

Availability of iron from iron-storage proteins to marine phytoplankton

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Abstract

We examined the bioavailability of iron-storage proteins—including representatives of maxi- and miniferritins—to various species of marine phytoplankton. Both eukaryotic and prokaryotic species were able to grow rapidly with horse spleen ferritin (HoSF) or deoxyribonucleic acid (DNA)-binding proteins from starved cells (Dps) from *Trichodesmium erythraeum* as the sole Fe source in the medium. In the presence of ethylenediaminetetra-acetic acid (EDTA), cells grown with HoSF or Dps maintained exponential growth rates similar to those obtained at the same concentration of FeEDTA. Growth was also observed in the absence of EDTA, showing that a complexing agent is not necessary for Fe availability. The bioavailability of Fe in these storage proteins apparently results from a spontaneous release of Fe(III) to solution with an effective first-order rate constant $\sim 0.15 \text{ d}^{-1}$. Genes coding for iron-storage proteins are common in DNA samples from seawater. In iron-deprived marine ecosystems, iron-storage proteins may be important constituents of the recycled iron pool and modulate its availability to phytoplankton.

Iron, an essential element for virtually all living organisms, is present at extremely low concentrations in the oxygenated waters of most oceanic regions because of the insolubility of Fe(III) (Bruland et al. 1994; Martin et al. 1994). Numerous field studies have demonstrated that Fe limits phytoplankton productivity in high-nitrate, low-chlorophyll (HNLC) regions of the ocean (Martin et al. 1994; Coale et al. 1996, 2004). The interaction of oxygen, light, and Fe leads to the production of reactive oxygen species that cause oxidative damage to cells. Iron-storage proteins are used by organisms to sequester and store intracellular iron in a safe manner and provide a source of iron that can be drawn on when external iron supplies are limited (Harrison and Arosio 1996; Andrews 1998). Ferritin, bacterioferritin (heme-containing ferritin), and deoxyribonucleic acid (DNA) binding protein from starved cells (Dps) compose a superfamily of iron-storage proteins that are found in both prokaryotes and eukaryotes. Although ferritin, bacterioferritin, and Dps proteins are distantly related they retain similar structural and functional properties. Ferritin and bacterioferritin are composed of 24

subunits, whereas Dps proteins are composed of 12 subunits (Andrews 1998; Stefanini et al. 1999). The subunits are assembled to form a spherical protein with a central cavity capable of storing a maximum of 4,500 Fe(III) atoms in ferritins, 2,000 Fe(III) atoms in bacterioferritins, and 500 Fe(III) atoms in Dps proteins. Iron is incorporated into the central cavity by the oxidation of Fe(II) followed by the formation of a microcrystalline ferrihydrite–phosphate core usually referred as the “iron core.” The oxidation of Fe(II) is carried out by the ferroxidase center that is located within the subunits of ferritins and bacterioferritins and between subunits in Dps proteins (Andrews 1998; Ilari et al. 2000). The physiological processes that lead to the incorporation of Fe into the core and its release from iron-storage proteins to other molecules in vivo remain not fully understood. The Fe core can vary in composition and crystallinity. Ferritin isolated from animals tends to contain ordered cores that resemble the ferrihydrite mineral and are low in phosphate (P_i : Fe 1 : 8), whereas bacterial and plant ferritin are high in phosphate (P_i : Fe 1 : 1 to 1 : 3) and amorphous (Treffry et al. 1987). The Fe core of bacterioferritins tends to be disordered and normally contains more phosphate (P_i : Fe 1 : 1 to 1 : 2) (Andrews 1998; Treffry et al. 1987). The core composition of Dps proteins is less known, but phosphate incorporation into the iron core of *Trichodesmium erythraeum* Dps protein has been reported (P_i : Fe 1 : 4) (Castruita et al. 2006).

The three types of iron-storage proteins can exist in the same bacterium and multiple copies of ferritin or bacter-

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ioferritin genes can be identified in organisms (Andrews 1998; Andrews et al. 2003; Keren et al. 2004). Although ferritins are ubiquitous to all forms of life, the study of these proteins in marine phytoplankton is limited. Genomic analysis of oceanic phytoplankton reveals the presence of iron-storage genes in several cyanobacteria and a Dps protein from *T. erythraeum* (Dps_{tery}) has been overexpressed and partially characterized (Castruita et al. 2006). But the role of iron-storage proteins in the marine environment remains elusive. Ferritins are known to be exceptionally stable proteins able to withstand elevated temperatures, denaturants, proteolytic enzymes, and low pH (Hofmann and Harrison 1963; Niitsu et al. 1973; Aisen and Listowsky 1980). As a result, it is plausible that upon cell lysis iron-storage proteins released to the environment would retain their integrity for a period of time of hours or days, long enough to play a role in the recycling of iron.

Dissolved iron in the ocean is found overwhelmingly bound to strong organic ligands (~99%) (Rue and Bruland 1995; Wells et al. 1995). However, the origin, chemical identity, and the bioavailability of this organically bound Fe are, to a large extent, unknown. These compounds are thought to be the products of inducible iron-uptake systems of prokaryotes (siderophore complexes) and strong Fe chelators present in phytoplankton cells (porphyrin complexes) that might be released by grazing, viral lysis, or by cell autolysis after death (Wilhelm and Trick 1994; Wu and Luther 1995; Witter et al. 2000). It is also possible that some of the Fe released from cells may be in Fe storage proteins. Chemists consider ferritins as dissolved proteins, although the core is sometimes referred to as a biomineral; they would be measured as part of the colloidal pool by many separation techniques used by oceanographers. The bioavailability of these Fe compounds to phytoplankton is of key importance in HNLC areas where the biological recycling of Fe plays an important role due to the limited external inputs of new Fe. Several studies have shown that marine phytoplankton are able to access the Fe from some chelates (Gobler et al. 1997; Hutchins et al. 1999; Poorvin et al. 2004; Kustka et al. 2005). Interestingly, results of both culture and natural assemblage experiments suggest that eukaryotes and prokaryotes compete for different organically bound iron species (Hutchins et al. 1999; Poorvin et al. 2004). This indicates that specialization in utilization of different Fe-organic compounds may minimize competition, allowing for niche separation.

