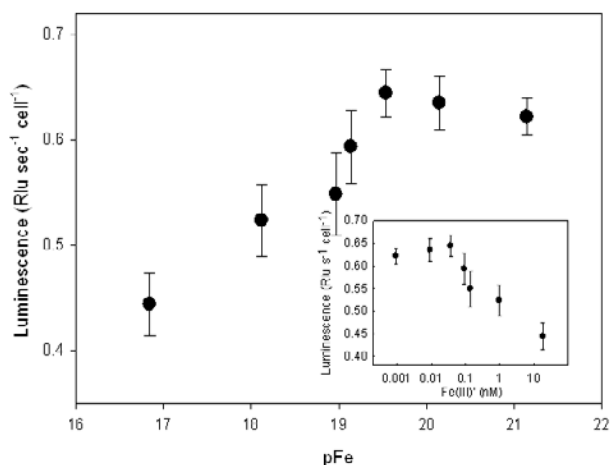


Figure 2. Dose-response characterization of the Fe bioreporter *P. putida* FeLux in the trace metal buffered BESAW medium. Cells preconditioned in Fe-deficient BESAW ($\text{Fe}^{3+} = 10^{-21.2} \text{ M}$) were inoculated in BESAW medium at various free ferric iron concentrations ($\text{Fe}^{3+} = 10^{-21.2} \text{ M}$, $10^{-20.2} \text{ M}$, $10^{-19.5} \text{ M}$, $10^{-19.1} \text{ M}$, $10^{-19.0} \text{ M}$, $10^{-18.1} \text{ M}$, $10^{-16.8} \text{ M}$). Bioluminescence was measured following 12 hours of incubation and normalized to the number of bioreporter cells mL^{-1} . Values reported represent the average value obtained for three experiments performed in identical conditions. Error bars represent the standard deviations of three experiments with triplicate samples analyzed in each experiment ($n = 9$). Regression analysis of the linear range pFe 16.8 ($\text{Fe}^{3+} = 10^{-16.8} \text{ M}$) to pFe 19.6 ($\text{Fe}^{3+} = 10^{-19.6} \text{ M}$) is shown ($R^2 = 0.935$; slope = 0.070 and y intercept = -0.684).

incubation experiments, analysis of variance (one-way ANOVA) was used to establish the statistical significances of variation among different treatments. In parallel, multiple comparison tests were performed to determine where the differences lay. Dunnett's test was used to analyze the significance of the variations of the means of a set of amended treatments relative to the control treatment mean [Corston and Colman, 2003]. The Tukey's honestly significant difference test (Tukey-HSD test) was used to establish the statistical significance of variations among a set of treatments means [Corston and Colman, 2003]. For all analyses, a 95% confidence interval was used. Results were considered significant at $p < 0.05$ unless otherwise stated.

3. Results

3.1. Laboratory Characterization

[15] To characterize the response of the bioreporter to Fe bioavailability, preconditioned *P. putida* FeLux cells were incubated for 12 hours in the trace-metal buffered synthetic seawater medium BESAW supplemented with various concentrations of Fe to obtain a calculated range of pFe 21.2 (an $\text{Fe(III)} = 0.9 \text{ pM}$) to pFe 16.8 (an $\text{Fe(III)} = 180 \text{ nM}$) (Figure 2). The Fe-chelating agent EDTA was used to buffer Fe^{3+} concentrations in BESAW culture medium. It

has previously been demonstrated that unlike other common xenobiotic ligands such as nitriloacetic acid (NTA), EDTA does not promote intracellular Fe incorporation in *Pseudomonas* species [Meyer and Hohnabel, 1992]. The use of this Fe chelating-agent ensured therefore that the bioluminescence signal could be related to the bioavailable Fe in the environment of the bioreporter cells. For the purpose of comparison between systems, the pFe of our model medium was used as metric to gauge Fe chemistry. Similar calculations (e.g., the sum of the inorganic Fe species, Fe^{I}) can also be made for the defined medium. Bioluminescence increased linearly between treatment pFe 16.8 and pFe 19.5, and reached a maximum for lower total Fe concentrations. All environmental samples tested were within the linear range of the calibration curve. A linear regression performed using the regression curve fitting of the Sigma Plot software (version 9.0, SPSS corporation) in the linear region of this calibration curve provided the following equation ($R^2 = 0.935$):

$$y = 0.0701x - 0.684, \quad (2)$$

where, y = luminescence per second per cell, and x = pFe.

