

Effect of hydroxamate ferrisiderophore complex (ferrichrome) on iron uptake and growth of a coastal marine diatom, *Chaetoceros sociale*

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Abstract

Recent studies point out that dissolved iron in seawater is bound to strong organic complexes that have stability constants consistent with microbially produced siderophores. In the present study, the growth and iron uptake of the coastal marine diatom *Chaetoceros sociale* were experimentally measured in culture experiments at 10°C in media containing a terrestrial fungal hydroxamate ferrisiderophore, desferriferrichrome (DFC)-Fe(III) (2 DFC : 1 Fe and 10 DFC : 1 Fe) complex with an Fe concentration of 100 nM. The DFC-Fe(III) (2 : 1) medium as well as EDTA-Fe(III) (2 : 1) medium in the previous study induced high cell yields and fast growth of *C. sociale*. The short-term iron uptake rate in the DFC-Fe(III) (2 : 1) medium aged for 9 d at 10°C was much faster than the uptake rates in the DFC-Fe(III) (2 : 1 and 10 : 1) media aged for 1 d and in the DFC-Fe(III) (10 : 1) medium aged for 9 d at 10°C. Furthermore, the DFC-Fe(III) (2 : 1) medium induced the fastest iron uptake rate in the organic-Fe(III) complexes [DFC-Fe(III), EDTA-Fe(III), citric-Fe(III) and fulvic-Fe(III)] and solid amorphous Fe(III) hydroxide [am-Fe(III)] media in the present and previous studies. The addition of excess DFC stopped iron uptake by *C. sociale*. The dissociation of DFC-Fe(III) complex in seawater was determined by simple filtration (0.025 μm). Fast Fe(III) release was observed over 6–12 d in the DFC-Fe(III) (2 : 1) complex at 10°C, resulting from the thermal and microbial decomposition of the DFC complexed with Fe(III). This result is consistent with the faster iron uptake rate and growth rate of *C. sociale* observed in the DFC-Fe(III) (2 : 1) medium at 10°C. These results show that the dissolved DFC-Fe(III) complex in seawater supplies biologically available inorganic Fe(III) species, which may determine the iron uptake rate in culture media by providing bioavailable Fe as the DFC decomposes. The thermal, photochemical, and microbial decomposition of organic chelators complexed with Fe(III) in aquatic environments is probably one of the most important mechanisms for providing bioavailable inorganic Fe(III) species into these environments.

Iron is one of the most important biological and geochemical trace elements in the ocean. Recently, many studies of iron speciation in seawater using competitive ligand equilibration/cathodic stripping voltammetry (CLE/CSV) (Gledhill and van den Berg 1994; Rue and Bruland 1995, 1997; van den Berg 1995; Wu and Luther 1995; Gledhill et al. 1998; Nolting et al. 1998; Witter and Luther 1998) and Fe(III) hydroxide solubility measurement (Kuma et al. 1996, 1998a) pointed out that iron complexation with organic ligands is prevalent in seawater. Rue and Bruland (1995, 1997) found two classes of Fe(III)-binding organic ligands in the central North Pacific and equatorial Pacific surface waters: a strong ligand class (L₁), with an average concentration of 0.3–0.5 nM and a conditional stability constant

$K'_{\text{FeL}_1, \text{Fe}^{3+}} = 10^{23.1-23.7} \text{ M}^{-1}$, and a weaker ligand class (L₂), with a conditional stability constant $K'_{\text{FeL}_2, \text{Fe}^{3+}} = 10^{21.5-22.8} \text{ M}^{-1}$. These ligands have stability constants consistent with microbially produced siderophores (Lewis et al. 1995; Rue and Bruland 1997). In addition, Kuma et al. (1996, 1998a) reported that Fe(III) hydroxide solubility in the eastern Indian and North Pacific surface mixed layer is generally high and variable (0.5–3.6 nM), sometimes corresponds with the depth of high chlorophyll *a* concentrations, and is highest (2.5–3.6 nM) in the boundary zone between subtropical and subarctic water masses in the northern North Pacific Ocean. The high Fe(III) hydroxide solubility in the surface waters is probably due to a higher concentration or stronger affinity of natural organic Fe(III) chelators, which may be released by some phytoplankton or cyanobacteria (Trick et al. 1983a; Benderliev and Ivanova 1994; Wilhelm 1995; Hutchins et al. 1999a).

Many bioassays conducted with algae have demonstrated that the uptake of trace metals such as Zn, Cu, Mn, Cd, and Fe is primarily a function of the free ion concentration (or activity) of metal, [M^{z+}], in the artificial external medium (Anderson et al. 1978; Anderson and Morel 1982; and others). These results lead to the formulation of the “free-ion activity model (FIAM)” (Tessier et al. 1994; Campbell 1995). According to this model, the physiological effect on an alga is proportional to the free-ion activity rather than to the total dissolved metal concentration, [M]_T, or to the complexed metal concentration, [ML]. Although the calculated free ferric ion activity, [Fe³⁺], provides a useful approxi-

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mation for the availability of iron for uptake in the presence of synthetic chelators, the quantity of total inorganic Fe(III) species [Fe(III)'], dominantly the hydrolysis products $\text{Fe}(\text{OH})_2^+$ or $\text{Fe}(\text{OH})_3^0$ represents the actual instantaneous availability of iron for uptake at a given pH (Hudson and Morel 1990). Therefore, according to the FIAM, the presence in surface seawater of natural organic Fe(III) chelators with a strong affinity for Fe(III) reduces the bioavailable [Fe(III)']. However, apparent exceptions to the FIAM exist; for example, specific transport ligands, such as siderophores or lipophilic metal complexes, may be directly utilized by cells. The diffusion across a membrane by lipophilic organic metal complexes is an uptake mechanism for certain metals, such as Cu and Ni, in addition to the uptake of free metal ions (Poldoski 1979; Phinney and Bruland 1994; Campbell 1995). In addition, two possible mechanisms by which [Fe(III)L] may be made generally accessible to phytoplankton are via a cell-surface reduction process, such as the use of a plasma membrane ferrireductase (Jones et al. 1987; Hutchins et al. 1999a), and photochemical cycling (Wells and Mayer 1991; Johnson et al. 1994; Kuma et al. 1995). In both cases, reduced Fe(II) readily dissociates from the ligand, L, and is then acquired by a porter site (Rue and Bruland 1997).

