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Direct use of inorganic colloidal iron by marine mixotrophic phytoplankton

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Abstract

Three species of photosynthetic flagellates capable of phagotrophy (mixotrophic species) were tested for their abilities to use inorganic iron colloids for growth. *Ochromonas* sp., *Chrysochromulina ericina* (a coastal strain), and *C. ericina* (an oceanic strain) were grown in iron-free seawater supplemented with 1 μM Fe-ferrihydrite (amorphous hydrous ferric oxide), magnetite (Fe_3O_4)/maghemite (γ-Fe_2O_3), hematite (α-Fe_2O_3), or goethite (α-FeOOH). Desferrioxamine B, an iron-binding siderophore, was used to reduce the concentration of dissolved iron in the colloid-amended media, and none of the flagellates were able to use its iron complex as an iron source under the conditions of the experiments. Both strains of *Chrysochromulina* grew at 35%–70% of their maximum rates with goethite, hematite, and magnetite/maghemite but were unable to use ferrihydrite. *Ochromonas* grew well with ferrihydrite but could not use any of the other forms. *Thalassiosira oceanica* (clone 1003) and *Thalassiosira pseudonana* (clone 3H), diatoms that could only take up dissolved forms of iron, were unable to use any of the colloids tested. The mechanism of iron acquisition by the flagellates appeared to involve ingestion of the iron colloids, because bacteria resident in the cultures were too iron poor to be a significant source of iron and were unable to use the iron contained in the colloids themselves. Variations in the sizes of the colloids were hypothesized to account for differences in their availability, independent of colloid chemical stability. The results provide the first strong evidence for direct use (i.e., without prior dissolution) of colloidal iron by mixotrophic phytoplankton and document a new pathway of iron acquisition that may be important for their survival in low-iron waters of the sea.

Bacteria and phytoplankton acquire iron through a complex series of extracellular reactions that are influenced by aqueous iron chemistry and speciation. Synthesis and transport of siderophores (Haygood et al. 1993; Wilhelm and Trick 1994; Granger and Price 1999), and the use of ferric chelate reductases (Lynnes et al. 1998; Xue et al. 1998; Maldonado and Price 1999, 2000) and high-affinity, inorganic iron transporters (Hudson and Morel 1990, 1993) are some of the mechanisms that they have evolved to procure iron in its various forms from the environment. Low concentrations of dissolved iron ($<$0.2–0.4 μm), which are characteristic of oceanic waters (Johnson et al. 1997), are often limiting to net primary productivity (Martin et al. 1994; Coale et al. 1996) and, at times, to secondary production (Pakulski et al. 1996; Tortell et al. 1996). Because of its requirement in photosynthetic and respiratory metabolism, iron is in high demand by all planktonic organisms and is the object of intense interspecific competition.

Dissolved iron exists largely as organic complexes (Gledhill and van den Berg 1995; Rue and Bruland 1995; Wu and Luther 1995) and colloidal forms (Wu and Luther 1994; Kuma et al. 1998) of uncertain composition. The inorganic species, which are minor components of the dissolved phase, are hydrolysis products [$Fe(OH)_2^+$, $Fe(OH)_4^-$] of low solubility. Uptake of the truly dissolved forms is thought to be the norm for phytoplankton, because even the smallest colloids, made up of as little as six iron atoms, are unavailable for transport (Rich and Morel 1990). Recently, however, a mixotrophic flagellate, a photosynthetic phytoplankton species capable of consuming particles, was shown to derive its iron ration by ingesting iron-containing bacteria (Maranger et al. 1998). An extrapolation of these results to the field suggested that, in iron-poor waters, some types of particulate iron might be important for growth of phytoplankton and that phagotrophy could represent a significant pathway of iron uptake by phototrophs.

Because of its low solubility, iron exists in particulate form (operationally defined as $>$0.2–0.4 μm) in oxygenated seawater, often at concentrations greater than the dissolved species. This is particularly true in regions where atmospheric inputs are large and in coastal waters receiving freshwater runoff from land (Johnson et al. 1997; Kuma et al. 1998). The particulates vary greatly in size, from 5 nm to 1 mm (McCave 1984; Wells and Goldberg 1994), and encompass a variety of forms, including iron in oxyhydroxides, aluminosilicates, organic/inorganic colloids, and living organisms (Price and Morel 1998). Although they are present in higher concentrations, many of the particulates are relatively refractory and not thought to be directly available for uptake by phytoplankton without prior dissolution.

Since the early 1930s, there has been considerable interest in understanding the availability of colloidal iron for phytoplankton growth (Cooper 1935; Harvey 1937*a,b;* Goldberg 1952). Experimental studies that used diatoms have established that a variety of well-defined iron colloids can be used as iron sources for growth. Their efficacy for plankton nutrition is related to their thermodynamic stability and photochemical lability (Rich and Morel 1990; Wells et al. 1991*a,b;* Kuma and Matsunaga 1995). Dissolution of the

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oxide phases determines the rate of supply of dissolved iron and hence its availability to phytoplankton. Highly crystalline forms of iron oxide (e.g., goethite) dissolve too slowly to fulfill the phytoplankton requirements, but amorphous forms (e.g., ferrihydrite) are readily available (Rich and Morel 1990; Kuma and Matsunaga 1995). No evidence for direct uptake of colloids by phytoplankton (i.e., without their prior solubilization) has been obtained, despite initial claims to the contrary (Goldberg 1952; Harvey 1937*a*).

Recently, a number of marine plankton have been shown to ingest particulate forms of iron and to assimilate and regenerate iron from these phases. Barbeau et al. (1996) demonstrated that heterotrophic flagellates were capable of solubilizing ferrihydrite, and Chase and Price (1997) showed that *Paraphysomonas imperforata* obtained iron from bacterial prey and was able to use freshly precipitated iron, adsorbed to the surfaces of bacteria, for growth. Similar results were obtained for a mixotrophic flagellate, *Ochromonas* sp. (Maranger et al. 1998), highlighting the potential importance of particulate iron for phytoplankton growth.

Little is known about the pathways of nutrient acquisition by mixotrophic phytoplankton, but their ability to exploit particulate forms of nutrients may be one reason for their ecological success in the sea. Colloids are among the most abundant phases of iron in the ocean (Cooper 1935; Mill 1980). They exist in crystalline and noncrystalline states and have been identified in aerosols over land and sea (Duce and Tindale 1991). The present study examines whether pure forms of inorganic iron colloids [ferrihydrite (amorphous hydrous ferric oxide), maghemite (γ-Fe_2O_3), hematite (α-Fe_2O_3), and goethite (α-FeOOH)] can be used as iron sources by some mixotrophic phytoplankton.

