

## Simultaneous determination of iron reduction and uptake by phytoplankton

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### Abstract

We have developed a new method to assess simultaneously the rates of biological reduction of Fe(III)-ligand complexes at nanomolar concentrations and iron uptake in marine phytoplankton. Other methods for measuring iron reduction require micromolar iron concentrations, which can saturate iron uptake in these organisms. In the present study, high sensitivity was obtained by combining radiometric techniques, ligand competition, and selective retention on solid phase extraction columns (C<sub>18</sub> Sep-Pak®). Following the addition of the Fe(II) specific ligand ferrozine (FZ) to seawater spiked with nanomolar concentrations of radiolabeled iron, the rate of iron uptake is determined by filtering aliquots of cultures, and the rate of Fe(II) binding to FZ by passing the filtrate through a column, which retains the Fe(II)FZ<sub>3</sub> complex. The method was calibrated using <sup>55</sup>Fe(II) standards in NaCl solution, yielding high recovery efficiency and linear behavior across the entire range tested from 3 × 10<sup>-11</sup> M to 2 × 10<sup>-6</sup> M Fe(II). Special attention was given to possible artifacts caused by reduction of Fe(III) in the presence of FZ and Fe(III) adsorption onto the column, which are critical when measuring picomolar concentrations of Fe(II). The method was successfully applied to *Thalassiosira weissflogii* cultures and to natural phytoplankton populations in the Bering Sea using ethylenediaminetetraacetic acid as a model ligand.

In the two decades since John Martin first proposed his iron hypothesis, various aspects of iron-phytoplankton interactions have been intensively studied from molecular mechanisms to global ocean-atmosphere interactions. Nonetheless, the fundamental mechanisms and regulation of iron acquisition by phytoplankton are currently the subject of debate and await new methodology. In this paper we present a method for simultaneous determination of iron uptake and reduction by microorganisms at nanomolar iron concentrations. This method is applicable to both laboratory cultures and natural populations and might help investigating the intriguing question of iron acquisition from organic complexes and colloids.

Laboratory data show that the rate of iron uptake by eukaryotic phytoplankton is dependent upon the concentration of Fe' (the sum of dissolved inorganic iron species)

(Anderson and Morel 1982; Hudson and Morel 1990; Sunda and Huntsman 1995). Based on kinetic considerations, other abundant iron species like iron(III) chelates and colloids are not thought to be directly available for uptake (Hudson and Morel 1993). Nevertheless most of the iron in seawater is bound to strong organic ligands that buffer remarkably low unchelated iron (Fe') concentrations (Rue and Bruland 1995; van Den Berg 1995; Wu and Luther 1995). Eukaryotes, however, do acquire adequate amounts of iron to allow them to grow reasonably fast in systems where calculated concentrations of Fe' are exceedingly low (Timmermans et al. 2001; Liu et al. 2002). There are a few indications from iron limited regions of the ocean that eukaryotic phytoplankton are able to acquire iron bound to strong organic complexes (Hutchins et al. 1999; Maldonado and Price 1999; Bowie et al. 2001). Whereas many prokaryotes possess siderophore-specific cell surface receptors capable of transport of Fe(III)-ligand complexes, it appears that diatoms such as *Thalassiosira oceanica* acquire organically bound Fe through a cell-surface reductase that reductively dissociates the iron from the organic ligand (Maldonado and Price 2000, 2001).

A number of spectrophotometry-based and chemiluminescence-based analytical methods have been developed to determine Fe(II) in natural waters (Elrod et al. 1991; King et al. 1991; O'Sullivan et al. 1991; King et al. 1995; O'Sullivan et al.

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1995; Croot and Laan 2002). The spectrophotometric methods use specific Fe(II) ligands such as FZ (ferrozine, 3-[2-pyridyl]-5,6-bis[4-phenylsulphonic acid]-1,2,4-triazine) or bathophenanthroline disulphonic acid that form Fe(II) complexes with characteristic absorption wavelengths (Blair and Diehl 1961; Stookey 1970). Both the spectrophotometry-based and the chemiluminescence-based methods are not sensitive enough to determine the rates of algal-mediated Fe(II) production in natural waters or in algal cultures under the low iron concentrations required for Fe limitation. Therefore, biological Fe reduction experiments are conducted at micromolar concentrations of Fe(III)L (Anderson and Morel 1980; Jones et al. 1987; Maldonado and Price 1996, 1999; Wegar 1999; Wegar and Espie 2000; Shaked et al. 2002). The results of these experiments must then be extrapolated back through three orders of magnitude because the iron concentration in the open ocean and the half saturation constant for uptake in laboratory cultures lie in the nanomolar range (Johnson et al. 1997).

In this paper we present a radiometric method that allows sub-nanomolar Fe(II) determination in phytoplankton cultures or natural waters. This method takes advantage of the simplicity and sensitivity of radiometric assay and combines it with the selective adsorption of the Fe(II)FZ<sub>3</sub> complex onto the C<sub>18</sub> column. This combination enables simultaneous determination of rates of phytoplankton-induced Fe(III) reduction and Fe uptake at low Fe(III)L concentrations.

## Materials and procedures

**Phytoplankton culturing and analytical reagents**—Cultures of *Thalassiosira weissflogii* (clone Actin) were grown at 20°C with the synthetic medium Aquil (Price et al. 1988/89), under continuous light at 150 μmol quanta m<sup>-2</sup> s<sup>-1</sup> supplied by VHO fluorescent tubes. The culture medium contained 10, 100, and 100 μM phosphate, nitrate, and silicate, respectively. Trace metals (Cu, Mn, Zn, and Co) were buffered with 100 μM ethylenediaminetetraacetic acid (EDTA) to approximate the free ion activities found in seawater. Total iron concentrations were varied to achieve iron limited growth (60 to 90 nM Fe) or iron-replete growth (280 nM Fe). Cells were counted using a Multisizer II Coulter Counter, and specific growth rates were then determined from the linear regressions of the natural log of cell density versus time. In most laboratory experiments conducted during the method evaluation high cell density (2 × 10<sup>5</sup> cell mL<sup>-1</sup>) was used to produce a strong signal. In later experiments cell density was reduced to 1 to 3 × 10<sup>4</sup> cells mL<sup>-1</sup>, in which minor pH shifts occurs and the uptake and reduction kinetics are slow enough to maintain the integrity of the EDTA buffer system. All solutions were prepared with double-distilled water (Milli-Q, Millipore, 18.2 mΩ) distilled acids, and analytical or higher-grade salts. Nutrients stocks, Aquil medium, and FZ were cleaned using Chelex 100 resin (Price et al. 1988/89). Sep-Pak cartridges packed with C<sub>18</sub> resin were obtained from Waters Association and preconditioned according to King et al. (1991). <sup>59</sup>FeCl<sub>3</sub> (specific activity 1.1 GBq mg<sup>-1</sup>)