Many aerobic and facultatively anaerobic microbes synthesize siderophores. Siderophores generally have a molecular weight less than 1,000, are synthesized only under iron-deficient conditions, and are capable of binding Fe(III) with high affinity and specificity. Many microbes can use not only endogenously produced siderophores, but also siderophores such as desferriferriochrome, coprogen, and rhodotorulic acid, which are produced by other organisms because they have specific transport systems to permit their passage through the cell envelop. Siderophores can generally be classified chemically as being either catecholates or hydroxamates, the most important functional differences between them being variations in stability and in affinity for Fe(III) (Earhart 1996). In addition, laboratory culture experiments have shown that several marine phytoplankton and bacteria can produce extracellular siderophores that have a strong chelating affinity for iron, copper, and other metals (Trick et al. 1983a,b; Haygood et al. 1993; Wilhelm and Trick 1994; Lewis et al. 1995; and others). Recent studies (Wells et al. 1994; Hutchins et al. 1999b; Wells 1999) have demonstrated that the addition of excess amounts of the fungal siderophore desferriferrioxime B (DFB) to Fe-replete waters greatly reduces the biological Fe uptake and, consequently, growth rate in phytoplankton. DFB is a hexadentate ligand with three hydroxamates and a terminal amine, which is protonated, and is a strong Fe(III)-complexing agent with a large thermodynamic stability constant between DFB and Fe^{3+} ($\log K = 31\text{--}32$) (Albrecht-Gary and Crumbliss 1998; Hudson et al. 1992). However, it has been suggested that a marine diatom clone (Soria-Dengg and Horstmann 1995), phytoplankton communities in the subarctic Pacific (Maldonado and Price 1999), and heterotrophic marine bacteria (Granger and Price 1999) can utilize DFB-bound Fe. Additionally, Hutchins et al. (1999a) have reported that iron bound to a variety of siderophores is more available to prokaryotic picoplankton (cyanobacteria) than to eukaryotic phytoplankton that

can efficiently assimilate porphyrin-complexed iron, suggesting that the two plankton groups exhibit fundamentally different iron-uptake strategies.

In the present study, we used hydroxamate siderophore from terrestrial microorganisms as a model for marine siderophore with similar iron-binding functional groups (Granger and Price 1999). We examined the effect of a hydroxamate-type ferrisiderophore complex [ferrichrome, DFC-Fe(III) complex] on the iron uptake and growth of the coastal marine diatom *Chaetoceros sociale* in laboratory culture. In particular, we investigated the iron uptake rate of phytoplankton and the dissociation of DFC-Fe(III) complex (2:1 and 10:1) media by comparing them with the rates in EDTA-Fe(III) complex (2:1 and 100:1) and solid amorphous Fe(III) hydroxide [am-Fe(III)] media previously reported (Kuma and Matsunaga 1995; Kuma et al. 1999).

Material and methods

Seawater collected from a coastal region near Hokkaido, Japan, in the northern Sea of Japan (salinity = 33.8) was used after being filtered through an acid-cleaned 0.45- μm Millipore cellulose membrane filter. The iron concentration in the filtered seawater was determined using a graphite furnace atomic absorption spectrophotometer after preconcentration by ammonium 1-pyrrolidinedithiocarbamate/diethylammonium diethyldithiocarbamate (APDC/DDDC) chloroform organic extraction (Landing and Bruland 1987). The iron concentration was ≤ 2 nM. Generally, dissolved iron concentrations ($< 0.45\text{-}\mu\text{m}$ fraction) in coastal waters in the northern Sea of Japan are seasonally and spatially variable, with a range of 1 to 10 nM (Kuma et al. 1998b, 1999). The concentrations of NO_3^- , PO_4^{3-} , and SiO_2 in the filtered seawater measured by a Technicon autoanalyzer were less than 0.5, 0.1, and 5 μM , respectively, which are negligible values compared with the concentrations added in the experiments (see below). Desferriferriochrome (iron-free ferrichrome) obtained from the rust fungus *Ustilago sphaerogena* was purchased from Sigma Chemical as a hydroxamate siderophore. The terrestrial fungal siderophore desferriferriochrome (DFC) is stable under desiccated conditions at 2–8°C and is a strong Fe(III)-complexing agent forming a 1:1 DFC-Fe(III) complex with a large thermodynamic stability constant ($\log K = 29$) (Fig. 1) (Lewis et al. 1995; Earhart 1996; Albrecht-Gary and Crumbliss 1998; Leong and Winkelmann 1998).

The following experimental procedure for DFC-Fe(III) was basically the same as that described for EDTA-Fe(III) (2:1 and 100:1) in Kuma et al. (1999).

Culture experiments—The filtered seawater was autoclaved for 20 min at 121°C (1.1 kg cm^{-2} pressure), and the culture medium was prepared by adding f/2 nutrient (Guillard and Ryther 1962) without trace metals and EDTA to the autoclaved filtered seawater. The f/2 medium contained 880 μM nitrate, 38 μM phosphate, and 35 μM silicate. The coastal marine cold-water diatom *C. sociale* (a single species) was grown in 1 liter of the f/2 media to which ferric iron stock solution ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 0.005 M HCl) was added to make an iron concentration of 100 nM. Cells were grown