Materials and methods

Phytoplankton species—Three photosynthetic flagellates and two centric diatoms, *Thalassiosira pseudonana* and *Thalassiosira oceanica*, were obtained from the North East Pacific Culture Collection and the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (Table 1). The flagellates, *Ochromonas* sp., *Chrysochromulina cf ericina*, and *C. ericina* were mixotrophic species, capable of ingesting bacteria and other particles (Jones et al. 1993; Maranger et al. 1998). *T. pseudonana* was maintained in axenic culture, but cultures of the other isolates contained low densities of bacteria ($\sim 10^4$–10^5 ml^{-1}).

Growth conditions and medium—All phytoplankton were grown in Aquil medium (Price et al. 1988/1989) with standard enrichments of phosphate (PO_4^{3-}) and silicate (SiO_3^{2-}) and with 300 μM NO_3^- and 50 μM NH_4^+. The nutrient enrichments were purified of trace metals by use of Chelex 100 ion-exchange resin (Bio-Rad Laboratories), according to the procedure of Price et al. (1988/1989). Media were microwave sterilized in acid-washed Teflon bottles and were enriched with filter-sterilized (0.22 μm Acrodisc) vitamins (B_{12}, thiamine, and biotin) and trace metals. Two types of trace metal enrichments were added to the media: in one of them, the trace metals (Co, Cu, Mn, and Zn) were added complexed to 100 μM ethylenediaminetetra-acetic acid (EDTA) as in the Aquil recipe, and, in the other, the trace metals were added without EDTA. Trace metal concentrations buffered with 100 μM EDTA were added to give free-ion concentrations of Co, Cu, Mn, and Zn of $10^{-10.88}$, $10^{-13.79}$, $10^{-8.27}$, and $10^{-10.88}$ M, respectively. These concentrations were calculated by use of the chemical equilibrium program MINEQL (Westall et al. 1976). This medium was designated ''−Fe Aquil.'' In media where the trace metals were not buffered with any EDTA, total concentrations of Co, Cu, Mn, and Zn added were $10^{-8.60}$, 10^{-9}, $10^{-7.64}$, and $10^{-8.40}$ M, respectively. This medium was designated ''−Fe, −EDTA Aquil.'' Molybdenum and Se concentrations in all media were 10^{-7} and 10^{-8} M.

Iron was added separately, either as a filter-sterilized (0.22 μm Acrodisc) iron-ligand complex with ETDA (1 : 1.05) or desferrioxamine B (DFB) (1 : 1,000, 1 : 100) or as an inorganic colloid. The iron-ligand complexes were prepared before their addition to the medium, as described in Maldonado and Price (1999). Total concentrations of iron in the ETDA-buffered media were 8.41 μM and 12.9 nM, corresponding to free ferric ion concentrations of $10^{-18.18}$ M (pFe18) and 10^{-21} M (pFe21) and inorganic iron concentrations of 41 nM and 25 pM. EDTA was omitted from all of the media enriched with colloids, to prevent photochemical dissolution of the particulate iron. To minimize contamination, all manipulations were performed in a sterile laminar flow hood. Media were equilibrated overnight before use and stored in sterile, acid-washed polycarbonate bottles rinsed with 18.2 M Ω cm Milli-Q water (Millipore).

Phytoplankton were grown in semicontinuous batch cultures at 20°C under continuous light (200 μE m^{-2} s^{-1}) in acid-washed 28-ml polycarbonate tubes. Biomass was monitored daily by measuring in vivo fluorescence with use of a Turner Designs 10AU fluorometer. Specific growth rates were determined from the slope of linear regressions of $\log_e$ in vivo fluorescence versus time during the exponential growth phase. Each species was acclimated in the test media prior to experiments until growth rates of successive transfers were similar.

Iron colloid preparation and analysis—Four colloidal iron forms, ferrihydrite, magnetite, hematite, and goethite, were synthesized according to published methods, as briefly described below (Schwertmann and Cornell 1991; Wells et al. 1991*b;* Cornell and Schwertmann 1996). Plasticware and glassware used in preparation and storage were acid washed and either autoclaved or microwave sterilized.

Ferrihydrite was synthesized from addition of Analar (BDH) ferric chloride ($FeCl_3 \cdot 6H_2O$) to Milli-Q water to a final concentration of 4.0×10^{-4} M. The solution was kept at room temperature for 50 min, and a 15 ml aliquot was subsequently heated in a polypropylene tube in a 90°C water bath for 5 min. The solution was cooled to room temperature and added directly to experimental media. Colloidal ferrihydrite was freshly prepared for each growth experiment.

Magnetite was prepared in a sealed 1-liter glass reaction vessel equipped with an airtight rubber stopper with three ports for a thermometer, a N_2 gas inlet, and a separatory funnel tip. A quantity of 80 g of Analar $FeSO_4 \cdot 7H_2O$ was dissolved in 560 ml of Milli-Q water previously flushed with

Table 1. Description of phytoplankton species.

Species	Strain No.	Provenance	Habitat designation	Cell diameter (μm)	Class
Chrysochromulina cf ericina	CCMP281	North Pacific Ocean 49°36′N, 140°37′W	Oceanic	6–8	Prymnesiophyceae
Chrysochromulina ericina	CCMP283	North Atlantic Ocean, Gulf of Maine 44°30′N, 62°0′W	Coastal	6–8	Prymnesiophyceae
Ochromonas sp.	NEPCC457	Northeastern Pacific Ocean, West San Juan Island, WA 48°30′N, 123°0′W	Coastal	6–9	Chrysophyceae
Thalassiosira oceanica (clone 1003)	CCMP1003	North Atlantic Ocean, Sargasso Sea 36°11′N, 69°35′W	Oceanic	5	Coscinodiscophyceae
Thalassiosira pseudonana (clone 3H)	CCMP1335	North Atlantic Ocean, Moriches Bay, Long Island, N.Y. 40.5°31′N, 72.5°45′W	Coastal	5	Coscinodiscophyceae

N_2. The reaction vessel was heated to 90°C in a water bath, whereupon 240 ml of N_2-flushed solution containing 6.46 g KNO_3 and 44.9 g KOH was added dropwise from the separatory funnel within 5 min. The solution was heated for another 30–60 min and cooled overnight. The precipitate was washed four times with Milli-Q water by decanting the supernatant after the magnetite particles settled.

Hematite was synthesized by adding 10.81 g of Analar ferric chloride ($FeCl_3 \cdot 6H_2O$) to 2 liters of 0.001 M HCl at 98°C. The solution was held in a sealed, high-density polyethylene container at 98°C in an oven for 10 d. It was transferred to 250-ml polyethylene centrifuge bottles and centrifuged four times at 960 *g* for 1.5 h each, after rinsing with Milli-Q water.