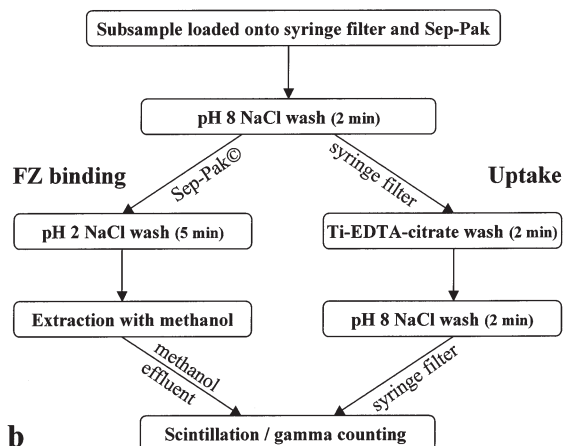
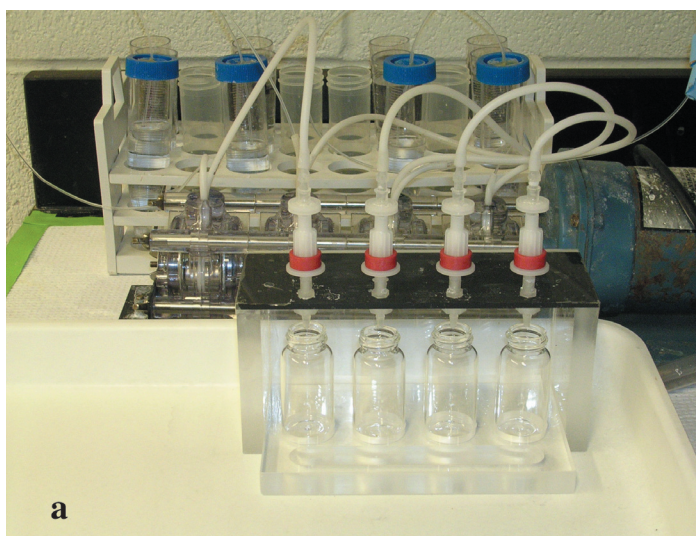
and <sup>55</sup>FeCl<sub>3</sub> (specific activity 1.2 GBq mg<sup>-1</sup>) were obtained from Perkin-Elmer Life Sciences. The two radioisotopes were tested to expand the applications of this method, where <sup>59</sup>Fe provides a lower detection limit as a result of low quenching. A stock solution of <sup>55</sup>Fe(II) was prepared from the <sup>55</sup>FeCl<sub>3</sub> solution by reducing the Fe(III) with ascorbic acid (1 mM). The concentration of <sup>55</sup>Fe(II) stock solution was checked with the FZ method (Stookey 1970). High-performance liquid chromatography-grade methanol (J.T. Baker) was used for Sep-Pak cleaning, and methanol for spectroscopy (Merck) was used for sample elution and spectrophotometric measurements.

**Theoretical background, terminology, and experimental conditions**—The method is based on the addition of the specific Fe(II) binding ligand, FZ, to seawater with radiolabeled Fe(III) ligand. The rate of iron uptake is determined simultaneously with the rate of Fe(II) binding to FZ, measured by the retention of the Fe(II)FZ<sub>3</sub> complex on C<sub>18</sub> Sep-Pak columns. Once reduced, Fe is complexed by FZ according to the rate law:

$$\text{FZ binding rate} = k_f \times \text{Fe(II)} \times [\text{FZ}]^3 \quad (1)$$

where the rate constant  $k_f$  can be estimated as  $2 \times 10^{11} \text{ M}^{-3} \text{ s}^{-1}$  on the basis of a kinetic study by Lin and Kester (1992). This study shows that 100 to 200 μM FZ efficiently out-competes oxygen for Fe(II) complexation at pH = 8.2 seawater, and hence Fe(II) oxidation is negligible in our laboratory experiments. In the field, Fe(II) oxidation rates could be accelerated by H<sub>2</sub>O<sub>2</sub>, which is found at concentrations up to 10<sup>-7</sup> M (Yuan and Shiller 2001). Nonetheless, for seawater with pH = 8.2 at 25°C, the effective first-order oxidation rate constants— $k_{\text{O}_2} = 2 \times 10^{-3} \text{ s}^{-1}$  (for saturating oxygen) and  $k_{\text{H}_2\text{O}_2} = 0.01 \text{ s}^{-1}$  (for 10<sup>-7</sup> H<sub>2</sub>O<sub>2</sub>)—are smaller than those for FZ binding,  $k_f \times [\text{FZ}]^3 = 0.2 \text{ s}^{-1}$  and 1.6 s<sup>-1</sup> for 100 and 200 μM FZ, respectively (Millero et al. 1987; Millero and Sotolongo 1989; Lin and Kester 1992). Lower temperatures, found in most of the world's oceans, slow down significantly the rates of Fe(II) oxidation by H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub> (Millero et al. 1987; Millero and Sotolongo 1989), whereas FZ complexation rates are only slightly slower (Lin and Kester 1992). At high FZ concentrations (400 to 1500 μM), the kinetics of FZ binding do not follow the above equation and its rate has no physiological significance (see "Effect of FZ concentration on iron reduction" in *Assessment*).