3.2. Background Conditions at the FeCycle Study Site

3.2.1. Dissolved Fe Concentrations in the Patch

[16] The FeCycle site had a surface mixed layer of $\sim 40\text{--}50 \text{ m}$ [Ellwood, 2004] characteristic of Austral summer thermal stratification in this region [Hutchins et al., 2001b; Boyd et al., 2001]. Concentrations of DFe at the patch center by the graphite furnace technique were low ($< 0.075 \text{ nM}$) for samples from surface waters throughout the study period (Figure 3). The initial vertical profile (5 February 2003) showed a minimum of 0.043 nM Fe at a depth of 32 m . Independent estimates of DFe concentrations from flow injection analysis provided different results and are discussed elsewhere (P. L. Croot et al., The effects of physical forcing on iron chemistry and speciation during the FeCycle experiment in the south West Pacific, submitted to *Global Biogeochemical Cycles*, 2005) (here-

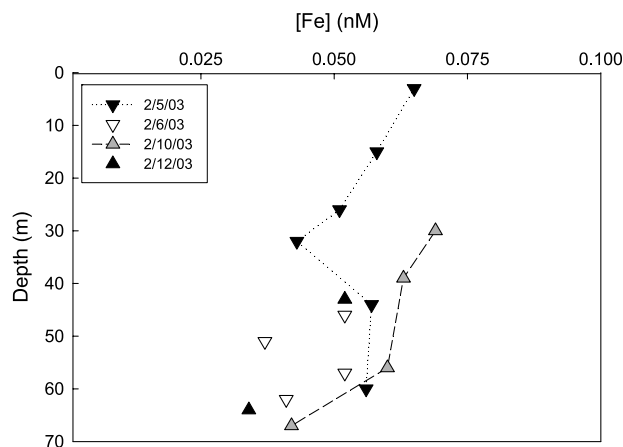


Figure 3. Depth profiles of total dissolved Fe ($< 0.2 \mu\text{m}$, nM) concentrations determined at four stations located at the center of the SF_6 -labeled patch.