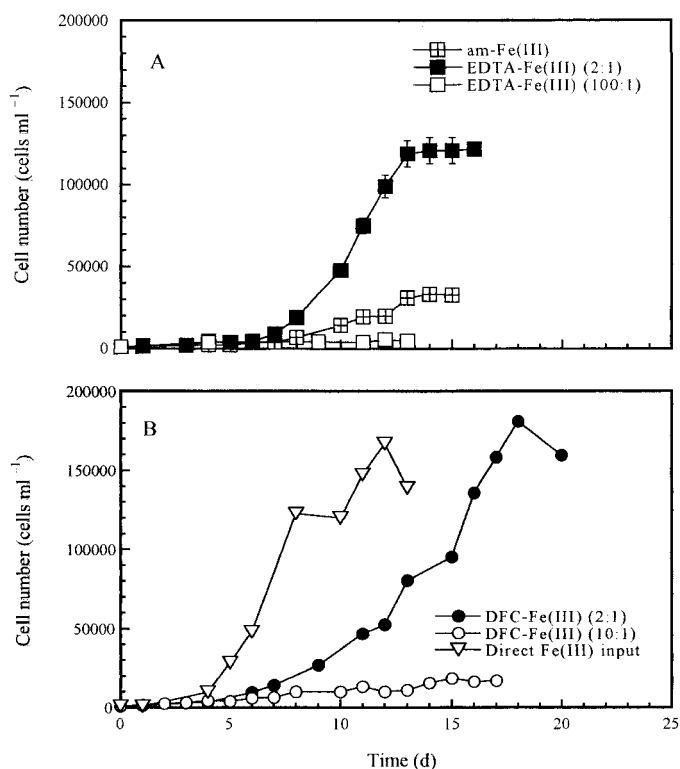


Fig. 2. Cell numbers of *C. sociale* cultures supplied with pre-mixed organic-Fe(III) complexes and solid am-Fe(III) (10°C). (A) Growth in EDTA-Fe(III) (2:1 and 100:1) complex and solid am-Fe(III) media in previous study (Kuma et al. 1999). Data on cell concentrations represent mean \pm 1 SD ($n = 3$); some error bars are covered by symbols. (B) Growth in DFC-Fe(III) (2:1 and 10:1) complex and direct Fe(III) input media. Data on cell concentrations represent mean ($n = 2$).

experiments. For studies of the direct iron input, the iron uptake experiment was started just after the direct addition of a small volume of stock ⁵⁹Fe(III) solution in the culture media (10°C), which already contained f/2 nutrient and *C.*

sociale culture. The cell concentration in the media was ca. 10,000 cells ml⁻¹. The nutrient concentrations were the same as those in the culture experiments described above. The iron uptake experiments were conducted in duplicate.

At each sample point (1, 3, 5 h), a 150-ml aliquot was transferred to a glass beaker containing 60 ml of 0.175 M Ti(III)-citrate-EDTA solution (0.175 M TiCl₃, Na₃citrate·2H₂O, and Na₂EDTA·2H₂O in filtered seawater, followed by an adjustment of the pH to 8 with NaOH solution) as demonstrated by Hudson and Morel (1989, 1990). The Ti(III) solution was used to rapidly dissolve am-Fe(III) precipitates and extracellularly adsorbed iron by reductive dissolution of Fe(III) without cellular damage to *C. sociale*. After the mixture was allowed to stand for 10 min, it was gently vacuum filtered through a quantitative filter paper (No. 5C, Advantec) that retains all precipitate $\geq 1 \mu\text{m}$ in size. The filter was rinsed with 30 ml of 0.05 M Ti(III)-citrate-EDTA solution. The drained filter was digested with 7 ml of conc. HNO₃:conc. HClO₄ (1:1) and then diluted to 20 ml. The γ -activity of 4 ml of diluted sample in a counting vial was measured using a scintillation counter, and the results were converted to amounts and rates of iron incorporation. In addition, iron uptake experiments for cell-free control media [DFC-⁵⁹Fe(III) (2:1) and direct ⁵⁹Fe(III)] at 10°C were conducted to ascertain the reductive dissolution of freshly precipitated and aged am-Fe(III) by the Ti(III) solution.

Results

Growth rate and cell yields—Figure 2 presents the results of the culture experiments for *C. sociale* in EDTA-Fe(III) (2:1 and 100:1) and solid am-Fe(III) media (10°C) from Kuma et al. (1999) (Fig. 2A), and in DFC-Fe(III) (2:1 and 10:1) and Fe(III) input media at 10°C in the present study (Fig. 2B). Growth rates (μ , d⁻¹) of cultures were determined from simple linear regressions of the natural log of cell concentration versus time (Fig. 2B) for the exponential growth phase (Table 1). In Kuma et al. (1999), the relative order for growth rates and maximal cell yields on different media was

Table 1. Growth rate, maximal cell yields in culture experiments (see Fig. 2) and iron uptake rates by *Chaetoceros sociale* (see Figs. 4 and 5) in media with organic-Fe(III) complexes [EDTA-Fe(III) (2:1 and 100:1) and DFC-Fe(III) (2:1 and 10:1)] and solid am-Fe(III). Iron uptake rate just after addition of ferric iron solution into culture containing *C. sociale*, 0; iron uptake rate in 1-d aged media, 1; iron uptake rate in 9-d aged media, 2.

Medium	Ratio L:Fe	Temper- ature (°C)	Growth rate	Maximal cell yields	Iron uptake rate
			(d ⁻¹)	(cells ml ⁻¹)	($\times 10^{-17}$ mol cell ⁻¹ h ⁻¹)
			(±1 SD for $n=3$ or range for $n=2$)		
Previous study ($n=3$, Kuma et al. 1999)					
solid am-Fe(III)	—	10	0.27±0.02 (0–13 d)	38,000±8,700	0.20±0.04 (1)
EDTA-Fe(III)	2:1	10	0.39±0.02 (0–13 d)	136,000±16,000	0.56±0.03 (1)
EDTA-Fe(III)	100:1	10	0.11±0.03 (0–12 d)	7,300±2,100	0.20±0.04 (1)
This study ($n=2$)					
DFC-Fe(III)	2:1	10	0.30–0.31 (0–18 d)	175,000–190,000	0.06–0.09 (1) 3.40–3.68 (2)
DFC-Fe(III)	10:1	10	0.21–0.23 (0–11 d)	17,000–19,000	0.03–0.04 (1) 0.29–0.63 (2)
Direct Fe(III) input	—	10	0.62–0.64 (0–8 d)	164,000–173,000	3.16–3.17 (0)

EDTA-Fe(III) (2:1) \gg solid am-Fe(III) > EDTA-Fe(III) (100:1) and all differences were significant ($P < 0.01$) (Fig. 2A, Table 1). In the present study (Fig. 2B), direct Fe(III) input to the culture solution induced the highest growth rate (0.62–0.64 d⁻¹) and very high maximal cell yields (164,000–173,000 cells ml⁻¹). DFC-Fe(III) (2:1) medium at 10°C resulted in the highest maximal cell yields (175,000–190,000 cells ml⁻¹) in this study, although the growth rate was approximately half of that for direct Fe(III) input. The growth rate and cell yields in DFC-Fe(III) (10:1) medium at 10°C were low and similar to those in am-Fe(III) medium at 10°C (Kuma et al. 1999).