Because of the great stability of iron-storage proteins, we hypothesized that once they enter the marine environment upon cell lysis, they become part of the pool of dissolved or colloidal iron and become a potential source of Fe to marine phytoplankton. We report here on the availability of Fe bound in Dps_{tery} or in the commercially available horse spleen ferritin (HoFS) to species of both eukaryotic and prokaryotic marine phytoplankton.

Methods and materials

Preparation of protein samples—Fe loading of Dps_{tery} was carried out as described in Castruita et al. (2006). To a 100 $\mu\text{g mL}^{-1}$ Dps solution in 20 mmol L^{-1} tris(hydroxy-

methyl)aminomethane hydrochloride (Tris-HCl) (pH 7.0), 150 mmol L^{-1} NaCl, 0.1 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA), 500 $\mu\text{mol L}^{-1}$ freshly prepared iron(II) ammonium sulfate hexahydrate was added, and the preparation was left at room temperature for 3 h. After incubation, the Fe-protein solution was washed for 10 min with a solution of 50 mmol L^{-1} EDTA and 100 mmol L^{-1} oxalate (pH 7.0) to remove unbound Fe (Tang and Morel 2006), followed by centrifugation at $3,000 \times g$ for 30 min in a Centricon 10 tube. The protein pellet was then rinsed twice by adding 1 mL of 50 mmol L^{-1} Tris-HCl (pH 7.0) buffer and centrifuged for an additional 30 min (repeated twice). HoSF obtained from Sigma (containing ~2,000 Fe atoms per protein molecule) was washed with EDTA-oxalate solution as described above to remove unbound Fe. For Fe quantification, the method of Stookey (1970) was modified for protein use. Protein sample (10 μL) was added to 50 μL of trace-metal-clean 1 mol L^{-1} HCl and incubated at room temperature for 1 h. Fifty microliters of 1 mol L^{-1} NaOH was added, followed by the addition of 500 μL of 2 mol L^{-1} sodium dithionite and incubated for 30 min. Finally, 250 μL of 0.4 mmol L^{-1} ferrozine and 250 μL of 0.5 mol L^{-1} sodium acetate were added and incubated for 18 h. The samples were measured on an ultraviolet (UV)-visible spectrophotometer at a wavelength of 562 nm.

Dialysis of Dps_{tery} against apotransferrin—A solution of Dps_{tery} (~2 mmol L^{-1}) was placed in Slide-A-Lyzer 10K cassette (10000 MWCO) and placed in a trace-metal-clean polycarbonate bottle that contained a 0.27 mmol L^{-1} solution of apotransferrin (Sigma), 1 mol L^{-1} Tris (2-amino-2-hydroxymethyl-1,3-propanediol), and 50 mmol L^{-1} NaHCO₃, at pH 7.4. The dialysis cassette was soaked in a solution of 1 mol L^{-1} EDTA for 3 d before the experiment to remove metal contamination, then rinsed and soaked in MilliQ water for 3 d. Dialysis was performed at 4°C with constant stirring in the dark. One-milliliter aliquots of the transferrin solution were measured at a wavelength of 465 nm with a Cary 100 Bio UV-visible spectrophotometer. An apotransferrin solution (0.27 mmol L^{-1}) in Tris buffer and lacking Dps_{tery} was followed spectrophotometrically as above to determine if Fe contamination could result in the formation of Fe(III)-transferrin.

Phytoplankton culturing—Cyanobacteria: Axenic cultures of *Prochlorococcus* sp. strain MED 4 and strain MIT 9313 were grown in a Gulf Stream seawater medium based on Saito et al. (2002), and modified for iron limitation. Acid-cleaned polycarbonate bottles were used and the cultures were grown at 20°C under continuous light at 80–120 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. The culture medium contained 58.5 $\mu\text{mol L}^{-1}$ EDTA, 50 nmol L^{-1} Se (Na₂SeO₃) and Ni, 40 nmol L^{-1} Zn, 4 nmol L^{-1} Co, 15 nmol L^{-1} Mo, 450 nmol L^{-1} Mn, 50 $\mu\text{mol L}^{-1}$ NH₄Cl, 100 $\mu\text{mol L}^{-1}$ urea, and 10 $\mu\text{mol L}^{-1}$ KH₂PO₄. Before the experiments, *Prochlorococcus* cultures were grown in low iron conditions (5 nmol L^{-1} FeEDTA) to deplete intracellular Fe. Exponentially growing *Prochlorococcus* MED 4 cells were transferred to medium amended with 10, 60, and 300 nmol L^{-1} Fe as FeEDTA or ferritin and 10 and

60 nmol L⁻¹ Fe as Dps_{tery}. *Prochlorococcus* MIT 9313 cells were transferred to medium amended with 300 nmol L⁻¹ Fe as FeEDTA or HoSF. The growth of the cultures was monitored daily by measuring chlorophyll *a* (Chl *a*) fluorescence in vivo on a PerkinElmer LS55 luminescence spectrometer and specific growth rates were then determined from the linear regression of the natural log of the observed fluorescence versus time. Sterile trace-metal-clean techniques were used for all culture and experimental manipulations; sterility was checked periodically by inoculation of cultures into LB broth.

Diatom and coccolithophores: Axenic cultures of *Thalassiosira weissflogii* (CCMP 1336) and *Emiliana huxleyi* (CCMP 374) were grown at 20°C at continuous light at 80–120 μmol photons m⁻² s⁻¹ with Aquil medium (Gulf Stream water) prepared and sterilized according to Price et al. (1988/1989). Aquil medium for *T. weissflogii* contains 10 μmol L⁻¹ NaH₂PO₄, 100 μmol L⁻¹ NaNO₃, Na₂SiO₃, and EDTA, 79.7 nmol L⁻¹ Zn, 121 nmol L⁻¹ Mn, 50.3 nmol L⁻¹ Co, 19.6 nmol L⁻¹ Cu, 100 nmol L⁻¹ Mo, 1 nmol L⁻¹ Se (Na₂SeO₃), 0.396 nmol L⁻¹ vitamin B₁₂, 2.5 nmol L⁻¹ biotin, and 296 nmol L⁻¹ thiamine. The composition of Aquil for *E. huxleyi* cultures differs from that for *T. weissflogii*: it does not contain silica, vitamin B₁₂, or biotin, and the final concentrations of NaNO₃ and thiamine are 150 μmol L⁻¹ and 59.3 nmol L⁻¹ respectively. Cell counts were determined using a Coulter Counter and specific growth rates were calculated by the slope of the regression of ln(cell number) versus time. Trace-metal-clean and sterile techniques were used as above.