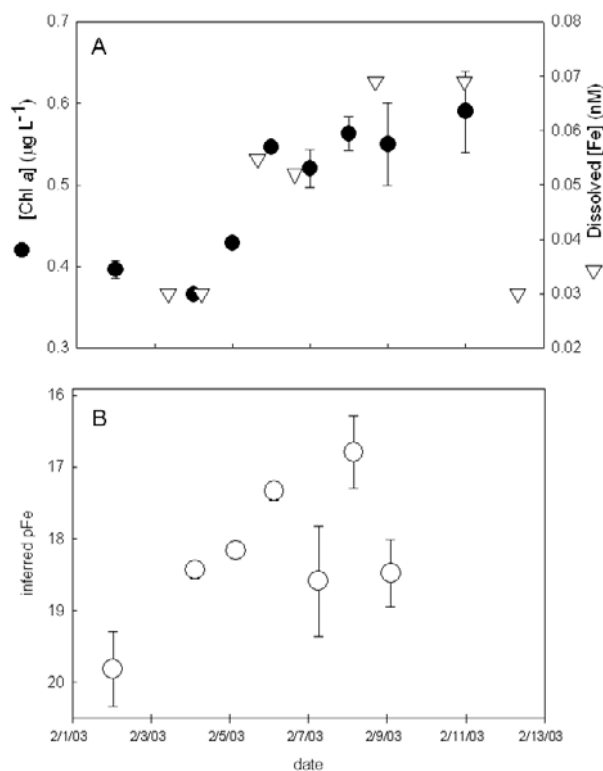


Figure 4. Comparison of changes in dissolved Fe concentration, Chl *a* and Fe bioavailability within the mixed layer of SF₆-labeled waters. (a) Dissolved Fe (open triangles, nM) and Chl *a* (solid circles, µg L⁻¹) concentrations within the FeCycle patch. (b) Bioavailable Fe level (estimated as pFe = $-\log[\text{Fe}^{3+}]$) predicted from *P. putida* bioreporter cells luminescence analyses (open circles). Cells preconditioned in Fe-deficient BESAW medium were used to inoculate seawater samples. Luminescence values normalized by cell density (cell mL⁻¹) were used to estimate the corresponding pFe based on the calibration curve obtained in the trace metal buffered BESAW medium. Error bars represent the standard deviations between replicates ($n = 3$).

inafter referred to as Croot et al., submitted manuscript, 2005).

[17] Within the mixed layer of the SF₆-labeled patch, initial DFe concentrations were very low (0.030 nM; Figure 4a). The DFe concentration doubled from 4 to 8 February, and reached a maximum of 0.069. It remained at that value until 10 February and then decreased to 0.03 nM on 12 February. These fluctuations in DFe concentrations are hypothesized to be driven by the entrainment of surrounding HNLC waters which different Fe concentrations (Croot et al., submitted manuscript, 2005) and chl *a* [Boyd et al., 2005]. In part this entrainment resulted from the increase in the areal extent of the patch (from 70 to >400 km²) over 10 days (Croot et al., submitted manuscript, 2005). The end results of these fluctuations in DFe concentrations provided a “natural laboratory” in which to rigorously test the Fe bioreporter.

3.2.2. Total Chlorophyll *a*

[18] Low DFe concentrations coincided with low initial chl *a* concentrations (<0.40 µg L⁻¹) in the mixed layer during FeCycle (Figure 4a). No change in chl *a* was observed during the two first days of the study. Total community chl *a* concentration increases (~60%) were concomitant with the increase in DFe observed between 4 and 6 February. These concentrations remained around 0.55 ± 0.02 µg L⁻¹ for the rest of the study period, suggesting that the increase in dissolved Fe concentration (~0.040 nM) may have been due to the entrainment of surrounding waters containing higher DFe and chlorophyll concentrations or that entrained DFe might have stimulated a modest increase in total chl *a*.

3.3. Assessment of Fe Bioavailability Over the Course of the FeCycle Voyage

[19] Relative bioavailable Fe levels (estimated from the response of the bioreporter in a standard, pFe defined medium) in the dissolved fraction (<0.2 µm) of surface seawater predicted from the *P. putida* FeLux bioluminescent reporter are represented on Figure 4b. Initial bioavailable Fe (2 February 2004) was lower than in the Fe-replete pFe 16.8 medium as bioreporters produced light levels equivalent to the highest concentrations of Fe on the calibration curve, suggesting that high-affinity Fe transport systems of *P. putida* FeLux cells were fully repressed. On the basis of bioluminescent reporter analyses, the evolution of Fe bioavailability over the study period can be divided in two main phases. Between 2 and 6 February, as dissolved Fe in surface water increased, inferred pFe decreased significantly ($p < 0.05$) and in a log linear fashion ($R^2 = 0.99$), dropping to a minimal pFe value of $\sim 17.4 \pm 0.9$. This observation suggests that the transient increases in dissolved Fe in the surface water were sufficient to be sensed (vis a vis altering the intracellular Fe status) by *P. putida* FeLux and to allow for the repression of high-affinity Fe transports. Dissolved Fe concentrations in surface waters were ~ 0.06 nM from 6 to 8 February (Figure 4a). Analysis of this water with the bioreporter provided results comparable to pFe 16.8–18.5 in our model medium (Figure 4b), suggesting that sufficient Fe was available for growth. On 9 February, Fe bioavailability decreased to the estimated levels of 4 February, indicative of a slight decrease in DFe, (although DFe concentrations were not determined at this time point). With the exception of the 7 February samples, the results suggest a strong correlation between the total DFe pool and the bioavailable Fe pool.