Rate of dissociation of Fe(III) complexes—Figure 3 presents the results of the Fe(III) release from premixed organic-Fe(III) complexes [EDTA-Fe(III): 2:1 and 100:1; DFC-Fe(III): 2:1 and 10:1] in seawater at 5, 10, and 20°C. Also shown is the hydrolytic precipitation rate of Fe³⁺ without any added organic ligands in seawater (10°C). In Figure 3A, the hydrolytic precipitation rate of Fe³⁺ in seawater (pH 8) at 10°C was extremely fast, resulting in extremely low 0.025- μ m filterable (dissolved) iron concentrations within short aging times (only 1.7 nM after 6 h of aging). The dissolved Fe(III) concentrations during aging for 1 to 25 d in the iron-only experiment were nearly constant, with a range of 0.6 to 0.8 nM. The constant dissolved iron concentrations over time are fairly consistent with the iron solubility values of Fe(III) hydroxide [am-Fe(III)] in seawater reported for previous studies (Kuma et al. 1996, 1998a, b). In contrast, the release of Fe(III) from organic Fe(III) complexes in seawater was relatively slow and depended on the ratio of Fe(III) to organic ligand, the class of organic ligands, and the water temperature. For EDTA-Fe(III) (100:1) at 10°C (Fig. 3A), Fe(III) release (loss from solution) was not observed, whereas some loss was observed for EDTA-Fe(III) (2:1) at the same temperature. For DFC-Fe(III) (2:1) complexes at 5, 10, and 20°C (Fig. 3B), higher temperatures induced a more rapid Fe(III) release, whereas little release was observed during aging for 23 d at 5°C. No Fe(III) release from DFC-Fe(III) complex at a ratio of 10 organic ligand:1 iron was observed during aging for 23 d at 5 and 10°C (Fig. 3C).

Iron uptake rate—After aging the media for 1 d in the dark (10°C), iron uptake rates by *C. sociale* (Fig. 4B) were measured in DFC-Fe(III) (2:1 and 10:1) complex media at 10°C. These experiments corresponded to the situations where no Fe(III) release was observed (Fig. 4A). Iron uptake rates (Fig. 4B) were also measured after aging the DFC-Fe(III) (2:1 and 10:1) media for 9 d in the dark. These experiments corresponded to the situation where rapid Fe(III) release from DFC-Fe(III) (2:1) medium was observed, and where no release from DFC-Fe(III) (10:1) medium was observed during 6–12 d (Fig. 4A). Titanium treatment was not completely effective in removing aged am-Fe(III) from the DFC-Fe(III) (2:1) after 9 d of aging at 10°C, although freshly precipitated am-Fe(III) from the am-Fe(III) medium after 1 h, 3 h, 5 h, and 1 d of aging was almost completely removed by the Ti(III) treatment. Therefore, the iron uptake rate by *C. sociale* (Fig. 4B) in 9-d aged

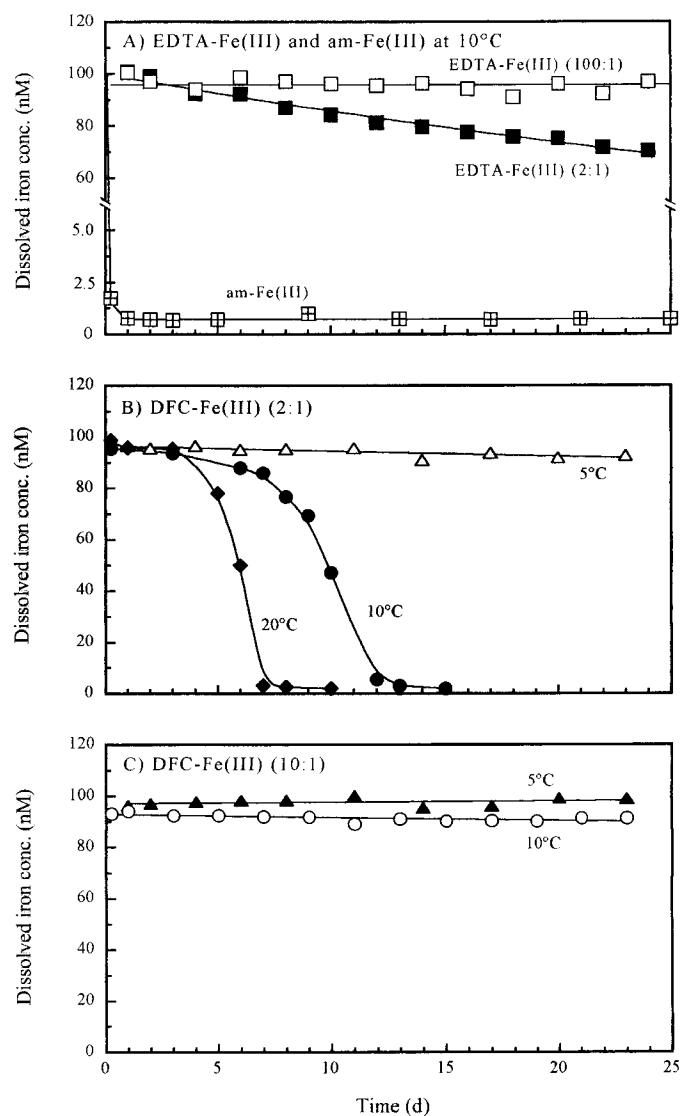


Fig. 3. Time-course of Fe(III) dissociative precipitation of premixed organic Fe(III) complexes and hydrolytic precipitation of Fe³⁺ [am-Fe(III)] in seawater at 5 and 10°C. (A) Dissociative precipitation of EDTA-Fe(III) (2:1 and 100:1) complex and hydrolytic precipitation of Fe³⁺ [am-Fe(III)] at 10°C. (B) Dissociative precipitation of DFC-Fe(III) (2:1) complex at 5, 10, and 20°C. (C) Dissociative precipitation of DFC-Fe(III) (10:1) complex at 5 and 10°C.