Goethite was prepared from a 100-ml solution of 1 M $Fe(NO_3)_3$, with use of Analar unhydrolyzed $Fe(NO_3)_3 \cdot 9H_2O$ dissolved in Milli-Q water. This solution was placed in a 2-liter polyethylene flask and mixed with a stir bar, and 180 ml of 5 M KOH was rapidly added. The resultant mixture was diluted to 2 liters with Milli-Q water and placed in a 70°C oven for 60 h. The precipitate was centrifuged at 670 *g* for 10 min in 250-ml polyethylene centrifuge bottles after each of four rinses with Milli-Q water.

Magnetite, hematite, and goethite were analyzed by qualitative X-ray diffraction on a Rigaku D/Max 2400 automated powder diffractometer and their spectra compared with pure standards from the International Centre for Diffraction Data (ICDD). A portion of each of the aqueous stock solutions was dried for 48–72 h at 100–110°C until constant mass was achieved. Identification was based on the position and intensity of the $K_{\alpha 1}$ peaks, by use of instrument software and the ICDD database. Size and structure of the synthesized colloids were determined by a combination of atomic force microscopy (AFM; Dimension 3000 atomic force microscope, Digital Instruments) and light microscopy (Zeiss). Samples were prepared for AFM by suspending acetic acid–cleaned glass slides in seawater solutions of colloids overnight. The glass slides were subsequently removed and the surfaces examined for colloids.

The molarities of the magnetite, hematite, and goethite solutions were calculated by use of the molecular weight of the synthesized minerals under the assumption of 100% purity. As described below, the synthesized magnetite (Fe_3O_4) was predominantly maghemite (Fe_2O_3), so the concentration of this iron colloid was underestimated by as much as a factor of 1.45. The concentration of each oxide in solution was determined by drying a portion of each magnetite, hematite, and goethite for 48–72 h at 100–110°C until constant mass was achieved. The molarity of ferrihydrite was assumed to equal the initial concentration of ferric chloride added.

Iron colloid medium—To determine whether the colloids could be used as a source of iron for phytoplankton growth, they were added to seawater medium at a concentration of 1 μM. Desferrioxamine B (10 μM) and a partially purified catecholate siderophore, PWF-L (5 μM), isolated from the marine bacterium PWF3 (Granger and Price 1999), were supplied in some treatments to reduce the concentration of dissolved inorganic iron present as a contaminant or added

with the colloids. Each of the photosynthetic flagellates was grown in duplicate or triplicate for a number of transfers in the test media. The diatoms were also grown in the same media to determine the quantity of dissolved iron that was present as a contaminant in the iron colloid–amended seawater (c.f. Wells et al. 1991*b*).

Mixotrophic grazing—The heterotrophic bacterium Jul88, isolated from Sta. S in the Sargasso Sea (Tortell et al. 1996), was acclimated for several transfers in iron-replete (pFe18) and iron-deplete (pFe21) Aquil enriched with 200 mg L^{-1} bactopeptone and 200 mg L^{-1} casein hydrolysate. A small inoculum of each culture was transferred to fresh medium in acid-washed, 400-ml polycarbonate centrifuge bottles and bubbled vigorously with acid-scrubbed (10% HCl) sterile air. Growth was monitored by absorbance at 600 nm (A_{600}) by use of a Cary 1E spectrophotometer, and, in late exponential phase, the bacteria were concentrated by centrifugation at 2700 *g* for 1 h and resuspended in seawater medium, lacking organic carbon and iron. Bacteria were heat killed in a water bath at 70°C for 30 min and the cultures left at room temperature overnight (Chase and Price 1997). The samples were centrifuged twice, rinsed with iron-free seawater, and the bacteria resuspended and diluted in iron-free seawater to achieve final densities of 5.5×10^7 and 2.5×10^6 cells ml^{-1}.

Growth of mixotrophic bacterial assemblage—Bacteria naturally present in the cultures of *C. ericina* (oceanic) were isolated by filtration through a 2-μm Nuclepore polycarbonate filter. Samples of the filtrate were inoculated into Aquil medium containing PO_4^{3-}, SiO_3^{2-}, NO_3^-, and NH_4^+, supplemented with an organic enrichment (200 mg L^{-1} bactopeptone and 200 mg L^{-1} casein hydrolysate). The bacteria that grew under these conditions were tested for their ability to use dissolved inorganic and organic iron and colloids. In two treatments, iron was added to −Fe Aquil to achieve pFe18 and pFe21. The remaining treatments used −Fe, −EDTA Aquil with the following enrichments: None, DFB (10 μM), Coll (1 μM hematite), Coll/DFB (1 μM hematite + 10 μM DFB), PWF-L (5 μM), and Coll/PWF-L (1 μM hematite + 5 μM PWF-L). Each medium was added to polystyrene cuvettes containing a Teflon stir bar and Teflon lid, all of which were acid washed and sterilized by microwaving in Milli-Q water. A small inoculum of the bacterial assemblage was added to each cuvette to initiate the experiment. Growth was measured spectrophotometrically, A_{600}, at a constant temperature of 20°C, by use of the kinetics application program of a Cary 1E Varian spectrophotometer. Growth was monitored for several transfers in each medium.

Results

Iron colloid identification—X-ray diffractograms of the synthesized colloids, magnetite, hematite, and goethite, matched the standard spectra reported in the ICDD database (Fig. 1). Two of the colloids, hematite and goethite, were identified as pure iron oxides; however, the magnetite preparation contained a mixture of magnetite and maghemite, with maghemite predominating. Examination of aqueous suspensions of the stock solutions by light microscopy re-