[20] Addition of 1.5 nM of Fe to seawater sampled when Fe concentrations peaked (~ 0.069 nM) within the patch resulted in significantly lower luminescence (t -test, $p < 0.05$) which is characteristic of Fe-replete bacterial cells (Figure 5). This result suggests that although bioreporter cells sensed an increase in bioavailable Fe in the surface seawater, their high-affinity transport systems were never completely repressed. The decrease in luminescence in Fe-amended relative to unamended treatments was of comparable magnitude for the three samples examined, averaging 0.19 ± 0.02 relative light units s⁻¹ cell⁻¹. Not only does this data demonstrate the reproducibility of the

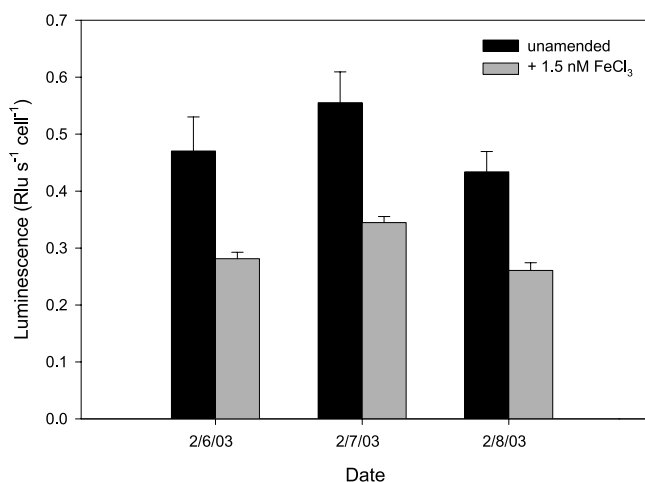


Figure 5. Influence of added FeCl_3 on perceived Fe bioavailability. Luminescence measures from bioreporter cells grown in $0.2\text{-}\mu\text{m}$ filtered surface water and Fe (1.5 nM) amended $0.2\text{-}\mu\text{m}$ filtered surface water from the center of the FeCycle patch for 6 through 8 February. Although Fe concentrations and Fe bioavailability peaked during this period, the results indicate that high-affinity transporters could still be further suppressed by added Fe, and as such the Fe availability was still in part constraining the community.

response of the bioreporter cells, it also demonstrates that the dynamic range of bioreporter response is well within the natural variation in Fe concentrations that can be observed in HNLC systems.

4. Discussion

4.1. Information Provided by the Fe-Dependent Heterotrophic Bioreporter

[21] This article is the first to describe the use of a prokaryotic Fe-specific bioluminescent reporter in seawater to directly estimate Fe bioavailability to heterotrophic bacteria. The use of *P. putida* FeLux strain as a diagnostic tool of Fe bioavailability presents several advantages. First, the regulation of Fe metabolism in response to Fe availability via the ferric-uptake regulator protein (FUR) is widespread among marine, freshwater, terrestrial, commensal and pathogenic heterotrophic bacterial strains [Escobar *et al.*, 1999; Andrews *et al.*, 2003]. Indeed, for pathogenic bacteria one of the most difficult parts of infecting a new host is scavenging Fe, which they accomplish using mechanisms similar to the one bacteria employ in seawater [Wilhelm, 1995; Weinberg, 1998]. And while we have no doubt that there are other potential Fe transport systems in marine environments, the FUR system is indeed among the most prominent; a BLAST analysis of the Sargasso Sea shotgun library [Venter *et al.*, 2004] demonstrates more than 100 quality hits to the FUR protein of *E. coli* (data not shown), implying it is well distributed within this marine system. As such, this transport system is representative of many Fe transport systems in the ocean. Moreover, while

specific bacteria may assimilate Fe from different Fe-organic pools and respond to Fe stress by producing different siderophores [Wilhelm, 1995], the regulation of this process within the majority of well-characterized microbial cells is by the FUR system, and as such conserved. Therefore bioreporters based on the FUR-regulated *fepA-fes* promoter fusion are an appropriate tool to estimate the Fe-status of many members of the heterotrophic bacterial component in environments such as seawater.