Before experimentation in medium containing various Fe concentrations as detailed in the results section, *T. weissflogii* cells were conditioned in Aquil medium with the usual EDTA and trace metals mix but no added Fe. For experiments in the absence of EDTA, seawater was enriched with filter-sterilized vitamins and chelexed major nutrients (10 μmol L⁻¹ PO₄³⁻, 100 μmol L⁻¹ NO₃⁻, and 100 μmol L⁻¹ SiO₂) but no added trace metals. In the experiments where the EDTA concentration was varied (200 μmol L⁻¹, 100 μmol L⁻¹, and 50 μmol L⁻¹), the concentration of the trace metal mix was varied proportionally with EDTA.

⁵⁹Ferritin uptake experiments—⁵⁹FeCl₃ (8 μmol L⁻¹) was reduced with 0.1 mol L⁻¹ ascorbic acid for 2 d (9 μL). The radiolabeled solution was mixed with non-labeled Fe(II) (ferrous ammonium sulfate hexahydrate) to yield a final Fe concentration of 0.48 μmol L⁻¹. The radiolabeled solution was added to 0.3 mg mL⁻¹ of apo-HoSF and incubated overnight. Finally the ⁵⁹Fe-loaded protein was carefully washed three times in a centrifugal filter with the oxalate-EDTA solution as explained above and Fe quantified by the method of Stookey (1970).

Short-term iron uptake of Fe-limited *T. weissflogii* cells was measured in both EDTA-containing and EDTA-free Aquil medium that contained 53 nmol L⁻¹ and 87 nmol L⁻¹ ⁵⁹Fe-labeled HoSF. Cells previously grown in low-Fe medium were harvested by gentle filtration onto a 0.2-μm Nucleopore filter and immediately resuspended in 10 mL of Aquil medium without EDTA and divided

among treatments. Final cell concentrations were ~80,000 cells mL⁻¹ (53 nmol L⁻¹ ⁵⁹Fe-HoSF), and ~30,000 cells mL⁻¹ (87 nmol L⁻¹ ⁵⁹Fe-HoSF). Experiments were conducted in the dark to avoid photoreduction. At each of four time points over 4 h, 20-mL aliquots from each treatment were loaded onto 0.8-μm syringe filters (13 mm, Versapor), treated with Ti-citrate-EDTA wash, and counted on a LS 6500 scintillation counter (Beckman) (Shaked et al. 2004).

Results

Iron-storage proteins as iron source to marine phytoplankton—The bioavailability of iron from Fe-storage proteins to Fe-deprived marine phytoplankton was tested in cultures of the diatom *T. weissflogii*, the coccolithophore *E. huxleyi*, and the cyanobacterium *Prochlorococcus* (both MED 4 and MIT 9313 isolates) using Dps_{tery} and HoSF in the presence of excess EDTA. In every case, the cultures were able to grow in the presence of either Dps_{tery} or HoSF (10–300 nmol L⁻¹ Fe-protein) with growth rates and final biomass comparable with those grown with FeEDTA (Table 1, Fig. 1).

Cultures of *T. weissflogii* acclimated to low Fe conditions grew almost as fast in medium amended with either Fe-Dps_{tery} or Fe-HoSF as in medium amended with FeEDTA at the same concentration (Fig. 1A,B and Table 1). At low concentrations of the Fe source (30 or 40 nmol L⁻¹) stable Fe-limited growth rates (0.63 to 0.88 d⁻¹) were observed in all the cultures. *E. huxleyi* grew at its maximum rate in the presence of 87 nmol L⁻¹ FeEDTA or Fe-Dps_{tery} (Table 1). The two isolates of the cyanobacterium *Prochlorococcus* that were tested, MIT 9313 and MED 4, also grew at their maximum rate in the presence of 300 nmol L⁻¹ FeEDTA or Fe-HoSF (Fig. 1C,D). Decreasing steady-state growth rates of the cyanobacteria were observed when the concentration of Fe-ferritin was lowered to 60 and 10 nmol L⁻¹.

Growth in the absence of EDTA—The mechanisms thought to be involved in dissolving the ferritin biomineral under physiological conditions include the chelation of Fe inside the protein, or transfer of Fe to a chelator outside the protein (or both) (Crichton et al. 1980; Theil et al. 2000; Liu et al. 2003). Since EDTA is an excellent chelator of Fe, there is the possibility that EDTA present in Aquil medium could enter the protein and dissolve the Fe core through chelation and thus make Fe available for uptake. To examine whether EDTA chelation is essential for Fe uptake from iron-storage proteins we conducted a growth experiment in Aquil lacking both EDTA and trace metals. These experiments are inherently more difficult since contamination by Fe and other trace metals is probable. We observed an initial growth rate of 0.91 d⁻¹ and 1.0 d⁻¹ for *T. weissflogii* cells grown in the presence of 40 nmol L⁻¹ Fe-Dps_{tery} and 120 nmol L⁻¹ Fe-HoSF respectively. In the presence of 30 nmol L⁻¹ Fe-HoSF an initial growth rate of 0.68d⁻¹ was observed for *T. weissflogii* and only modest growth in the control cultures with no Fe added (Fig. 2, Table 1).

Table 1. Growth rates (d^{-1}) of marine phytoplankton in the presence of Dp_{stery} , ferritin, and FeEDTA. (Standard deviations for growth rates are shown in parentheses.)