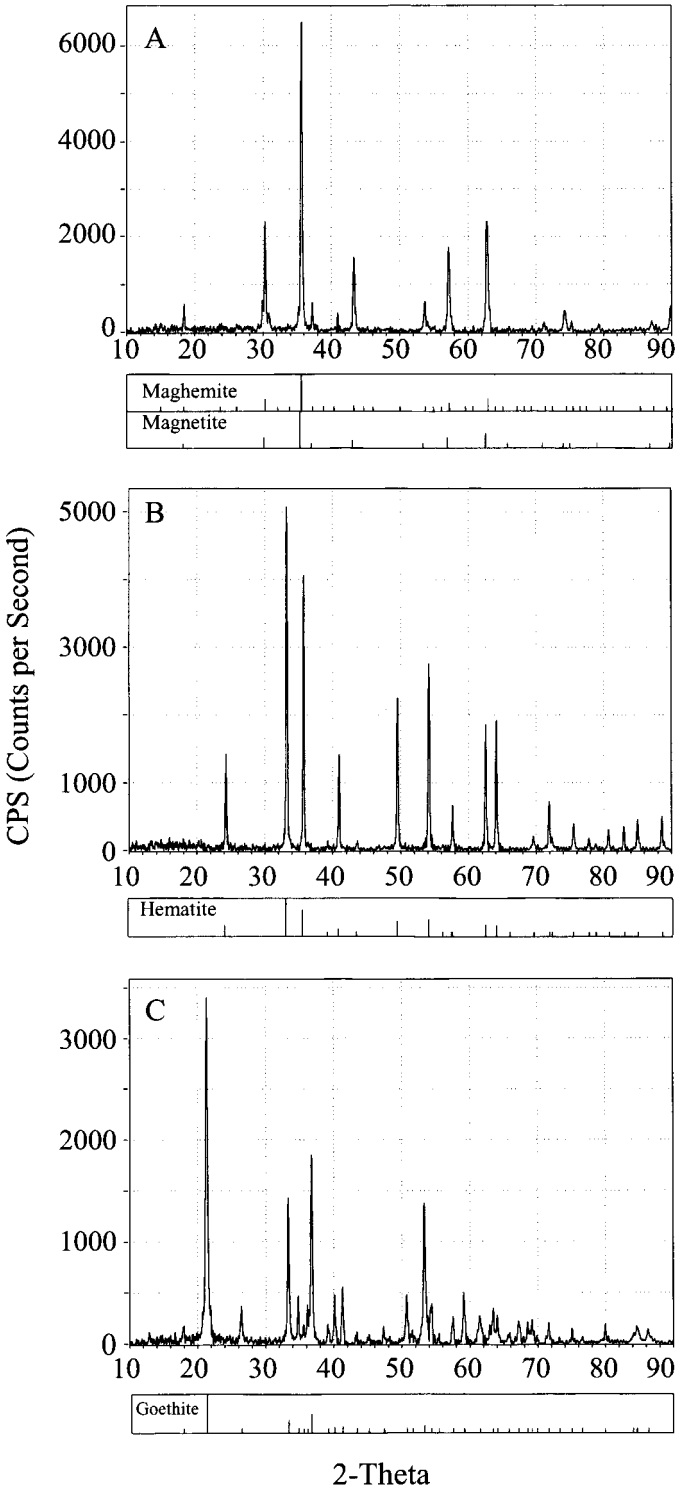

Fig. 1. X-ray diffractograms of synthesized colloids of (A) magnetite/maghemite (the colloid was a mixture of magnetite and maghemite with maghemite predominating), (B) hematite, and (C) goethite. For comparison, a matching stick diagram from the ICDD is provided for the identified phases in each sample.

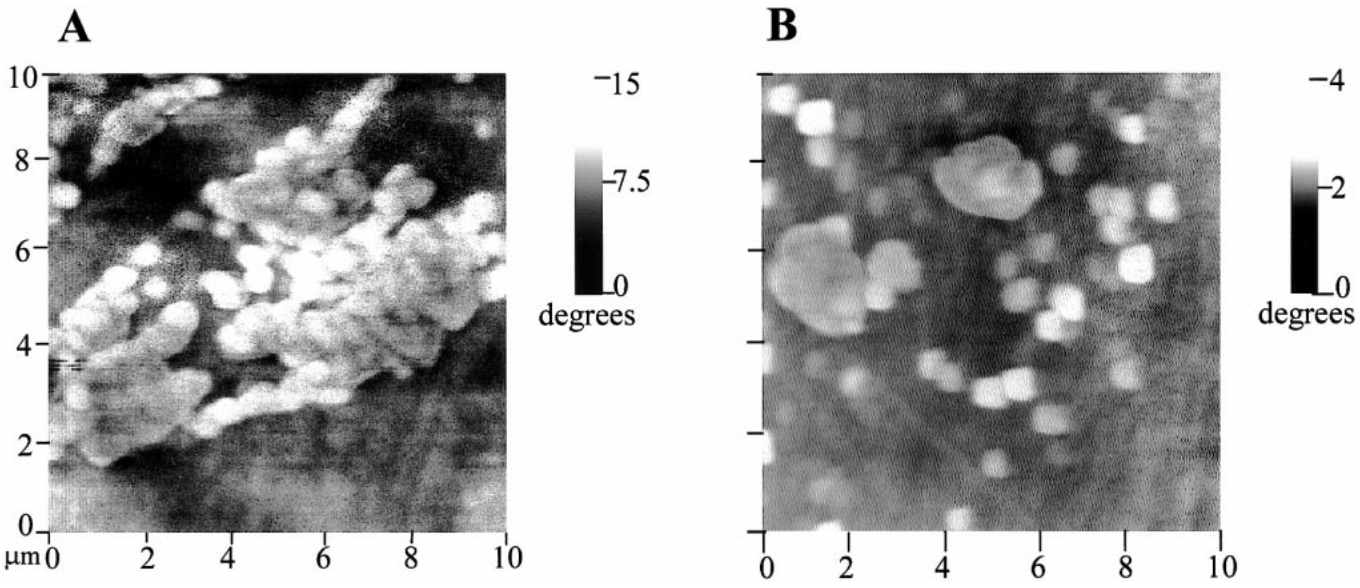

Fig. 2. Atomic force micrograph phase images of (A) magnetite/maghemite and (B) hematite. The degree scales refer to the difference in phase between the frequency imposed on the cantilever and its response after the interaction with the solid.

vealed a typical physical structure for each colloid. Subunits of hematite were hexagonal-shaped particles, those of goethite were needle shaped, and those of magnetite/maghemite were cube shaped. In seawater, at a concentration of 1 μM, the colloid subunits existed as discrete particles and as small aggregates. Hematite subunits were roughly 500 nm in diameter, and those of the magnetite/maghemite mixture were roughly 600 nm (Fig. 2). The size of goethite subunits was estimated by light microscopy to be 3–3.5 μm, with an acicular, needle-like appearance. Similar sizes were previously recorded by use of electron microscopy (Schwertmann and Cornell 1991). Goethite could not be observed by AFM because it did not adhere to the glass slides for observation. X-ray diffraction was not used to examine the structure of the amorphous ferrihydrite because it would change as samples were dried for analysis. A subunit size of 5–10 nm was assumed on the basis of previous measurements obtained by scanning electron microscopy (Barbeau et al. 1996).