DFC-Fe(III) (2:1) medium was obtained by subtracting the amount of insoluble iron in cell-free medium from the amount of iron uptake in medium containing cells. Short-term iron uptake rates (mol cell⁻¹ h⁻¹) were determined from the slope of iron uptake (mol cell⁻¹) versus time using linear regression (Table 1). The iron uptake rates by *C. sociale* in DFC-Fe(III) (2:1 and 10:1) media aged for 1 d at 10°C were extremely low (0.03–0.09 $\times 10^{-17}$ mol cell⁻¹ h⁻¹; Table 1). The iron uptake rate in DFC-Fe(III) (10:1) medium aged for 9 d was also low, whereas that in DFC-Fe(III) (2:1) aged for 9 d was the highest (3.40–3.68 $\times 10^{-17}$ mol cell⁻¹ h⁻¹) among the iron uptake rates observed in both the present and previous (Kuma et al. 1999) studies of iron complexes.

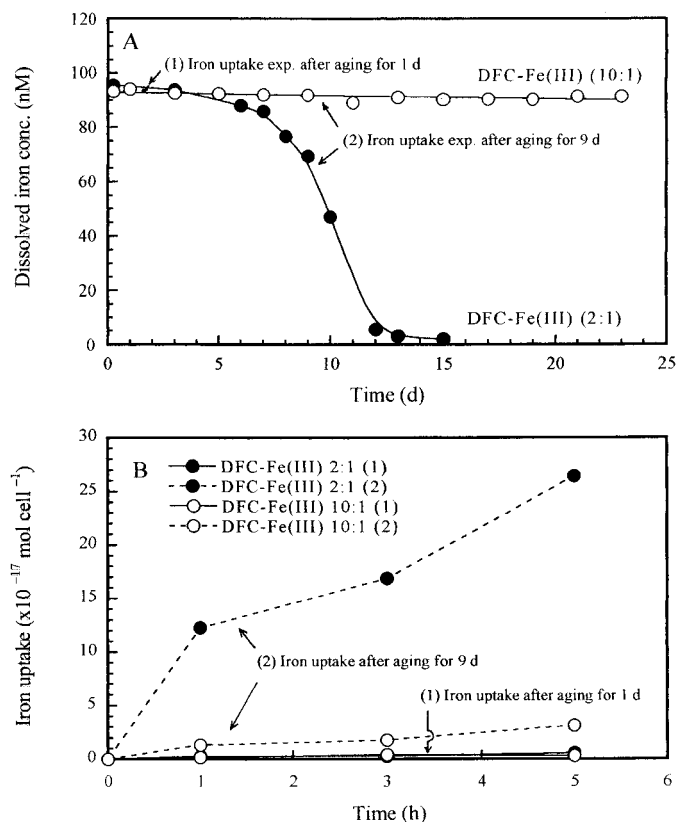


Fig. 4. Time-course of Fe(III) dissociative precipitation of DFC-Fe(III) (2:1 and 10:1) complex in seawater at 10°C and short-term iron uptake by *C. sociale* in the DFC-Fe(III) (2:1 and 10:1) complex media at 10°C. (A) Dissociative precipitation of DFC-Fe(III) complex indicating (1) iron uptake experiments in DFC-Fe(III) media after aging for 1 d and (2) iron uptake experiments in the media after aging for 9 d. (B) Iron uptake rates in (1) 1-d aged and (2) 9-d aged DFC-Fe(III) media. Data on iron uptake represent mean ($n = 2$).

In Kuma et al. (1999), the iron uptake rate by *C. sociale* was greater in EDTA-Fe(III) (2:1) than in EDTA-Fe(III) (100:1) (Fig. 5A, Table 1). The iron uptake rates by *C. sociale* in DFC-Fe(III) (2:1) medium aged for 9 d at 10°C and direct Fe(III) input medium were remarkably high and nearly the same, with values of $3.16\text{--}3.68 \times 10^{-17}$ mol cell $^{-1}$ h $^{-1}$ (Fig. 5B, Table 1).

Discussion

In oxic seawater, iron colloids may exist in a number of noncrystalline and crystalline ferric oxide forms and may have widely varying origins, chemical compositions, and sizes. The principal phases commonly found in natural waters are amorphous Fe(III) hydroxide [am-Fe(III)], α -Fe $_2$ O $_3$ (hematite), γ -FeOOH (lepidocrocite), and α -FeOOH (goethite) (Murray 1979). Because the internal structure of iron colloids affects their solubilities and dissolution rates, it should also influence the uptake rate of iron by phytoplankton. In a previous study (Kuma and Matsunaga 1995), highest maximal cell yields of coastal marine phytoplankton (a