FZ addition has no adverse effect on the photosynthetic capacity of the cells, as observed by measurements of variable to maximum fluorescence (Fv/Fm) using the PSII inhibitor DCMU (dichlorophenyl dimethyl urea; Geider et al. 1993). Experiments were conducted in the dark to avoid FeEDTA photo-reduction in pre-equilibrated (at least 24 h) <sup>59</sup>Fe or <sup>55</sup>Fe labeled Aquil (Price et al. 1988/89). Total iron concentrations used in the laboratory experiments were in the range 90 to 115 nM, which is known to induce iron limitation of *T. weissflogii* in Aquil. Exponentially growing laboratory cultures were harvested by gentle filtration onto acid-cleaned 5 μm polycarbonate (MSI Poretics) filters and resuspended in the pre-equilibrated



**Fig. 1.** Experimental setup and procedure. (a) Major features of the system—Sample vials, tubing, peristaltic pump, syringe filters, and Sep-Pak cartridges. (b) Schematic illustration of the experimental procedure.

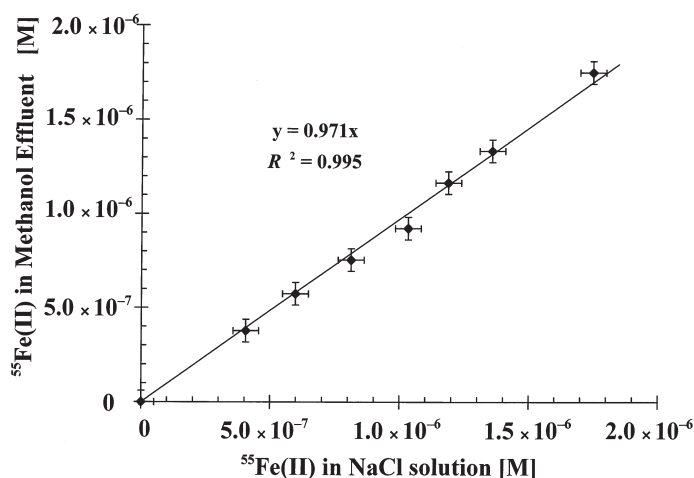
experimental medium at a density of  $3 \times 10^4$  to  $3 \times 10^5$  cells  $\text{mL}^{-1}$ . The sample was then split into individual treatments, each receiving a different FZ concentration. In the Bering Sea experiments, 20 nM Fe pre-equilibrated with 100  $\mu\text{M}$  EDTA was used. The Bering Sea experiments were run in the dark under ambient temperatures of  $10^\circ\text{C}$  to  $12^\circ\text{C}$ ,  $\text{pH} = 8.0$ , and saturating levels of oxygen. Field populations were gently filtered onto 5  $\mu\text{m}$  membrane (which was replaced frequently to prevent clogging and speed the filtration) and re-suspended in filtered seawater. This was done under trace metal clean conditions, inside a Class 100 Hood. Ten to 20 L of ambient seawater were collected in a 20-L fluorinated HDPE carboy (previously acid cleaned, rinsed, and conditioned with high nutrient low chlorophyll [HNLC] seawater). The carboy spigot was attached to an in-line Teflon (Saville Corp.) filter holder, using a pre-cleaned 5  $\mu\text{m}$  47 mm polycarbonate filter. A subsample was removed and processed for flow cytometry-based

measurements of cell size distribution, particle surface area, and an estimate of bacterial biomass.

**Experimental setup**—The experimental setup for the combined uptake and reduction experiments is shown in Fig. 1a and is schematically illustrated in Fig. 1b. Trace metal clean protocols were applied to all procedures to prevent contamination with cold iron. Four sampling positions were installed on a four-head peristaltic pump to allow simultaneous processing of treatments. Each position was equipped with a pre-wetted 0.2  $\mu\text{m}$  syringe filter (Acrodisc<sup>®</sup> HT Tuffryn<sup>®</sup> membrane) for uptake measurements, followed by a Sep-Pak column for reduction measurements. At chosen time intervals, gravimetrically quantified aliquots of the radiolabeled experimental medium (20 to 30 mL cultures and 40 to 50 mL for field samples) were loaded at a flow rate of  $5 \text{ mL min}^{-1}$ . Then, the filters and columns were washed for 2 min with a 0.5 M solution of NaCl buffered with 0.005 M  $\text{NaHCO}_3^-$  (pH 8 NaCl hereafter) to remove the dead volume of radiolabeled medium from the filter and the columns. The filters were removed, placed on another peristaltic pump, and washed for 2 min with Ti-EDTA-citrate solution to remove adsorbed extracellular Fe (Hudson and Morel 1989). The filters were washed again for 2 min with pH 8 NaCl, followed by a rinse of the filter housing before  $\gamma$  counting the syringe filter. Meanwhile, the Sep-Pak columns, left in their original position, were washed with a 0.5 M solution of NaCl buffered with 0.005 N HCl (pH 3 NaCl hereafter) for 5 min at  $5 \text{ mL min}^{-1}$  to remove any adsorbed Fe(III) species from the column. After purging the retention volume of pH 3 NaCl from the Sep-Pak, the Fe(II)FZ<sub>3</sub> complex was eluted from the column by methanol pumped at a flow rate of three  $\text{mL min}^{-1}$ . For experiments using  $^{59}\text{Fe}$ , 20 mL of methanol effluent was collected in vials and counted in 1480 Wizard<sup>™</sup> 3"  $\gamma$  counter.