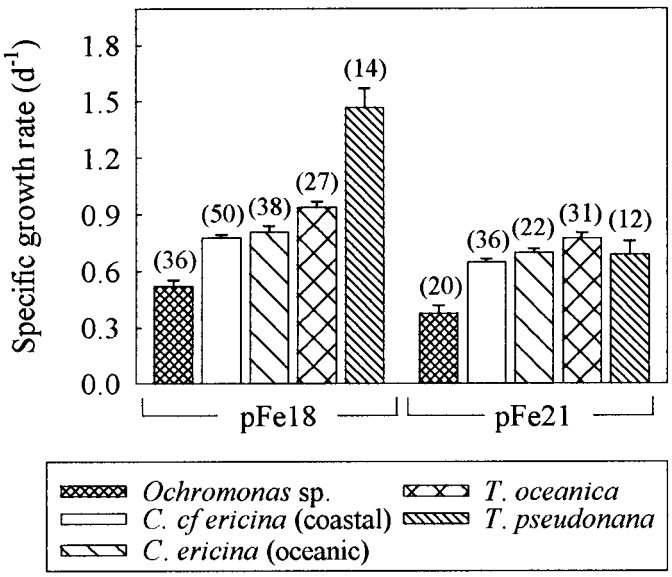

Fig. 3. Growth rates of phytoplankton in high (pFe18, [Fe]′ = 41 nM) and low (pFe21, [Fe]′ = 25 pM) iron media. Values reported are mean rates ±1 SE. The numbers of replicates for each treatment are in parentheses.

Iron dependence of growth—Growth rates of all species decreased in iron-deplete (pFe21), compared with iron-replete (pFe18), media by 20%–50% (Fig. 3). The greatest reduction was observed in the coastal isolate *T. pseudonana.* Surprisingly, the coastal and oceanic isolates of *C. ericina* grew at similar iron-limited rates in pFe21 medium. Because EDTA was omitted from the colloid experiments, the medium was enriched with a lower concentration of essential trace metals (Co, Zn, Cu, and Mn) than the standard Aquil recipe (*see Methods*). To assess whether these metals were sufficiently concentrated to allow good growth, rates were measured in both the high and low trace metal media and compared. When iron was added complexed 1 : 1.05 with EDTA at the same total concentration as in pFe18 medium, *Ochromonas* sp., *T. pseudonana,* and *T. oceanica* grew rapidly at rates similar to those observed in Aquil containing the normal complement of trace metals. Both strains of *C. ericina* grew at slightly yet significantly slower rates in the low trace metal medium not buffered with EDTA (78% μ_{pFe18} and 77% μ_{pFe18}, respectively) (1-way ANOVA, $P < 0.001$ for both species), possibly because one of the other metals was limiting or toxic.

The background concentration of iron in the basal medium without EDTA (−Fe, −EDTA Aquil) was very small, judging from the limited growth of phytoplankton in the absence of iron addition (Fig. 4). Final biomasses of all species were significantly lower in medium lacking iron and EDTA (−Fe, −EDTA Aquil) than in pFe21 (2-way ANOVA, $P < 0.001$).

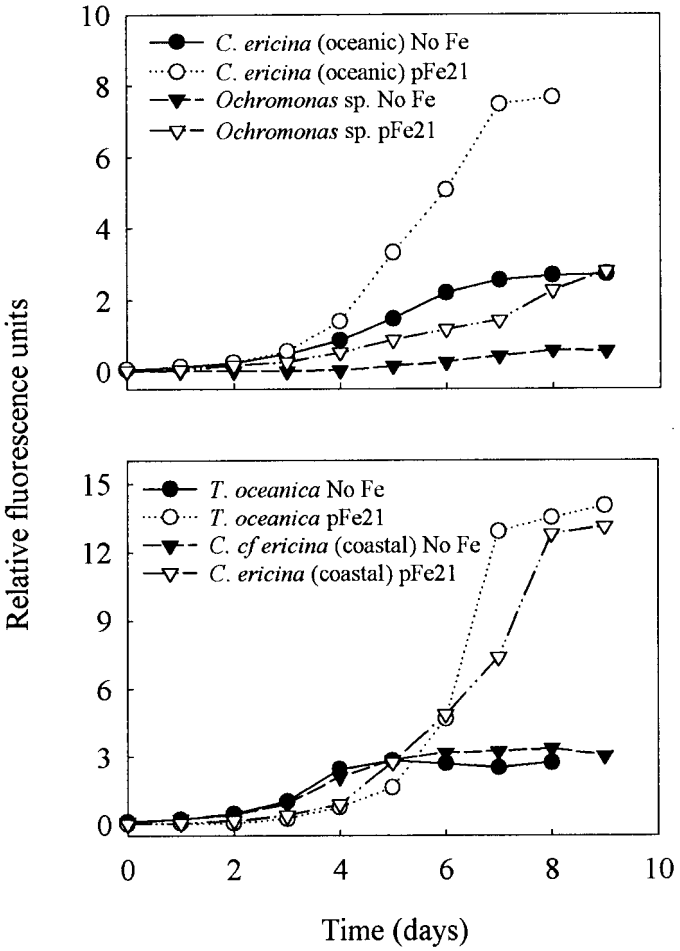

Fig. 4. Growth of phytoplankton in iron-free medium without EDTA (No Fe) and in pFe21 medium (pFe21).

Even *T. oceanica*, an oceanic isolate known for its ability to grow in low iron medium with high concentrations of EDTA (Maldonado and Price 1996), was severely limited.

Growth on iron colloids—Because bioassays were used to assess the availability of the colloids, we had to rule out the possibility that phytoplankton could use the low levels of dissolved inorganic iron for growth that were inadvertently added with the colloids. To accomplish this, very high concentrations of DFB (10 μM), a strong iron-binding ligand, were added to the medium, to ensure that all free, dissolved iron was rapidly complexed. We examined whether FeDFB itself could be used for growth of the flagellates by adding 10 and 100 nM iron bound to DFB or 10 μM EDTA to Aquil with trace metals buffered with 10 μM EDTA. The results demonstrated that, under these conditions, none of the flagellates were able to use iron bound to DFB for growth (Fig. 5).