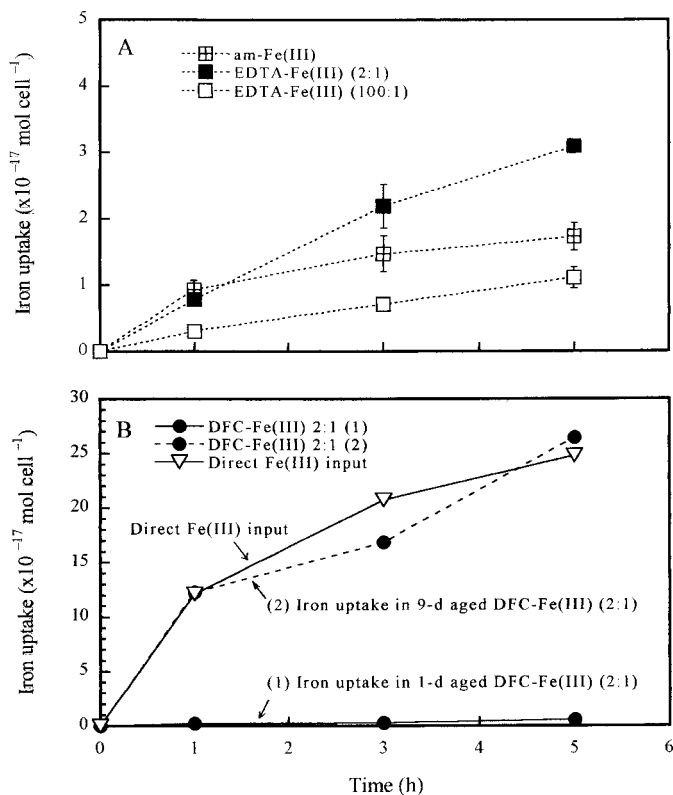


Fig. 5. Short-term iron uptake by *C. sociale* in cultures supplied with premixed organic Fe(III) complexes, solid am-Fe(III), and direct Fe(III) input (10°C). (A) Iron uptake rates in EDTA-Fe(III) (2:1 and 100:1) and solid am-Fe(III) media in the previous study (Kuma et al. 1999). Data on iron uptake represent mean ± 1 SD ($n = 3$); some error bars are covered by symbols. (B) Iron uptake rates in (1) 1-d aged and (2) 9-d aged DFC-Fe(III) (2:1) media and in direct Fe(III) input medium. Data on iron uptake represent mean ($n = 2$).

coastal marine diatom, *Phaeodactylum tricornutum*, and two red tide marine flagellates, *Heterosigma akashiwo* and *Gymnodinium mikimotoi*) were observed with additions of am-Fe(III) followed by γ -FeOOH, then Fe $_5$ O $_7$ (OH)·4H $_2$ O (hydrated ferric oxyhydroxide polymer), and lowest rates were obtained with α -FeOOH. This order is consistent with iron solubilities and estimated dissolution rate constants of these ferric oxides in seawater. Therefore, the ability of colloidal iron to provide a source of iron for phytoplankton is related to the thermodynamic stability and kinetic lability of the colloidal ferric oxide phases, which probably control the uptake rate of iron by phytoplankton and, hence, their growth rate (Wells et al. 1983; Rich and Morel 1990; Kuma and Matsunaga 1995).

The hydrolytic precipitation rate of Fe $^{3+}$ in seawater (pH 8) was extremely fast, resulting in an extremely low 0.025- μ m filterable (dissolved) iron concentration with short aging times (Fig. 3A) (Kuma et al. 1999). Solid am-Fe(III) additions to cultures of *C. sociale* induced only a small increase in cell number (Fig. 2A) compared with the additions of organic-Fe(III) complexes [such as EDTA-Fe(III) (2:1) and

DFC-Fe(III) (2:1)], probably because of slower iron uptake rates in the am-Fe(III) medium (Fig. 5A).

Wells et al. (1983) reported that the addition of freshly precipitated am-Fe(III) to culture experiments with the coastal marine diatom *Thalassiosira pseudonana* produced better cell yields than the addition of aged and heated amorphous phases because of the increase in the thermodynamic stability of ferric oxide after aging and heating. In addition, the dissolution rates of the amorphous phase decreased rapidly with aging time because of the slow conversion to more stable phases (Wells et al. 1983; Wells and Mayer 1991). Our results showed that freshly precipitated am-Fe(III) (aged for 1 d at 10°C) was almost completely dissolved by the Fe(III) reductive dissolution with Ti(III) treatment. However, aged am-Fe(III) produced in a DFC-⁵⁹Fe(III) (2:1) cell-free medium during aging for 9 d at 10°C (Fig. 4A) was incompletely reductively dissolved by Ti(III) solution because of the much slower dissolution of aged am-Fe(III). This result suggests that the internal structure of ferric oxide in culture solution changed to more highly ordered phases with time during the 20- to 30-d culture experiments, resulting in a decreased dissolution rate with time.

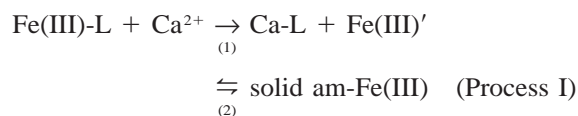
Kuma et al. (1999) reported that the addition of organic-Fe(III) complexes such as EDTA-Fe(III) (2:1 and 100:1), citric-Fe(III) (100:1), and fulvic-Fe(III) (0.1–1 ppmC) into seawater (pH 8) retards the precipitation of am-Fe(III) because of the slow dissociation of organic-Fe(III) complexes in seawater. This rate depends on the ratio of organic ligand to Fe(III) and the complexing ability of organic ligand with Fe(III) [Fig. 3A for EDTA-Fe(III)]. The solution equilibrium between ferric ion and organic ligand is further affected by inorganic complexation of the ferric ion with seawater anions (such as OH⁻, Cl⁻, and CO₃²⁻), and by organic ligand association with protons and major cations (such as Ca²⁺ and Mg²⁺). These side reactions can reduce the free ferric ion and free organic ligand concentrations, and must be considered when defining a conditional stability constant for a specific set of solution conditions, such as those in seawater (Ringbom 1963). The conditional stability constant (assuming 1:1 complexation) with respect to Fe³⁺ can be formulated as follows:

$$K'_{\text{Fe(III)L}} = [\text{Fe(III)L}]/[\text{Fe}^{3+}][\text{L}'] \quad (1)$$

$$\alpha_{\text{Fe}^{3+}} = [\text{Fe(III)'}]/[\text{Fe}^{3+}] \quad (2)$$

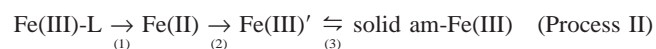
where [Fe(III)L] is the concentration of Fe(III) complexed by organic ligand, [L'] is the concentration of ligand not complexed with iron, free [L'] and complexes of L with the major cations, protons, and possibly other trace metals in solution, and [Fe(III)'] is the concentration of Fe(III) not complexed with organic ligand [including all inorganic Fe(III) species]. The value of $K'_{\text{Fe(III)L}}$ is conditional upon the solution composition. Values for the inorganic side reaction coefficient ($\alpha_{\text{Fe}^{3+}}$) at pH 8 range from 10¹⁰ (Hudson et al. 1992) to 10¹¹ (Byrne et al. 1988). The following exchange reaction between organic-Fe(III) complex and major alkaline earth metals (such as Ca²⁺ and Mg²⁺) in seawater is one of the most important processes resulting in slow dissociation of organic-Fe(III) complexes and subsequent