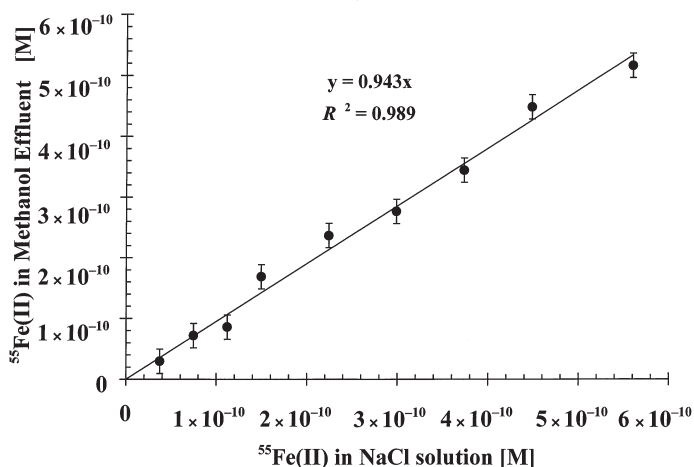
### Assessment

**Fe(II) recovery and method calibration**—The efficiency of Fe(II) extraction by the Sep-Pak cartridges was first determined using relatively high  $^{55}\text{Fe(II)}$  concentrations (0.4 to 1.8  $\mu\text{M}$ ), so that the concentrations of  $^{55}\text{Fe(II)}$  in the standards and in the methanol extract could be concurrently measured by spectrophotometry using the standard FZ method (Stookey 1970; Fig. 2). A series of  $^{55}\text{Fe(II)}$  standards in NaCl solutions (0.6 M) containing 40  $\mu\text{M}$  FZ at  $25^\circ\text{C}$  were loaded onto Sep-Pak cartridges and were subjected to the same wash and elution procedures used for the cells. The calibration curve between the  $^{55}\text{Fe(II)}$  standards and the radiometric measured  $^{55}\text{Fe(II)}$  concentrations in the methanol effluent followed linear behavior ( $R^2 = 0.995$ ) and yielded good recovery of  $^{55}\text{Fe(II)}$  (a slope of 0.97, i.e., a 97% recovery; Fig. 2). Another calibration curve for low  $^{55}\text{Fe(II)}$  concentrations of  $4 \times 10^{-11}$  to  $6 \times 10^{-10}$  M (below the range that can be measured by a spectrometer) was conducted to assess the method accuracy and sensitivity. This calibration curve yielded good linear behavior ( $R^2 = 0.989$ ) for the entire concentration range and 94% recovery (Fig. 3). The analytical

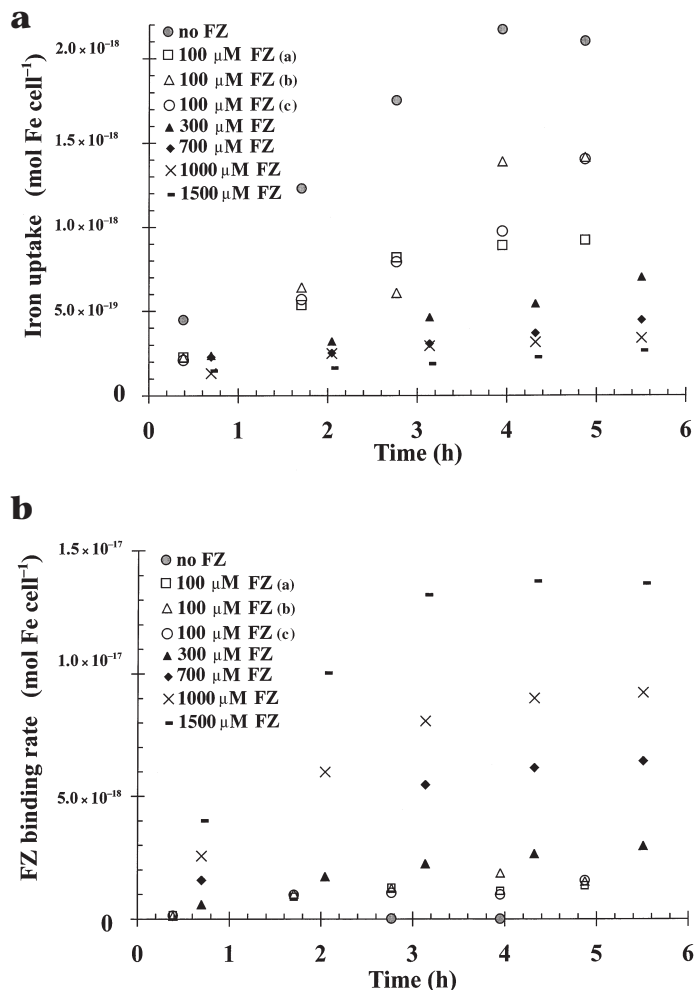


**Fig. 2.** Calibration curve between  $^{55}\text{Fe(II)}$  standards and  $^{55}\text{Fe(II)}$  extracted from the Sep-Pak. The calibration curve was conducted at micromolar  $^{55}\text{Fe(II)}$  to allow spectrophotometric detection of the Fe(II) in the NaCl standards prior and post extraction. The extraction of Fe(II) by the Sep-Pak followed linear behavior for the concentration range tested ( $R^2 = 0.995$ ;  $P < 0.0001$ ) and yielded good recovery (97%). Error bars represent average duplicate SD for the spectrometric and radiometric measurements.

detection limit of the method is dictated by the instrument ( $\gamma$  or scintillation liquid counters) blank (empty or scintillation cocktail containing vials respectively). Analytic detection limits of  $3 \times 10^{-11}$  M for  $^{55}\text{Fe}$  and  $2 \times 10^{-14}$  M for  $^{59}\text{Fe}$  were obtained based on three standard errors of the instrument blank. The limit of detection for FZ binding rates varies according to the method's application and the choice of experimental condi-



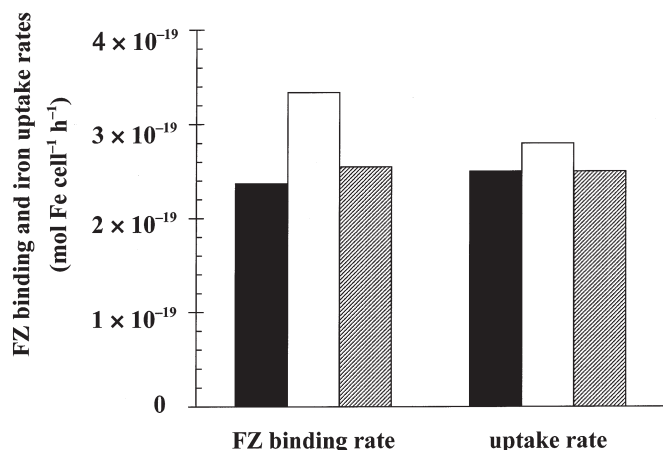
**Fig. 3.** Calibration curve between  $^{55}\text{Fe(II)}$  standards and  $^{55}\text{Fe(II)}$  extracted from the Sep-Pak. This calibration curve was conducted at sub nanomolar  $^{55}\text{Fe(II)}$  concentrations to evaluate the sensitivity and accuracy of the Sep-Pak extraction procedure at low Fe(II) concentrations. The extraction of Fe(II) by the Sep-Pak followed linear behavior at concentrations of  $3 \times 10^{-11}$  M (at the detection limit) to  $6 \times 10^{-10}$  M ( $R^2 = 0.989$ ;  $P < 0.001$ ) and yielded good recovery (94%). Error bars represent duplicate error of 11% for the radiometric measurements.