Organism	Medium conditions	FeEDTA		Dp_{stery}		Ferritin	
	EDTA ($\mu\text{mol L}^{-1}$)	Total [Fe] (nmol L^{-1})	Growth rate (d^{-1})	Total [Fe] (nmol L^{-1})	Growth rate (d^{-1})	Total [Fe] (nmol L^{-1})	Growth rate (d^{-1})
<i>T. weissflogii</i>	0					30	0.68 (0.11)
	50					30	0.88 (0.14)
	100	30	0.70 (0.09)			30	0.64 (0.11)
	200					30	0.43 (0.05)
	0				40	0.91 (0.08)	
	0				120	1.00 (0.17)	
<i>Prochlorococcus</i> MIT 9313	100	40	0.88 (0.05)	40	0.63 (0.05)		
	100	120	1.10 (0.04)	120	0.97 (0.08)	120	1.09 (0.05)
<i>Prochlorococcus</i> MED 4	58.5	300	0.47 (0.05)			300	0.45 (0.09)
	58.5	300	0.41 (0.08)			300	0.41 (0.09)
<i>E. huxleyi</i>	58.5	60	0.36 (0.05)	60	0.38 (0.06)	60	0.34 (0.09)
	58.5	10	0.28 (0.05)	10	0.26 (0.06)	10	0.29 (0.09)
	100	87	1.37 (0.25)	87	1.34 (0.17)		

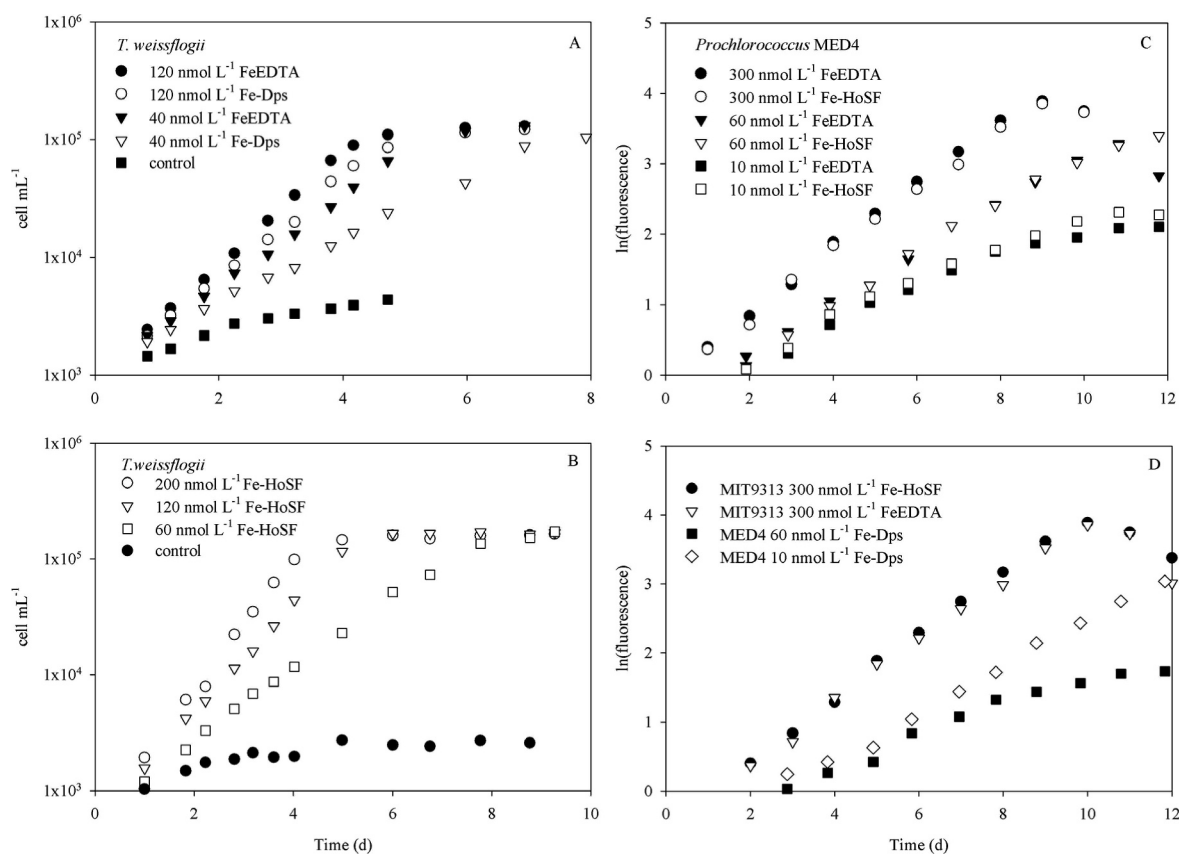


Fig. 1. Growth of marine phytoplankton in the presence of FeEDTA, Fe-HoFS, or Fe- Dp_{stery} in EDTA-containing Aquil medium. Before experimentation cells were acclimated in low-Fe medium and then transferred to Aquil containing FeEDTA, Fe- Dp_{stery} , or Fe-HoFS as the iron source. (A) *T. weissflogii* in Aquil medium containing $100 \mu\text{mol L}^{-1}$ EDTA and 120 or 40 nmol L^{-1} Fe- Dp_{stery} or FeEDTA. Control cells are in medium lacking any added Fe. (B) *T. weissflogii* in Aquil medium containing $100 \mu\text{mol L}^{-1}$ EDTA and 200, 120, or 60 nmol L^{-1} Fe-HoFS. Control cells are in medium lacking any added Fe. (C) *Prochlorococcus* sp. MED 4 in medium containing $58.5 \mu\text{mol L}^{-1}$ EDTA and 300, 60, or 10 nmol L^{-1} Fe-HoFS or FeEDTA. (D) *Prochlorococcus* sp. MED 4 in Aquil medium containing $58.5 \mu\text{mol L}^{-1}$ EDTA and 10 or 60 nmol L^{-1} Fe- Dp_{stery} ; *Prochlorococcus* sp. MIT 9313 in Aquil medium containing $58.5 \mu\text{mol L}^{-1}$ EDTA and 300 nmol L^{-1} Fe-HoSF or FeEDTA.