[22] Second, owing to the halotolerance of *P. putida* FeLux, this strain can be used to detect Fe bioavailability in a wide range of environments (e.g., along a salinity gradient). Previously, this bioreporter had been employed to determine Fe availability in a freshwater system (Lake Erie) and to elucidate the impact of various Fe chelators in freshwater culture medium [Mioni *et al.*, 2003]. In this study, we have demonstrated the applicability of this bioreporter to open ocean HNLC waters where heterotrophic bacteria play a dominant role in both Fe uptake and in Fe regeneration via virus-mediated lysis and/or grazing of bacterial cells [Strzepek *et al.*, 2005]. Using this bioreporter strain and an incubation time of 12 hours in a synthetic seawater medium, the bioluminescent reporter cells responded to increasing bioavailable Fe in a dose dependent manner within the range of pFe values of 19.5 to 16.8. Although these values may appear relatively high, results from water samples collected within the FeCycle patch fell within the linear portion of our calibration curve, allowing us to estimate corresponding bioavailable Fe levels. Furthermore, the results indicate that our bacterial reporter was sensitive enough to detect subnanomolar changes in bioavailable Fe in this environment. Effectively, a transient increase of $\sim 0.040\text{ nM}$ Fe was sufficient to increase Fe bioavailability to the *P. putida* FeLux reporter strain.

[23] The data also suggest that this strain can provide insight regarding the relationship between bioavailable Fe and total DFe concentrations. During the transient increase in total dissolved Fe (2–6 February), if the character of the Fe-organic complexes also changed (e.g., as new organically complexed Fe was entrained), they were at least partially available to *P. putida* FeLux since inferred bioavailable Fe levels tracked the changes in DFe concentrations within the SF6 labeled patch. Although, using a single strain of bacteria should be cautiously extrapolated to the natural populations (as Fe requirements and uptake of alternate substrates vary between species), the data suggest that the transient increase in dissolved Fe may have been sufficient to stimulate production by the prokaryotic component of the microbial community. It is important to note that the calibrations values are valid against the standard medium only, and not predictions of Fe speciation (e.g., pFe, Fe(III)). The importance of these values is that the response at these concentrations is highly reproducible, and as such they can serve as a relative comparison at different locations and across different times.

4.2. Comparing the Response of Bacteria and Algae to Changes in DFe During FeCycle

[24] In contrast to our indicator of the heterotrophic bacterial Fe-status, the response of the resident phytoplank-

ton assemblage to fluctuations in dissolved Fe concentrations during FeCycle was not evident. Although chl *a* concentrations increased over time in the patch (potentially due to entrainment of phytoplankton or in response to the elevated Fe concentration), photosynthetic efficiencies remained low ($F_v/F_m \sim 0.2$; [McKay *et al.*, 2005]). Moreover, the Ferredoxin Index (the ferredoxin to flavodoxin ratio in plankton) ranged from 0.07 to 0.25 over the course of FeCycle, again suggesting that a strong physiological Fe-limitation (typically denoted by ratios <0.5 [Strzepek and Harrison, 2004]) prevailed in the resident phytoplankton community [McKay *et al.*, 2005]. Several scenarios could explain this disparity between the responses of photosynthetic plankton community and the heterotrophic reporter to the transient Fe increase: (1) Fe from entrained waters was rapidly complexed by prokaryotic siderophores, making it unavailable to phytoplankton but potentially available to the bioreporter; (2) the speciation of the entrained Fe may have been more bioavailable to the bacterioplankton than phytoplankton, resulting in a stronger physiological limitation of the photosynthetic assemblage relative to the heterotrophic bacterium; (3) the entrained water may have introduced a different plankton population that biologically altered the system's Fe chemistry; and (4) the different approaches employed here to assess Fe stress may yield to differing sensitivities. Indeed, the algal photosynthetic competence (F_v/F_m) and Ferredoxin ratio approaches may not be as sensitive to increases in Fe bioavailability. Although we are unable to resolve the differences observed between the heterotrophic bioreporter and the proxies for phytoplankton Fe-status [see McKay *et al.*, 2005], our bioreporter data demonstrate an increase in Fe bioavailability concurrent to a subnanomolar increase in dissolved Fe concentrations.