All of the phytoplankton were able to grow in −Fe, −EDTA Aquil enriched with colloids, but only some of the species were able to do so with some of the colloids in the presence of DFB (Fig. 6). The diatoms, *T. oceanica* and *T. pseudonana*, which are known to use only dissolved iron, were used as controls. Their growth rates were slower than

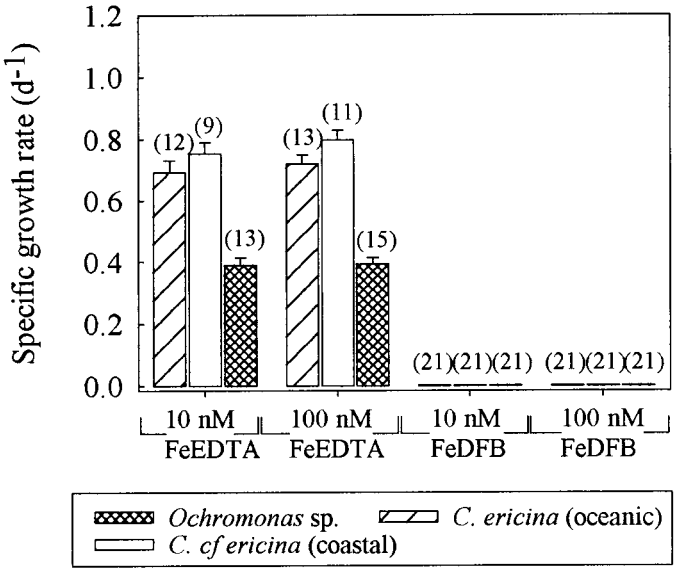

Fig. 5. Growth rates of photosynthetic flagellates in media containing 10 and 100 nM iron complexed with 10 μM EDTA or 10 μM DFB. Values reported are mean rates ±1 SE and the number of replicates for each treatment are in parentheses.

or equal to those observed in pFe21, confirming that the colloid-based media contained very low concentrations of dissolved Fe, ≤25 pM. In the hematite-enriched medium, for example, *T. oceanica* grew at 0.06 d^{-1}, the slowest iron-limited rate thus far reported for this species, corresponding to <10% of its maximum rate at full iron. Growth, however, was completely inhibited by addition of DFB to this and the other colloid-amended media, suggesting that extremely low levels of contaminating dissolved iron added with the colloids were sufficient to allow growth. Although *T. oceanica* (clone 1003) is able to acquire iron from ferrioxamine B (Maldonado and Price 2000), it is unable to do so at fast rates at such a high concentration of DFB (Wysote et al. unpubl. data) and such a low concentration of FeDFB (Maldonado and Price 2000).

Ferrihydrite supported rapid rates of growth of *Ochromonas* sp., even in the presence of DFB, but neither of the *Chrysochromulina* strains could grow under these conditions (Fig. 6). Mean growth rates of *Ochromonas* sp. were remarkably fast with ferrihydrite, significantly greater than those observed in either pFe21 or pFe18 media (1-way ANOVA, $P < 0.001$) (c.f. Fig. 3). Results opposite to those with the ferrihydrite experiment were observed when the other colloids were provided as an iron source (Fig. 6B–D). *Ochromonas* sp. was unable to grow with magnetite/maghemite, hematite, or goethite, but the *Chrysochromulina* strains grew well.

To validate the ferrihydrite result, *Ochromonas* sp. was provided ferrihydrite alone and in the presence of another organic ligand, PWF-L, isolated from a heterotrophic marine bacterium (Granger and Price 1999). PWF-L, a partially purified catechol siderophore, is estimated to have an equilibrium constant with respect to iron of 10^{29} M^{-1}. Like DFB, good growth was observed when PWF-L was added with ferrihydrite. A mean rate of 0.95 ± 0.13 d^{-1} was observed,

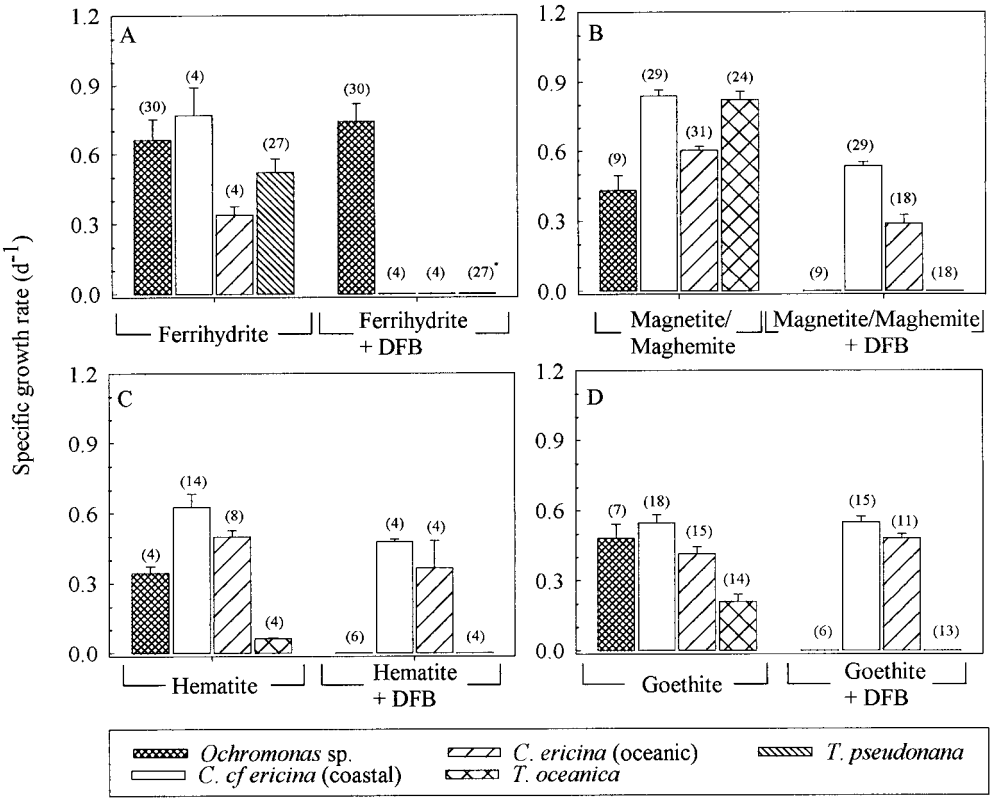

Fig. 6. Growth rates of phytoplankton in media enriched with one of four iron colloids in the presence and absence of 10 μM DFB: (A) ferrihydrite, (B) magnetite/maghemite, (C) hematite, and (D) goethite. Values are mean rates ± 1 SE and the numbers of replicates of each treatment are in parentheses. *In the case of *T. pseudonana*, 8 of the 27 replicates for the ferrihydrite + DFB treatment grew at an average rate of 0.06 d^{-1}.

significantly faster than the growth rate of *Ochromonas* sp. in pFe18 medium (1-way ANOVA, $P < 0.001$).