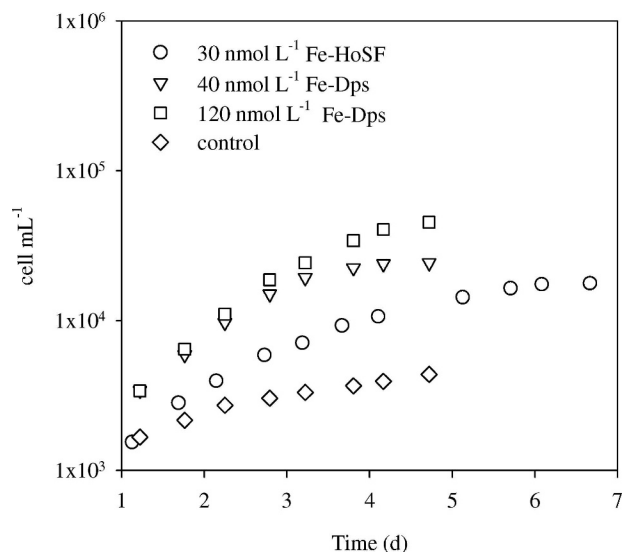


Fig. 2. Growth of iron-limited *T. weissflogii* inoculated into an EDTA-free Aquil medium amended with 30 nmol L⁻¹ Fe-HoSF, 40 nmol L⁻¹ Fe-Dps_{tery}, or 120 nmol L⁻¹ Fe-Dps_{tery}. The medium does not contain EDTA or added trace metals. Control cells are in medium lacking any added Fe.

Effect of EDTA on growth rates—In a well-buffered medium containing a large excess of EDTA, Fe(III) is predominantly found in a 1 : 1 complex with EDTA. A low concentration of unchelated Fe, Fe', is thus maintained by the formation and dissociation of FeEDTA, and its value is fixed by the ratio of the concentrations of total Fe and total EDTA in the medium. Under these conditions, Fe' has been shown to determine the rate at which phytoplankton acquire Fe. The similar growth rates observed in cultures grown at similar concentrations of FeEDTA and Fe-Dps_{tery} or Fe-HoSF suggest that the corresponding growth media may contain similar Fe'. If this is true, it should be possible to decrease Fe' and hence the growth rate of the cultures by increasing the concentration of EDTA in the medium. Indeed, in medium containing 30 nmol L⁻¹ Fe-HoSF, the growth rate of *T. weissflogii* decreased systematically from 0.88 d⁻¹ to 0.43 d⁻¹ as the concentration of EDTA was increased from 50 to 200 μmol L⁻¹ EDTA (Fig. 3 and Table 1).

Short-term Fe uptake—To gain insight into the mechanisms of Fe release from Fe-storage proteins and Fe uptake by the cells, we carried out short-term Fe uptake experiments with ⁵⁹Fe-radiolabeled ferritin. Fe-deficient cultures of *T. weissflogii* were transferred to medium amended with 87 and 53 nmol L⁻¹ ⁵⁹Fe-HoSF, in the presence and absence of excess EDTA. The uptake rate of ⁵⁹Fe-HoSF was measured by the method of Shaked et al. (2004) and the results are shown in Fig. 4 and Table 2. In the absence of EDTA, the total cellular uptake rate per liter of culture was roughly proportional to the amount of Fe added, increasing 1.4-fold between the low- and high-Fe conditions. The addition of excess EDTA to the medium resulted in a decrease in uptake rate by a factor of two to three (Fig. 4), suggesting that EDTA may be competing with the cells for unchelated Fe.

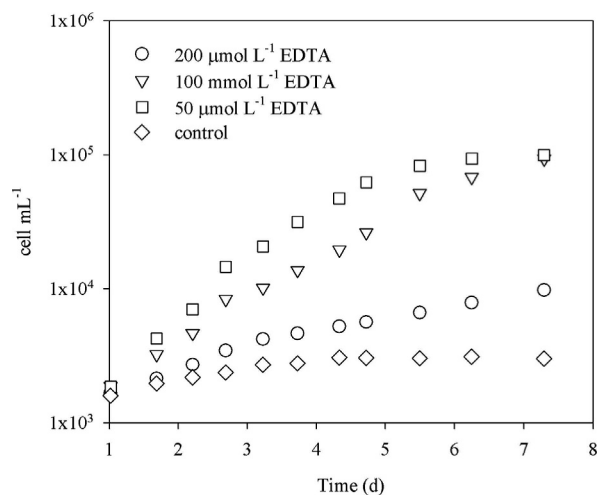


Fig. 3. Growth of *T. weissflogii* in Aquil medium containing 30 nmol L⁻¹ Fe-HoSF and 200 μmol L⁻¹, 100 μmol L⁻¹, or 50 μmol L⁻¹ EDTA. Growth rates are dependent on EDTA concentration; maximum growth rate was observed for the cells in 50 μmol L⁻¹ EDTA. Control cells are in medium lacking any added Fe.

Possible mechanism of iron release—Growth experiments show that EDTA is not required to make Fe in iron-storage proteins available to the cells. Results of the short-term uptake experiments where the cells are filtered before the experiments and resuspended in new medium make it improbable that extracellular metabolites released by the cells are involved in Fe release from the protein. Unless the cells themselves somehow interact with the proteins, it thus seems that Fe is released to the medium by spontaneous dissolution of the Fe core. This mechanism is also consistent with the decrease of both growth and uptake rates observed in the presence of EDTA. To establish if Fe release indeed occurs in ferritins without the direct aid of a chelator, we performed an experiment in which Dps_{tery} was placed in a dialysis bag immersed in an apotransferrin solution to sequester Fe(III). Direct interaction of the proteins is prevented by the dialysis bag because of their high molecular mass. As shown in Fig. 5, Fe(III) did become complexed by transferrin over time. A similar experiment with HoSF yielded similar results (Castruita et al. 2007). Because the transfer of Fe from Dps_{tery} to transferrin requires diffusion through the dialysis bag, such an experiment does not provide useful kinetic information. But it establishes that the release of Fe(III) from Fe-storage proteins is indeed spontaneous and does not require a reducing agent or a chelator.