**Fig. 4.** Iron uptake and FZ binding by Fe-deplete *T. weissflogii* at varying FZ concentrations. Experiments were conducted in the dark using pre-equilibrated  $^{59}\text{FeEDTA}$ . The same assay was used to determine both Fe uptake and FZ binding rates. Cell density =  $2.6 \times 10^5$  cells  $\text{mL}^{-1}$ ,  $\text{Fe(III)}_T = 90$  nM, EDTA = 100  $\mu\text{M}$ . (a) FZ binding rates, determined in the Sep-Pak methanol effluent. Increased FZ concentration resulted in elevated FZ binding rates. (b) Iron uptake rates, determined by cell collection on 0.2  $\mu\text{m}$  syringe filters. Increased FZ concentration resulted in uptake inhibition.

tions such as sample volume, light level, and so on. In the presence of cells, the detection limit for FZ binding rates is dictated by the rates of FZ binding in the absence of cells (see "Effect of FZ concentration on iron reduction").

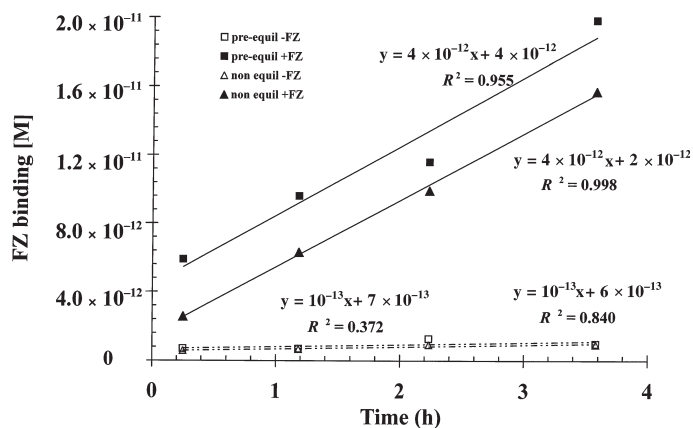
*Simultaneous iron uptake and FZ-binding experiments with Thalassiosira weissflogii laboratory cultures*—Typical short-term iron uptake and FZ binding rates were determined simultaneously for iron-limited *T. weissflogii* at increasing FZ concentrations (Fig. 4a and 4b). Sub-samples were pumped through the system and loaded on the filters and columns every hour for 6 h. In all instances, FZ binding and iron uptake followed linear behavior for 2-3 h, and their rates were calculated using a linear regression (Fig. 4a and 4b). It is likely that saturation



**Fig. 5.** Triplicate rate measurements of iron uptake and FZ binding by Fe-deplete *T. weissflogii* at 100  $\mu\text{M}$  FZ. Rates are calculated from the linear range of the data presented in Fig. 4. The standard deviations of the FZ binding and uptake rates are 19% and 9%, respectively.

would occur at high cell densities, and, therefore, it is recommended to measure uptake and FZ binding rates several times throughout the experiment to obtain robust rates or use lower cell density. Triplicate treatments were conducted at 100  $\mu\text{M}$  FZ to evaluate the method reproducibility (Figs. 4a, 4b, and 5). The average FZ binding rate of triplicate treatments was  $2.8 \pm 0.52 \times 10^{-19}$  mol Fe cell $^{-1}$  h $^{-1}$  (19% CV), and the average uptake rate was  $2.6 \pm 0.17 \times 10^{-19}$  mol Fe cell $^{-1}$  h $^{-1}$  (9% CV; Fig. 5). These standard deviations are significantly smaller than the differences between treatments with various FZ concentrations (Fig. 4a and 4b). The increase in FZ concentration resulted in elevated FZ binding rates and inhibited uptake rates (Fig. 4a and 4b). Uptake becomes negligible above 300  $\mu\text{M}$  FZ, whereas FZ binding rate continues to increase with increasing FZ concentration. The nature and significance of this phenomenon is addressed in “Effect of FZ concentration on iron reduction”

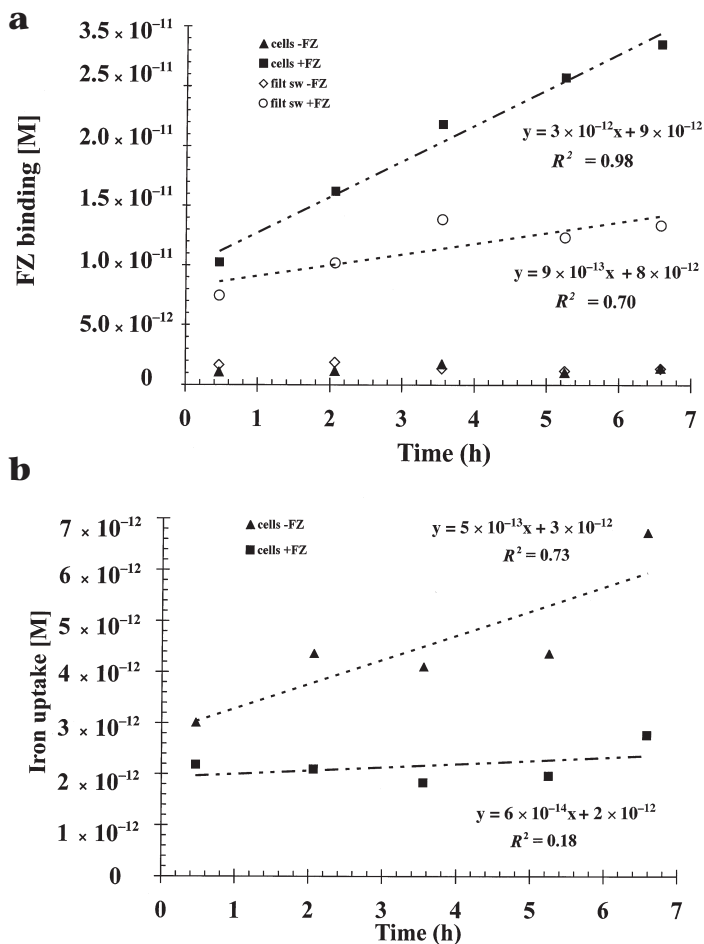
*Simultaneous iron uptake and FZ-binding experiments with natural phytoplankton assemblages in the Bering Sea*—The method was also applied to study iron uptake and reduction by natural phytoplankton assemblages in the HNLC (high nutrient low chlorophyll) region of the Bering Sea using 20 nM  $^{59}\text{FeEDTA}$  and 100 to 200  $\mu\text{M}$  FZ. This environment is analytically challenging because of the low phytoplankton biomass (0.3  $\mu\text{g L}^{-1}$  Chlorophyll *a*), low dissolved iron concentrations (50 to 80 pM; Buck and Bruland, pers. comm. unref.), and the presence of strong organic iron ligands (found in excess of dissolved iron concentrations; Buck and Bruland, pers. comm. unref.). Such strong ligands may extract  $^{59}\text{Fe}$  from the  $^{59}\text{FeEDTA}$  and stick to the Sep-Pak, resulting in high signal in the non-FZ treatment. In addition, the binding of the radio-labeled Fe by natural ligands may affect the FZ binding and uptake rates over the course of the experiment. To address this concern, we measured the FZ binding rate in cell-free solu-