Mixotrophic grazing of bacteria—Because it was shown elsewhere that *Ochromonas* sp. could derive its iron ration by ingesting bacteria (Maranger et al. 1998), we examined whether growth of the other flagellates in −Fe, −EDTA Aquil could be supported similarly. When heat-killed bacteria were provided as the sole source of iron, *Ochromonas* sp. and both strains of *C. ericina* grew well (Fig. 7). Growth rates of all the species were considerably faster when they were provided iron-rich compared with iron-poor prey, and the final biomasses, as estimated by fluorescence, were 1.5–3-fold higher. Calculations based on published values of the iron content of the prey bacterium, Jul88 (Granger and Price 1999), showed that at the densities added to the flagellate cultures the particulate iron concentrations in these media were roughly 0.4 and 0.013 nM, respectively. When the concentration of bacteria was varied from 2.5×10^6 to 5.5×10^7 cells ml^{-1}, there was little response in growth rate or final biomass of either strain of *C. ericina. Ochromonas* sp. was unable to grow with iron-poor bacteria at any concentration but grew well when supplied iron-rich bacteria at all concentrations with little or no change in growth rate or final biomass.

Growth of the flagellates in cultures with heat-killed bacteria supplemented with colloidal iron increased, compared with those with only heat-killed bacteria (Fig. 8). *C. cf ericina* (coastal) showed a small increase in growth when hematite was added, and *C. ericina* (oceanic) showed a more marked response, with a final fluorescence two times that of the cultures that were only supplied bacteria. *Ochromonas* sp. did not grow with iron-poor bacteria alone and was not able to use hematite (as observed in the initial assays) in the presence of the bacteria. When ferrihydrite was added to these samples, growth immediately resumed (Fig. 8). This species also showed enhanced growth in samples containing iron-rich bacteria and ferrihydrite in contrast to iron-rich bacteria alone (Fig 8).

Growth of mixotrophic bacterial assemblage—Bacteria naturally present in the flagellate cultures could potentially be a source of iron to the phytoplankton if they were able to assimilate the colloidal iron during the experiments. To assess this possibility we cultivated the bacteria isolated from *C. ericina* (oceanic) in −Fe, −EDTA Aquil containing colloidal iron. The results showed no significant difference in the final biomass yield among the treatments amended with colloids, with DFB, and with colloids + DFB (1-way ANOVA, $P < 0.001$) (Table 2), and none of these treatments were significantly different from the −Fe, −EDTA Aquil control. No growth was observed in cultures amended with

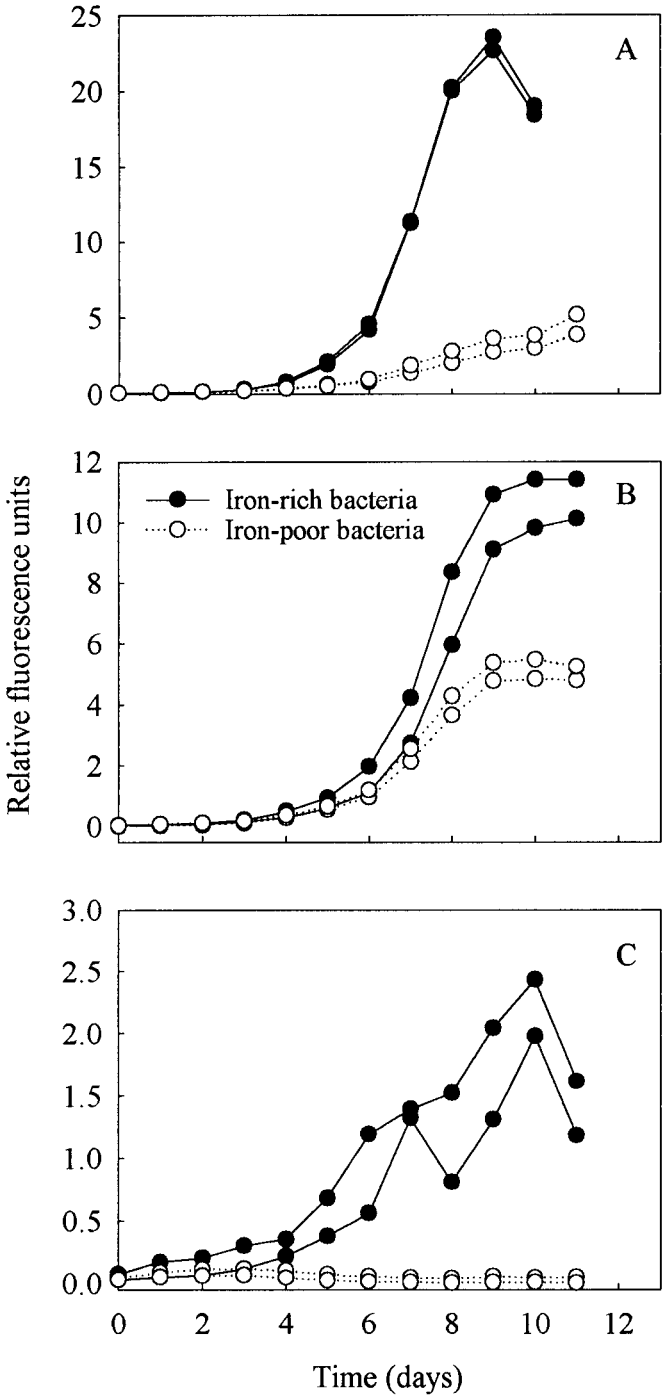

Fig. 7. Growth of photosynthetic flagellates in −Fe, −EDTA Aquil containing heat-killed bacteria, strain Jul88, at a density of 2.5×10^6 ml^{-1}—(A) *C. cf ericina* (coastal), (B) *C. ericina* (oceanic), and (C) *Ochromonas* sp. Control data showing flagellate growth in No Fe medium are reported in Fig. 4. Bacteria were first grown to stationary phase in high (pFe18) and low (pFe21) iron medium enriched with bactopeptone and casein hydrolysate. Particulate iron concentrations in the iron-rich and iron-poor treatments were estimated to be 0.4 and 0.013 nM, respectively.