Estimation of rate of Fe release—short-term uptake experiments—EDTA-free Aquil: On the basis of our results, it appears that Fe is released from Fe-storage proteins before being taken up by phytoplankton. In the absence of EDTA, we can schematically describe these processes by the following reactions:



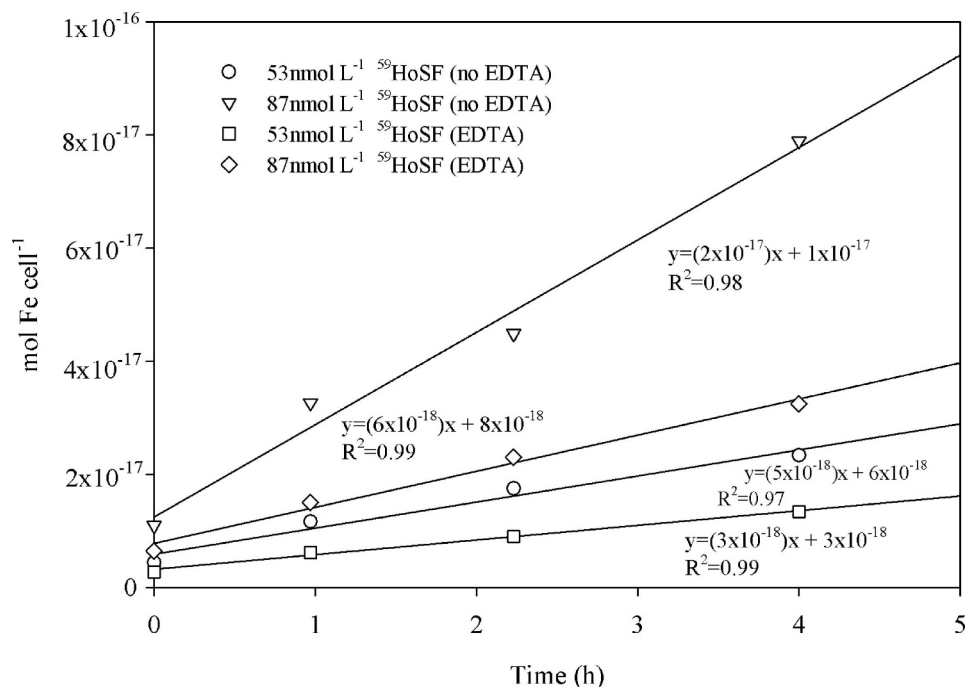


Fig. 4. Short-term uptake of ^{59}Fe -HoSFs by *T. weissflogii* in the presence and absence of EDTA. Experiments were conducted in the dark at Fe concentrations of 53 and 87 nmol L^{-1} . A concentration of 100 $\mu\text{mol L}^{-1}$ EDTA was used for the EDTA-containing medium. The slopes of the solid lines (which are linear regressions of moles of Fe per cell and time) represent the uptake rate of Fe by the cells.



A steady uptake rate is obtained when the release from the Fe core is equal to the rate of uptake. If the rate of release from the core is proportional to the Fe in the protein (Fe release rate = $k_{\text{release}} [\text{Fe-protein}]$), we can readily calculate the release rate constant from the total uptake rate:

$$k_{\text{release}} = \frac{\text{total uptake rate}}{[\text{Fe-protein}]} \quad (3)$$

where the total uptake rate is equal to the cellular uptake rate multiplied by the total cell number. From the results of the short-term uptake experiment with ^{59}Fe -HoSF in the absence of EDTA, we calculated reasonably similar values

Table 2. k_{release} , Fe' concentration, and total uptake rate for short-term experiments.

	Short-term culture			
	EDTA		EDTA-free	
Fe-ferritin (nmol L^{-1})	53	87	53	87
k_{release} (d^{-1})	1.37–0.16	1.97–0.16	0.18	0.15
Fe' (nmol L^{-1})	0.52–0.03	1.30–0.07	0.92–0.05	3.2–0.18
Total uptake rate (nmol $\text{L}^{-1} \text{d}^{-1}$)	4.90	4.70	9.53	13.40
Cell number (mL^{-1})	78,160	30,688	85,992	34,160
Cellular uptake ($\text{fmol cell}^{-1} \text{d}^{-1}$)	0.062	0.015	0.11	0.39

of k_{release} , $\sim 0.15 \text{ d}^{-1}$ and 0.18 d^{-1} for the 87 nmol L^{-1} and 53 nmol L^{-1} concentrations, respectively (Table 2).

EDTA system: The addition of EDTA to the short-term uptake experiments should have two effects: (1) an additional dissolution of Fe by direct interaction of EDTA with the core, and (2) a complexation of dissolved Fe by

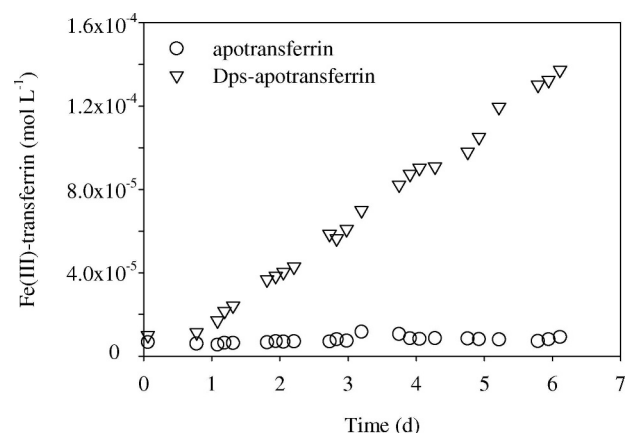


Fig. 5. Time course of the formation of Fe-transferrin complex in the presence of Fe-Dps_{terry}. Self-dissolution of the Fe core (2 mmol L^{-1} Fe-Dps_{terry}) releases Fe(III) that diffuses across a dialysis membrane and is then complexed by transferrin (0.27 mmol L^{-1}). The formation of Fe(III)-transferrin complex was followed spectrophotometrically at 465 nm. An apotransferrin solution lacking ferritin was used as a control to verify that Fe contamination was not responsible for the observed formation of Fe(III)-transferrin.

Table 3. $k_{\text{dissociation}}$, $k_{\text{complexation}}$ for FeEDTA, and k_{uptake} .