**Fig. 6.** Blanks—FZ binding and Fe(III) binding determination in 0.2  $\mu\text{m}$  filtered HNLC Bering seawater. Seawater was freshly filtered.  $^{59}\text{FeEDTA}$  was added to one set and allowed to equilibrate for three hours prior to the experiment. Background FZ binding rates (with no cells) were obtained in the pre-equilibrated ( $\blacksquare$ ) and the non-equilibrated ( $\blacktriangle$ ) filtered seawater with 100  $\mu\text{M}$  FZ. A possible artifact of Fe(III) binding to the columns was tested in filtered seawater without FZ ( $\square, \triangle$ ).

tions (0.2  $\mu\text{m}$  filtered seawater) where  $^{59}\text{FeEDTA}$  was either (a) allowed to equilibrate for 3 h (offering time for exchange with natural ligands) or (b) was added to the seawater immediately before commencement of FZ binding measurements (Fig. 6). Identical rates of FZ binding were measured for the non-equilibrated and pre-equilibrated treatments amended with 100  $\mu\text{M}$  FZ (3.9 to 4 pM h $^{-1}$ ; Fig. 6). The rates were linear throughout the experiment ( $R^2 = 0.955$  and  $0.998$ ; Fig. 6). Similar and very low (0.12 pM h $^{-1}$ ) background rates were obtained for both non-FZ amended treatments (Fig. 6). These observations demonstrate that naturally occurring strong organic ligands had no effect on our experiments. The agreement between the two FZ binding rates further demonstrates the reproducibility of the method.

Simultaneous uptake and reduction rates for natural phytoplankton assemblages in the Bering Sea were successfully obtained, despite the low ambient biomass (0.3  $\mu\text{g L}^{-1}$  Chlorophyll *a*; Fig. 7). This was achieved by pre-concentrating the cells using filtration through 5- $\mu\text{m}$  membrane filter. This pre-concentration step has the advantage of preferentially concentrating eukaryotic phytoplankton, which are of major interest for the bio-reduction studies. Further increase in signal was attained by increasing the volume of the sub-samples pumped through the system. While the significance of those results is beyond the scope of this manuscript, it is important to note that these are the first bio-reduction rates measured at lower than micromolar iron addition. The biomass used in the field experiment was 400-fold lower than that of the *T. weissflogii* cultures and hence special attention was paid to Fe(II) complexation by FZ in the absence of cells. For each experiment, a filtered seawater (SW) treatment was added and the rate of FZ binding in the absence of cells was measured (Fig.



**Fig. 7.** Iron uptake and FZ binding by natural phytoplankton in the HNLC water of the Bering Sea.  $\text{Chl } a = 1.4 \mu\text{g L}^{-1}$ ,  $\text{Fe(III)}_{\text{T}} = 20 \text{ nM}$ , EDTA = 100  $\mu\text{M}$ , FZ = 100  $\mu\text{M}$ . (a) FZ binding rates of cells ( $\blacksquare$ ) and filtered seawater ( $\circ$ ). Fe(III) binding to the columns was also tested in treatment of cells without FZ ( $\blacktriangle$ ) and filtered seawater without FZ ( $\diamond$ ). (b) Iron uptake rates obtained in the absence of FZ ( $\blacktriangle$ ) and with 100  $\mu\text{M}$  FZ ( $\blacksquare$ ).

7a, Table 1). The FZ binding rates obtained in the Bering Sea for cell-free filtered SW match those of synthetic SW in the laboratory (Table 1). These rates, however, are significant compared to the algal-mediated rates measured in the field (Fig. 7a; Table 1). This implies that in many environments the cells have to be preconcentrated to obtain an adequate signal above the blank FZ binding in the absence of cells (which needs to be subtracted from the data). Once normalized to chlorophyll, the blank corrected FZ binding rates measured in the Bering Sea match those of *T. weissflogii* cultures. Iron uptake rates obtained in the Bering Sea experiments were somewhat slower than FZ binding rates and were inhibited by FZ addition, as observed for *T. weissflogii* cultures (Fig. 7b).