Fe(III) hydrolytic precipitation (Brezonik 1994; Stumm and Morgan 1996):



where reaction (1) represents metal exchange of Fe(III)-L by Ca²⁺ and (2) is Fe(III) hydrolytic precipitation. Fe(III)-L and Ca-L are dissolved Fe(III) and Ca complexed by organic ligand L (such as EDTA and fulvic acid), respectively, and Fe(III)' is dissolved Fe(III) not complexed with L and includes all the inorganic Fe(III) species. The high concentration of alkaline earth cations in seawater probably caused the dissociation of organic Fe(III) complexes through the metal exchange reaction. In estuarine and coastal waters, the natural dissolved organic-Fe(III) complexes supplied by riverine input such as fulvic-Fe(III) may play an important role in the supply of biological available iron by heightening the dissolved inorganic Fe(III) concentration through the dissociation of organic-Fe(III) complexes during mixing with seawater (Kuma et al. 1999).

Other factors influencing iron release from organic-Fe(III) complexes include cell-surface reduction (Jones et al. 1987; Hutchins et al. 1999a; Maldonado and Price 1999) and photochemical reduction cycling (Wells and Mayer 1991; Johnson et al. 1994; Kuma et al. 1995). In addition, photoproduced bioavailable Fe(II) in seawater is rapidly oxidized and hydrolyzed to the bioavailable inorganic [Fe(III)'] species (Millero et al. 1987; Kuma et al. 1995). The mechanism can be summarized as



where reaction (1) represents cell-surface reduction or photoreduction of organic-Fe(III) complexes, (2) represents oxidation of Fe(II) and then hydrolysis, and (3) is Fe(III) hydrolytic precipitation.

Although there is no evidence that siderophores are used in situ by marine microorganisms, terrestrial microbial iron availability is largely determined by the coordination chemistry associated with the siderophores, a class of organic compounds that have a high and specific chelating affinity for Fe(III). The important chemical events in the processes involved in siderophore-mediated microbial iron transport are selective chelation of Fe(III) in the presence of other environmentally prevalent metal ions, molecular recognition of the Fe(III)-siderophore complex, transport of the iron in complexed form across the cell membrane, and deposition of the iron at an appropriate site within the cell (Albrecht-Gary and Crumbliss 1998). The terrestrial fungal siderophore DFC used in this study is unstable at high temperature (>8°C), resulting in the rapid Fe(III) release from DFC-Fe(III) (2:1) media at 20 and 10°C after aging for 3 and 6 d, respectively (Fig. 3B), whereas no Fe(III) release from DFC-Fe(III) (2:1) at 5°C and DFC-Fe(III) (10:1) at 5 and 10°C was observed, even during aging for as long as 23 d (Fig. 3B, C).

DFC is a very powerful natural complexing agent forming a 1:1 complex with iron. At pH 8.0–8.2, the solubility product of solid Fe(III) hydroxide for seawater from the open

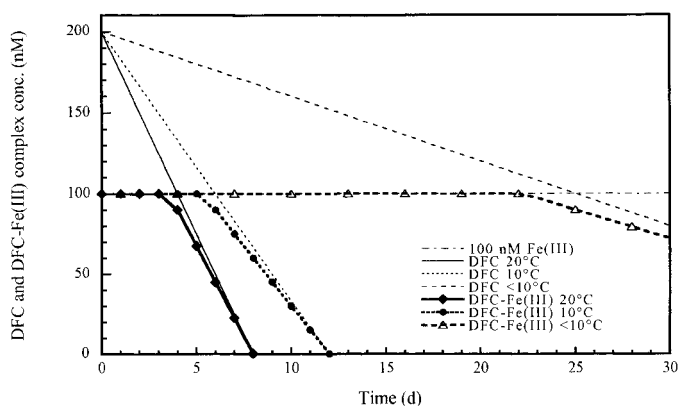


Fig. 6. The thermal decomposition rate model of DFC (thin lines) and DFC-Fe(III) complex (thick lines) with 200 nM DFC (initial concentration at day 0) and 100 nM Fe(III) at <10, 10, and 20°C.

ocean has a $\log^*K_{so} = \log[\text{Fe}^{3+}][\text{H}^+]^{-3} = 4.4-4.6$ (Kuma et al. 1996). Therefore, $[\text{Fe}^{3+}]$ in seawater is about 10^{-20} M. Regrettably, we cannot determine the conditional stability constant $[K'_{\text{Fe(III)-DFC}}]$ in seawater because the metal-ligand stability constants for DFC association with major cations in seawater are not known. However, assuming that $K'_{\text{Fe(III)-DFC}}$ in seawater is $\geq 10^{21} \text{ M}^{-1}$, about 90% of the DFC will be present as DFC-Fe(III) complex using Eq. 1 ($K'_{\text{Fe(III)-DFC}}[\text{Fe}^{3+}] = [\text{Fe(III)L}]/[\text{L}']$). We suggest that the Fe(III) release from DFC-Fe(III) (2:1) media at 10 and 20°C was not observed after short-term aging (Fig. 3B) because the equilibrium concentration of inorganic Fe(III) species was not limited by Fe(III) hydroxide solubility in organic ligand-free seawater. The solubility value in organic-free seawater at pH 8 is estimated to be 0.1–1 nM $[\text{Fe(III)}']$ using $[\text{Fe}^{3+}] = 10^{-20}$ M and $\alpha_{\text{Fe}'} = [\text{Fe(III)}']/[\text{Fe}^{3+}] = 10^{10-11}$. Simple equilibrium calculations using $K'_{\text{Fe(III)-DFC}} \geq 10^{21} \text{ M}^{-1}$ show that almost all Fe(III) in media containing DFC-Fe(III) at a ratio of DFC/Fe(III) of >1.1 and total Fe equal to 100 nM Fe(III) should exist as soluble DFC-Fe(III) complex, and that Fe(III) hydroxide precipitation will not occur in seawater. However, if the complex is broken down by thermal decomposition (or other mechanisms like photochemical and microbial decomposition), especially if the medium has a lower DFC-Fe(III) ratio, Fe(III) precipitation will be enhanced. In addition, a small amount of Fe tied up in particulate size classes may be incorporated into bacteria contained in culture solution.