	Units	Value	Conditions	Data source
FeEDTA, $k_{\text{dissociation}}$	d^{-1}	0.13	pH 8.13	Sunda and Huntsman (2003)
FeEDTA, $k_{\text{complexation}}$	$\text{mol L}^{-1}\text{d}^{-1}$	1.3×10^6	pH 8.26	Hudson et al. (1992)
k_{uptake}	$\text{L cell}^{-1}\text{d}^{-1}$	1.2×10^{-7} – 2.2×10^{-6}	pH 8	Shaked et al. (2005)

EDTA, which effectively competes with the cell for Fe'. As explained later, the transfer of Fe from the protein core to EDTA should effectively have no effect on the rate of release of unchelated Fe to the medium and thus on the rate of cellular uptake. The chelation of dissolved Fe by EDTA should decrease the rate of cellular uptake but without affecting the dissolution of the core if the back reaction leading to the reinsertion of Fe into the core is negligible.

To interpret the short-term uptake data quantitatively we take into account the formation of FeEDTA, in addition to the release of Fe from the protein (Eq. 1) and its uptake by the phytoplankton (Eq. 2):



At steady state, the rate of Fe release from the core is equal to the sum of the uptake rate by the phytoplankton and the rate of complexation by EDTA, which is determined by the second-order rate constant, $k_{\text{complexation}}$. The first-order rate constant for the release from the core, k_{release} , can be then be calculated as:

$$k_{\text{release}} = \frac{(\text{total uptake rate} + k_{\text{complexation}}[\text{EDTA}][\text{Fe}'])}{[\text{Fe-protein}]} \quad (5)$$

The calculation of Fe release rate constant for this experiment is intrinsically imprecise because it requires knowing the rate of complexation by EDTA, which depends on Fe', whose value can only be estimated by the rate of Fe uptake by the cells ($\text{Fe}' = \text{uptake rate}/k_{\text{uptake}}$, where k_{uptake} is taken from the literature; see Table 3). The range of values obtained, $k_{\text{release}} = 0.16$ – 2.0 d^{-1} , is somewhat high compared with the k_{release} calculated in the absence of EDTA. These high values may indicate that reinsertion of Fe' into the core, or possibly precipitation of ferric hydroxide, are in fact not negligible. Some precipitation is indeed likely since the calculated Fe' concentrations are above or near the saturation value $\sim 0.3 \text{ nmol L}^{-1}$ (Table 2).

Discussion

Uptake of Fe from iron-storage proteins by phytoplankton—Both our growth and short-term uptake experiments indicate that Fe from representatives of both maxi- and miniferritins are highly bioavailable to marine phytoplankton of diverse families. All our data are consistent with a mechanism whereby Fe(III) is spontaneously released from the proteins and then taken up by the phytoplankton. Addition of EDTA resulted in a decrease in uptake rates in short-term experiments, pointing to a competition between EDTA and the cell for the released Fe. According to the literature, chelating agents such as EDTA can also promote dissolution of Fe from the core by direct reaction inside the

protein (Crichton et al. 1980), but this effect would effectively be invisible in our experiments since Fe would be transferred directly from the ferritin to the chelated pool. We now examine the coherence of the results from our growth and short-term uptake experiments and discuss the significance of our results to the recycling of Fe in marine ecosystems.

The results of the long-term growth experiments in which Fe-HoSF or Fe-Dps_{tery} was supplied as a Fe source in a medium containing EDTA in excess are surprising in two ways: (1) they exhibit stable, Fe-limited, exponential growth as is typical of Fe-buffered growth media (Fig. 1); and (2) in most instances, the actual growth rate is practically the same as observed when the same Fe concentration is supplied as FeEDTA in the presence of the same excess EDTA concentration (Table 1). In the case of Fe-HoSF, these results are explained by the similarity in the rate of dissociation of FeEDTA and the rate of release of Fe from the ferritin core: 0.13 d^{-1} versus 0.15 – 0.18 d^{-1} (see Tables 2 and 3). In effect, regardless of whether Fe is added as Fe-HoSF or FeEDTA, for given total concentrations of Fe and EDTA, the unchelated Fe in the medium is buffered at the same concentration, Fe', by the release from ferritin or dissociation from EDTA and by recomplexation by EDTA. As expected, when the concentration of EDTA is increased or decreased, the growth rate decreases or increases in response to the change in Fe' in the medium, as it does in an EDTA-buffered system. One can infer from the similar data obtained in the presence of Fe-Dps_{tery} that the rate of spontaneous Fe release from the Dps_{tery} core must be reasonably similar to that from the HoSF core.

According to the foregoing discussion, one might expect that the growth rate of cultures supplied with either Fe-HoSF or Fe-Dps_{tery} in the absence of EDTA might be very high. Indeed, the calculated rate of release of Fe from the corresponding Fe protein core is much higher than needed for the cells to grow at maximum rate. For example, a cell concentration of $5,000 \text{ cells mL}^{-1}$, a maximum growth rate of 1.4 d^{-1} , and a corresponding quota of $500 \text{ amol cell}^{-1}$ (Price 2005) would result in a cellular demand ($5 \times 10^6 \text{ cells L}^{-1} \times 5 \times 10^{-16} \text{ mol cell}^{-1} \times 1.4 \text{ d}^{-1} = 3.5 \text{ nmol L}^{-1} \text{ d}^{-1}$) that is less than the rate of Fe release from 30 nmol L^{-1} Fe-HoSF ($30 \text{ nmol L}^{-1} \times 0.15 \text{ d}^{-1} = 4.5 \text{ nmol L}^{-1} \text{ d}^{-1}$). The relatively modest growth rates observed in these experiments must thus result from a cause other than Fe limitation (Fig. 2, Table 1). This is confirmed by the similarity in the growth rates observed in the *T. weissflogii* cultures at two different Fe-Dps_{tery} concentrations: 0.91 d^{-1} for 40 nmol L^{-1} and 1.0 d^{-1} for 120 nmol L^{-1} . It seems likely that these cultures are growing at a less-than-optimum rate because of either toxicity or limitation by some trace metal(s) other than Fe.