**Artifacts and blank measurements: Fe(III) adsorption on the Sep-Pak cartridges**—One possible artifact of the proposed method is the adsorption of Fe(III) on the Sep-Pak cartridge. This Fe(III) is

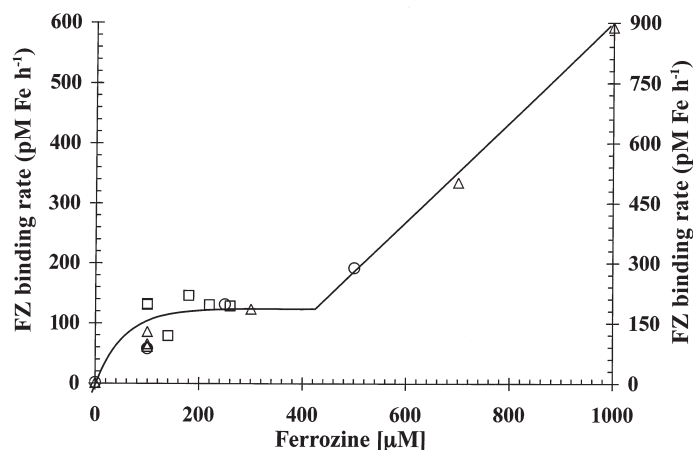
**Table 1.** Dark FZ binding rates in cell-free synthetic and natural seawater

Total Fe* (nM)	FZ ( $\mu\text{M}$ )	FZ binding rate ( $\text{pM h}^{-1}$ )
Laboratory experiments: synthetic seawater (Aquil)		
160	80	9.0
160	160	12
160	480	7.9
Experiments in Bering Sea: filtered seawater (0.2 $\mu\text{m}$ )		
20	100	4.0
20	100	3.9
20	100	0.9
20	100	0.7
20	100	2.3
20	200	0.9
20	200	1.6

\*100  $\mu\text{M}$  EDTA

later extracted by methanol and can mistakenly be interpreted as Fe(II). We tested the effect of different washes on Fe(III) removal from the column and found that a 5-min wash at 5 mL  $\text{min}^{-1}$  with pH 3 NaCl efficiently removed all the inorganic Fe(III) species off the Sep-Pak. We verified, using  $^{59}\text{Fe(II)}$  standards, that the HCl did not remove any of the Fe(II)(FZ)<sub>3</sub> complex from the Sep-Pak cartridge. It is possible, however, that in the presence of cells, iron bound to cellular exudates might stick to the column and will not be washed away by the acid. We routinely tested the extent of this possible artifact in the non-FZ-added treatments. In those treatments no Fe(II) is expected to be present because it is either taken up by the cells or oxidized, and hence any signal obtained in the columns effluent is the result of Fe(III) adsorption to the Sep-Pak. In the laboratory experiments, this artifact accounted at most for 2% of the iron extracted by the Sep-Pak in the presence of FZ (e.g., Fig. 4a). In our experiments with Bering Sea HNLC water, the low signal obtained in the absence of FZ treatments accounted for 1% to 15% of the rate measured in its presence (e.g., Fig. 7a).

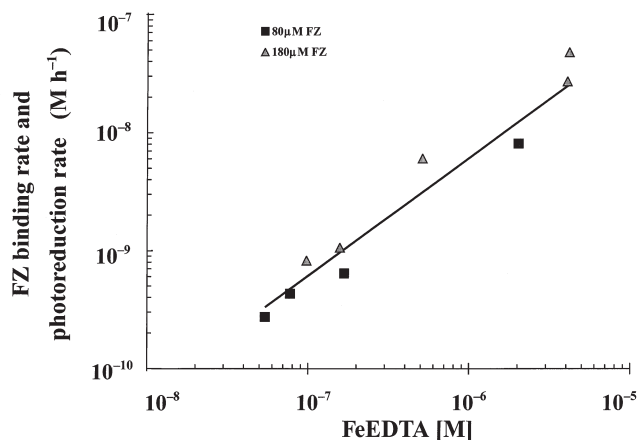
**Effect of FZ concentration on iron reduction**—Fe(III) reduction by specific Fe(II) complexing agent like FZ (or bathophenanthroline disulphonic acid) is a major concern in Fe(II) measurements in natural aquatic systems (O'Sullivan et al. 1991; Hudson et al. 1992). We therefore tested the FZ binding rates in the presence of cells over a wide range of FZ concentrations (Fig. 8). Three distinct regions in the kinetics of FZ binding are apparent from these data. At low FZ concentrations 0 to 150  $\mu\text{M}$ , FZ binding rates increase with increasing FZ concentrations (Fig. 8). Concomitant with this increase in FZ binding rate is a decrease in the rate of iron uptake (Fig. 4b). In this range of FZ concentrations, the rates of FZ binding and uptake are on the same order of magnitude and at 100  $\mu\text{M}$  FZ they become roughly equal (Fig. 5). At intermediate FZ concentrations of 150 to 300  $\mu\text{M}$ , FZ binding rates reach a plateau and do not increase with increasing FZ concentrations (Fig. 8). At those FZ concentrations



**Fig. 8.** FZ binding rates obtained for Fe-deplete *T. weissflogii* at varying FZ concentrations. This figure combines data from three sets of experiments, conducted at  $\text{Fe(III)}_T = 90$  nM and EDTA = 100  $\mu\text{M}$ . Triangles represent FZ binding rates calculated from the linear range of the data presented in Fig. 4. Rectangles and circles represent FZ binding rates obtained for experiments with  $3 \times 10^4$  cells  $\text{mL}^{-1}$  and  $2.6 \times 10^5$  cells  $\text{mL}^{-1}$  respectively (raw data not shown). Slower rates of FZ binding obtained in the high density experiments ( $\circ, \Delta$ ; left axis) might result from their more moderate iron limitation during growth (grown on 90 nM Fe vs. 60 nM for the low cell density) or from kinetic limitations on the supply of iron from the FeEDTA buffer system. The line connecting the points is drawn by hand.

the measured uptake rates are very low (Fig. 4b). Based on the constancy of FZ binding rates over this range, we deduce that FZ itself does not contribute to the measured FZ binding rate. At high FZ concentrations of 400 to 1000  $\mu\text{M}$ , FZ binding rates increase proportionally with FZ concentration at a slope of about 1 (Fig. 8). This slope indicates a pseudo-first-order reaction that does not match the third-order kinetics of Fe(II) binding by FZ (Eq. 1) that was determined for low FZ concentrations (up to 200  $\mu\text{M}$ ) by Lin and Kester (1992). At these high FZ concentrations, FZ greatly exceeds EDTA concentration and in the presence of reducing agents (such as cell exudates), it may promote the reduction of Fe(III)EDTA through ternary complex formation. We therefore recommend using 100 to 200  $\mu\text{M}$  FZ, and we do not assign any physiological significance to FZ binding rates measured above 400  $\mu\text{M}$  FZ.