where reaction (1) represents the thermal decomposition of DFC and (2) is Fe(III) hydrolytic precipitation. Figure 6 shows a model of the thermal decomposition rate of DFC alone (thin lines) and DFC-Fe(III) complex (thick lines) in a system containing 200 nM DFC and 100 nM Fe(III) at <10, 10, and 20°C. This model assumes that the thermal decomposition rate of DFC is constant at constant temperature and is slower at lower temperatures, and that the concentration of inorganic Fe(III) species, $[\text{Fe(III)}']$, is negligibly low. In this model, by assuming that the conditional stability constant $[K'_{\text{Fe(III)-DFC}}]$ is about 10^{21} M^{-1} , approxi-

mately 90% of the DFC should exist in soluble DFC-Fe(III) complex form from Eq. 1. Therefore, DFC-Fe(III) (2:1) media at low temperature will be stable for a long time (e.g. ≥ 23 d at 5°C). If inorganic iron is the form taken up by phytoplankton, this will probably result in a delay of phytoplankton growth and lower growth rates. At a given temperature, the time period at which the complex is stable will depend on the DFC concentration at the start of the experiment. Dissociation of DFC-Fe(III) complex by thermal decomposition will heighten the concentration of biologically available inorganic Fe(III) species, which could be an important factor determining the iron uptake rate, above the equilibrium concentration in media. This is supported by the fact that the higher Fe(III) loss from DFC-Fe(III) complexes in media aged for 9 d at 10°C (Fig. 4A) resulted in a higher iron uptake rate by *C. sociale* (Fig. 4B) that is almost the same as for direct Fe(III) input. In the DFC-Fe(III) (2:1) medium at 10°C, the delayed Fe(III) loss from the complex during the middle of the experiment (day 6–12 in Fig. 4A) resulted in the retardation of phytoplankton growth, in contrast with the rapid growth that occurred in the direct Fe(III) input medium (Fig. 2B). In addition, in cases where no Fe(III) release was observed [EDTA-Fe(III) (100:1) and DFC-Fe(III) (10:1) at 10°C (Fig. 3)], little or no growth of *C. sociale* was seen (Fig. 2, Table 1).

Wells et al. (1994) used a terrestrial fungal siderophore DFB to diminish iron availability in seawater samples containing natural populations. The conditional stability constant $[K'_{\text{Fe(III)-DFB}}]$ with respect to Fe^{3+} in seawater is $\sim 10^{25-26} \text{ M}^{-1}$, which is high enough for added DFB to effectively complex any inorganic Fe species in seawater up to the concentration of the added DFB (Wells et al. 1994). In the presence of excess DFB in short-term ^{59}Fe uptake experiments, iron was unavailable for uptake by phytoplankton and bacteria (Wells et al. 1994; Hutchins et al. 1999b; Wells 1999). According to the FIAM, the presence of organic Fe(III) chelators with strong affinity reduces the amount of bioavailable $[\text{Fe(III)}']$, although specific transport ligands, such as siderophores or lipophilic metal complexes, may be directly utilized by cells. It has been reported that iron transport by siderophores in aquatic environments is a two-step process; iron bound to extracellular hydroxamate-type siderophores is scavenged by the catechol-type siderophores associated with the cell surface, and the catechol-siderophore complex is then transported into the cell (Wilhelm and Trick 1994; Wilhelm et al. 1998). However, Granger and Price (1999) reported that a catechol-produced strain of heterotrophic marine bacteria could not take up iron bound to DFB. This experimental evidence suggests that iron uptake by the strain may be mediated by surface-associated catechol siderophores that scavenge inorganic Fe(III) species, as well as iron bound to weaker complexes, such as EDTA. It seems that iron bound to DFC used in this study cannot be utilized by phytoplankton. Several studies, on the other hand, suggest that a marine diatom clone, *P. tricornutum* (Soria-Dengg and Horstmann 1995), phytoplankton communities in the subarctic Pacific (Maldonado and Price 1999), the majority of heterotrophic marine bacteria strains (Granger and Price 1999), and prokaryotic picoplankton (cyanobacteria) (Hutchins et al. 1999a) can utilize iron bound to DFB. These studies dem-

onstrated that these phytoplankton assimilate iron through the biologically mediated Fe(III) reduction process, such as Process II, and that bacteria have outer membrane receptors that recognize iron bound to their own siderophores as well as specific receptors for siderophores produced by other species. In addition, the assimilation of Fe by *C. sociale* in this study may be potentially the result of biological transformation of Fe by bacteria that makes organically complexed Fe more available to the eukaryotic phytoplankton (Wilhelm 1995; Hutchins et al. 1999a).

Although the thermal stability of marine siderophores and the uptake mechanism of iron bound in siderophore-Fe(III) complexes in seawater remain unknown, siderophore-Fe(III) complexes are important in providing bioavailable inorganic Fe species ([Fe(II)'] and [Fe(III)']) in seawater. Our results suggest that the thermal and microbial decomposition of organic ligand complexed with Fe(III) in aquatic environments may play an important role in supplying biologically available inorganic Fe(III) species.

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