Since no EDTA has been added to the medium, a metal such Cu can easily become toxic to phytoplankton; vice versa, a metal such Zn can easily become limiting if not added in the medium.

The rate of spontaneous Fe release from ferritin should presumably be on the same order as the rate of dissolution of ferrihydrite, whose structure has been found to be similar to that of the protein core (Fig. 4; Table 2) (StPierre et al. 1996; Andrews 1998; Nichol et al. 2003). In seawater (pH 8.1), amorphous ferric oxyhydroxide (AFO) has been shown to dissolve with rate constants that are dependent on the age of the precipitate (Rose and Waite 2003). Using an excess of siderophore (5 mmol L⁻¹ desferrioxamine B) to trap the dissolved Fe, Rose and Waite (2003) obtained first-order rate constants of 1.47 d⁻¹ and 0.41 d⁻¹ for 48-h- and 1-week-old AFO respectively, though others have reported somewhat slower rates of AFO dissolution (Kuma et al. 1992). A further study of the rate of Fe release from HoSF and Dps_{tery} yielded first-order rate constants for spontaneous release of 0.15 d⁻¹ and 0.25 d⁻¹, respectively, by extrapolation of ligand-assisted release rates to zero ligand concentrations (Castruita et al. 2007). These rates are remarkably similar to those calculated on the basis of the phytoplankton uptake data. The rate of spontaneous Fe release from iron-storage proteins is apparently not affected by the age of the core but depends on its physicochemical characteristics, decreasing with its nuclearity (size) and increasing with its P content. In our samples of Dps_{tery} the smaller core size apparently more than compensates for a P content that is only half that of HoSF (Castruita et al. 2007). The high reactivity of the ferritin cores may be important in cellular functions; in our experiment, it supports high phytoplankton growth rates.

Abundance of iron storage proteins in the marine environment—The role and importance of iron-storage proteins in marine phytoplankton remain unknown, but the storage of Fe from episodic inputs may be advantageous to some, particularly photosynthetic, marine organisms. Genes for putative iron-storage proteins have been identified in four of the five available cyanobacterial genomes: one bacterioferritin and one Dps in *T. erythraeum*, *Crocospaera watsonii* WH8501, *Prochlorococcus* MIT 9313, and *Synechococcus* CC9311; two bacterioferritins in *Prochlorococcus* MED 4; and none in *Synechococcus* WH8102. A putative ferritin gene has been identified in the diatom *Phaeodactylum tricornutum* and none in the *Thalassiosira pseudonana* genome. Recently Parker et al. (2007) have found evidence that several pennate diatoms possess a ferritin gene.

To have a better assessment of the likely abundance of iron-storage proteins in the marine world, we examined the Venter et al. (2004) database of the Sargasso Sea pooled DNA. Using the amino acid residues that act as ligands to the ferroxidase center in Dps for positive identification, we found 19 Dps-like genes (Castruita 2006). Using the amino acids involved in the ferroxidase center in bacterioferritin, we identified 36 putative bacterioferritin genes (Castruita 2006). The alignments showed a low level of identity at the amino acid level with the sequences from the cultured

organisms. For example, although *Prochlorococcus* biotypes represented more than 90% of the cyanobacterial-like scaffolds found in the environmental sample, all the environmental sequences coding for putative Fe-storage proteins had low similarity to the MIT 9313 Dps protein or MED 4 bacterioferritin sequences.

Since there is apparently a low sequence similarity among marine Dps and bacterioferritin sequences, it is likely that there are in fact many more such sequences in the Venter et al. (2004) database than we could identify on the basis of a few known sequences. We tentatively conclude that Fe-storage proteins, or at least their genes, are common in the marine environment.

Recycling of iron-storage proteins—Marine viruses have been recognized as important factors in microbial communities (Fuhrman 1999; Wilhelm and Suttle 1999). Cyanobacteria of the genera *Synechococcus* and *Prochlorococcus* commonly found at concentrations of 10⁷ cells L⁻¹ are known to be infected by cyanophages and may be lysed at a rate of 10% d⁻¹ (Suttle and Chan 1994; Wilhelm and Suttle 1999). Poorvin et al. (2004) have shown that virus-mediated lysis of heterotrophic bacteria and cyanobacteria releases intracellular Fe that is highly bioavailable to model marine phytoplankton. Iron-storage proteins could potentially be a significant fraction of that recycled pool.

The importance of Fe recycling from ferritins depends on the fraction of cellular Fe in storage proteins and the rate of release of Fe from the proteins in solution. A major fraction of the cellular Fe can be sequestered in ferritins, as demonstrated for example in *Synechocystis* sp. (Keren et al. 2004). It is likely that this fraction is particularly large after a transient Fe input. The rate of release of Fe from the proteins (0.15 d⁻¹) that we observed is slower than the typical growth rate of phytoplankton in the oceans. The net result is that the release of ferritin upon cell lysis could prolong the biological utilization of a transient Fe input.

Upon release from ferritin, the free Fe may be taken up by the biota, complexed by ligands in solution, or precipitated as amorphous ferric oxide. Precipitation is unlikely, unless the unchelated Fe concentration reaches nanomolar concentrations (Rose and Waite 2003). Whether the Fe released from ferritin would be directly taken up by phytoplankton or first complexed by ligands in seawater depends on the relative kinetics of the two processes. The available field data (e.g., Price et al. 1994; Rue and Bruland 1997) do not provide a clear-cut answer. The second-order rate constant for complexation is 1–2 × 10⁺⁶ L mol⁻¹ s⁻¹, whereas the apparent second-order rate constant for uptake appears to be in the range 5 × 10⁻⁵–2 × 10⁻³ (μg L⁻¹ Chl *a*)⁻¹ s⁻¹. The two rates are thus likely about equal at a ligand concentration ca. 1 nmol L⁻¹ and a Chl *a* concentration of a few micrograms per liter. The initial fate of the Fe released from ferritin (or produced from any source) should vary with ambient conditions, including the type, physiological status, and concentration of phytoplankton as well as the Fe-binding ligand concentration. Much remains to be elucidated regarding the biological recycling of Fe in surface seawater, an important source of Fe for primary production, in which ferritins may play a significant role.

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