In addition, we investigated whether FZ can reduce Fe(III) in the absence of cells at the FZ concentrations of interest. This was done in the dark using synthetic seawater (Aquil) at 160 nM iron and 80 to 480  $\mu\text{M}$  FZ. We found that the FZ binding rates in the absence of cells were independent of FZ concentration and ranged from 8 to 12  $\text{pM h}^{-1}$  (Table 1). These FZ binding rates were much lower than the rates of FZ binding in the presence of iron-limited *T. weissflogii* culture, accounting at most for 17% of the signal. Similar results were obtained for 0.2  $\mu\text{m}$  filtered Bering Sea water, where FZ binding rates were independent of the FZ concentration used in the experiment (Table 1). In the field experiments the FZ binding rates in the



**Fig. 9.** FZ binding rates and calculated FeEDTA photo-reduction rates in illuminated ( $85 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ) Sigma seawater amended with 50 to 4000 nM FeEDTA. FZ binding rates were measured in the absence of cells with 80  $\mu\text{M}$  FZ (squares) and 180  $\mu\text{M}$  FZ (triangles). Fe(II) formation by EDTA photo-reduction (continuous line) was calculated according to Eq. 2.

absence of cells were similar to or lower than those measured in Aquil ranging from 0.7 to 4  $\text{pM h}^{-1}$  (Table 1). These rates, however, were significant compared with the rates obtained in the presence of cells, accounting on average for 41% of the signal, and required blank correction.

We have also tested whether FZ can reduce Fe(III)EDTA in the absence of cells in the light. We conducted a series of experiments with Sigma Seawater illuminated with 85  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  light (supplied by VHO fluorescent tubes) at 25°C and containing a large range of concentrations of iron (50 to 4000 nM), EDTA (20 and 100  $\mu\text{M}$ ), and FZ (80 and 180  $\mu\text{M}$ ) (Fig. 9). We then compared the FZ binding rates with the rates of Fe(II) formation by photo-reduction described by the equation:

$$\frac{d\text{Fe(II)}}{dt} = k_{\text{hv}} \times [\text{FeEDTA}] \quad (2)$$

where the photo-reduction rate constant ( $k_{\text{hv}}$ ) is dependent on the intensity and quality of light. We have used  $k_{\text{hv}} = 1.7 \times 10^{-6} \text{ s}^{-1}$  after Anderson and Morel (1982) for 95  $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ . Fe(II) produced from FeEDTA by photo-reduction could be then oxidized by oxygen or complexed by FZ. In the absence of FZ mediated reduction the rates of FZ binding should be similar to the Fe(II) formation rates (Eq. 2), since Fe(II) oxidation is negligible at 25°C and 100 to 200  $\mu\text{M}$  FZ. No systematic increase in the rates of FZ binding in the light compared with the calculated photo-reduction rates was observed (Fig. 9), indicating that FZ does not promote FeEDTA reduction in the light.

### Comments and recommendations

*Choice of radioisotopes and radiometric counter*—Several adaptations are required to use  $^{55}\text{Fe}$  rather than  $^{59}\text{Fe}$ . These include the use of removable membrane filters for uptake experiments (the 6 keV energy maximum will not pass through the Acrodisc

membrane housing), evaporation of the methanol effluent on a hotplate to decrease quenching and studying the quenching effect of each matrix. It is difficult to procure  $\gamma$  counters aboard research vessels, where scintillation counters are more common. Scintillation counting of the  $^{59}\text{Fe}$  is possible and even recommended due to its high  $\beta$  emission. For liquid scintillation counting the methanol effluent should be evaporated on a hotplate. Uptake measurements can be done using removable membrane filters or 13-mm syringe filters that fit into the scintillation vials. Regardless of the counter used, we found that the 13-mm syringe filters yielded more consistent uptake rates, probably as a result of a more thorough wash of extracellular iron with the Ti-EDTA-citrate reagent.

**Sensitivity enhancement, biomass, and differential uptake**—There are several possibilities to increase the method sensitivity. These include carrier-free radioisotopes, pumping of larger volumes, longer experiments, and preconcentration of the cells. Each of these possibilities has some potential drawbacks, either in practical terms such as cost and time, or in more serious aspects such as cell breakdown and changes in the medium chemistry caused by dense concentrations of cells. The optimal cell density for the experiments is determined by the reduction capabilities of the organism as well as the background FZ binding in the absence of cells (Table 1). In the light, higher cell density is required because photo-reduction of the FeEDTA results in a high background of FZ binding (Fig. 9). But, high cell densities may lead to insufficient buffering of the iron and are difficult to obtain in the field. Therefore it is strongly recommended to run the reduction-uptake experiments in the dark or use a non-photo-labile ligand such as diethylene triamine penta-acetic acid. Uptake rates of different size fractions can be measured in natural assemblages by using a series of in-line syringe filters with different pore sizes.

**Use of other Fe ligands**—We have described a method to measure the rate of uptake and reduction in the presence of excess concentrations of the artificial chelator EDTA. EDTA is commonly used as a model ligand that buffers iron availability in laboratory cultures and is therefore of interest for physiological-mechanistic studies (e.g., Hudson and Morel 1990; Sunda and Huntsman 1995). In principle, this method can be adapted to study the kinetics of uptake and reduction of iron bound to a variety of artificial and natural organic ligands. However, the  $\text{C}_{18}$  silica resin is not specific, and it would undoubtedly bind many of those ligands. Different resin would then have to be used to bind  $^{59}\text{Fe(II)FZ}_3$  and not the  $^{59}\text{Fe(III)ligand}$ . In all cases, the effect of FZ addition on cellular uptake can be measured as described.

**Other applications**—Regardless of uptake rate measurements, the combination of the radiometric assay with the selective adsorption of the  $\text{Fe(II)FZ}_3$  complex onto a  $\text{C}_{18}$  column, provides a sensitive, relatively simple, fast, and reliable method to evaluate sub-nanomolar Fe(II) concentrations, which might be applicable for iron photo-reduction rate measurements. An example of such application is seen in Fig. 9, showing FZ bind-

ing rates measured in the light (at  $85 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ) in cell-free Sigma Seawater. The FZ binding rates are in good agreement with calculated rates of FeEDTA photo-reduction (Eq. 2; Fig. 9), demonstrating the potential of this method for measuring reduction rates of photo-labile iron complexes.

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