[25] One parameter not considered in the above information is the potential for Fe to have been shunted from the particulate Fe pool to the dissolved Fe pool during our observations. The abundant cyanobacterial and heterotrophic prokaryote populations in the FeCycle patch [Strzepek *et al.*, 2005] represented a significant pool of particulate Fe. Recent studies suggest that the virus-mediated lysis of prokaryotes can lead to a release of significant organically complexed Fe [Wilhelm and Suttle, 1999; Poorvin *et al.*, 2004]. Indeed, when taken in light of the companion studies and previous work cited above, our results suggest that Fe-ligand complexes present in the surface waters of the patch (derived from viral-mediated lysis, grazing byproducts and/or siderophore production) were available to our bioreporter but may not have been available to eukaryotic phytoplankton.

4.3. Significance at the Ecosystem Level

[26] The current tenets of oceanography assume that picoplankton dominate HNLC areas because they are less physiologically limited by Fe availability than large phytoplankton species such as diatoms [Sunda and Huntsman, 1997; Price *et al.*, 1994] or, in many cases, because they maintain high-affinity transport systems [Wilhelm and Trick, 1994]. However, diagnostic probes/indices currently available have so far failed to confirm this hypothesis and instead

have suggested that the resident picoplankton-dominated community is also Fe-limited [Greene *et al.*, 1994]. The majority of diagnostic markers for environmentally imposed stress are generally optimized for, or specific to, the photosynthetic and mostly eukaryotic component of HNLC assemblages [Beardall *et al.*, 2001; Firme *et al.*, 2003]. At this time, no other diagnostic marker specific to heterotrophic bacteria is available. The heterotrophic bioluminescent reporter represents the first diagnostic tool specifically optimized to estimate the degree of Fe limitation of a component of the marine heterotrophic community.

[27] If we cautiously assume the sensitivity of the indigenous heterotrophic bacteria in HNLC waters is represented by the bioreporter, then we can extrapolate and conclude that the heterotrophic bacterial community responded differently than the phytoplankton community during the subtle shift in DFe supply that occurred during FeCycle (i.e., the Fe was more bioavailable to the bacteria than to the phytoplankton). The data presented here further suggest that the heterotrophic bioreporter is differentially sensitive (relative to the photosynthetic plankton) to natural variations in Fe bioavailability. Although the heterotrophic bioreporter and the phytoplankton (as diagnosed by both F_v/F_m and the ferredoxin diagnostic index [McKay *et al.*, 2005]) were Fe-limited at the beginning of the survey, only the bioreporter bacterial cells demonstrated a relaxation of their Fe-limited state during the transient elevation in Fe availability. Moreover, our bioluminescent reporter suggested that 1.5-nM Fe amendments of the seawater samples completely alleviated Fe-limitation in the heterotrophic bacteria, while comparable Fe amendments to on-deck incubation experiments, while resulting in enhanced chl *a* production, were not sufficient to completely alleviate nutrient stress of resident phytoplankton cells (as reflected by F_v/F_m values below the theoretical maximum of ~ 0.65 [McKay *et al.*, 2005]).

5. Conclusions

[28] This work has explored the use of bioreporters as a novel and sensitive biotechnology tool to estimate the bioavailability of Fe in marine environments; indeed, experiments concerning microbial Fe assimilation and bioavailability have been suggested as critical future needs by recent international working groups [SOLAS, 2005]. At this time, no other diagnostic marker of Fe availability specific to the heterotrophic bacterial community is available. By employing this tool we have generated data that suggest that small changes in total dissolved Fe concentrations, at the limit of chemical detection, could result in significant changes to the bioavailable Fe pool and thereby induce important changes in the microbial food web. Although such small-scale experiments need to be interpreted with caution, and constructus need to be introduced into more marine "relevant" bacteria, these results have important implications for the study of the biogeochemical cycling of Fe as they demonstrate that heterotrophic bacteria are key players in the "microbial ferrous wheel" [Kirchman, 1996].

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