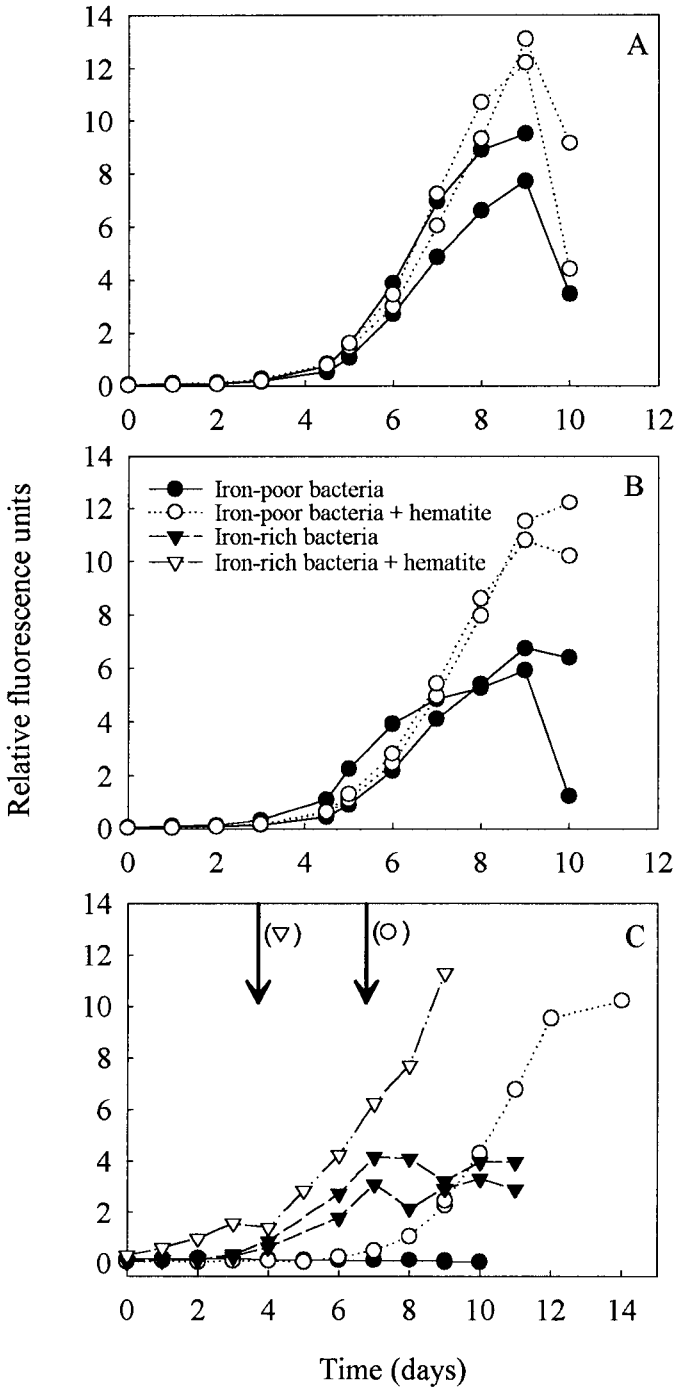

Fig. 8. Growth of photosynthetic flagellates in −Fe, −EDTA Aquil containing heat-killed bacteria at a density of 5.5×10^7 ml^{-1} with and without hematite—(A) *Chrysochromulina cf ericina* (coastal), (B) *C. ericina* (oceanic), and (C) *Ochromonas* sp. Arrows indicate the addition of 1 μM ferrihydrite to the hematite-enriched samples. Bacteria were prepared as described in Fig. 7. Particulate iron concentrations in the iron-rich and iron-poor treatments were estimated to be 8.8 and 0.28 nM, respectively.

Table 2. Comparison of final biomass yield of bacterial flora present in cultures of *Chrysochromulina ericina* (oceanic). Bacteria were separated from *C. ericina* by filtration and grown in seawater medium enriched with bactopeptone and casein hydrolysate. Biomass was measured by absorbance at 600 nm in a spectrophotometer equipped with a temperature-controlled cuvette holder. The effects of varying Fe concentration and source were examined: −Fe = no iron addition, DFB = 10 μM DFB, Coll = 1 μM hematite, Coll/DFB = 1 μM hematite and 10 μM DFB, PWF-L = 10 μM PWF-L, and Coll-PWF-L = μM hematite and 5 μM PWF-L. Statistically significant differences in biomass yields of the treatments are indicated by different alphabetical superscripts ($p < 0.05$).

Treatment	Final biomass (absorbance units ± 1 SD)	Sample size (*n*)
pFe18	0.230 ± 0.02[b]	7
pFe21	0.167 ± 0.02[a,c]	5
−Fe	0.189 ± 0.02[c,d]	17
DFB	0.156 ± 0.03[a]	20
Coll	0.164 ± 0.03[a,d]	6
Coll/DFB	0.157 ± 0.03[a]	18
PWF-L	0.031 ± 0.03[e]	6
Coll/PWF-L	0.031 ± 0.03[e]	3

PWF-L or PWF-L + colloids. Collectively, the results suggest that the bacteria present in the *C. ericina* culture could not use FeDFB, FePWF-L, or colloidal iron for growth.

Discussion

Precedent for colloidal iron use by phytoplankton—Much of what we know about the availability of colloidal iron for phytoplankton growth has been discovered from bioassay experiments with marine-centric and pennate diatoms (Harvey 1937*a;* Goldberg 1952; Rich and Morel 1990; Kuma and Matsunaga 1995). Altogether, they have shown that the colloids must dissolve before the iron is used for growth and that direct uptake of even the smallest colloids is not possible. The conclusions are well supported by the data (c.f. Goldberg 1952) but are only pertinent to our understanding of the nutrition of diatoms. Since many photosynthetic flagellates are capable of particle ingestion (the so-called mixotrophic species), they may possess different capabilities for iron use. Recent studies, for example, show that phagotrophic flagellates dissolve and regenerate some types of particulate iron during digestion (Barbeau et al. 1996; Chase and Price 1997; Barbeau and Moffett 1998, 2000) and use it as a nutritional supplement (Chase and Price 1997; Maranger et al. 1998). These observations suggest that alternative strategies of iron uptake may exist among related photosynthetic taxa. As discussed below, the results reported here provide the first strong evidence for the direct use (i.e., without prior dissolution) of colloidal iron by phytoplankton. We believe they highlight the potential biological importance of colloidal iron in the sea and illustrate a novel mechanism of iron acquisition that may be advantageous for the survival of mixotrophs in iron-poor waters.

Evidence for dissolved iron use by mixotrophic phytoplankton—The three flagellates examined in this study were mixotrophic phototrophs that had been shown to ingest bacteria (Jones et al. 1993; Maranger et al. 1998) and to use them as their sole iron source in growth (Figs. 7, 8). Initial experiments established that they grew well in the presence of high concentrations of dissolved iron and became limited as the concentration was reduced to 12.9 nM (pFe21 medium; Fig. 3). The reduction in growth rate at low iron was surprisingly less than had been expected for coastal flagellates, which are thought to have higher iron requirements for growth than species from the open sea (Ryther and Kramer 1961; Maldonado and Price 1996). At the moment, we are unable to explain this observation. Judging from their growth in pFe21 medium, the flagellates are not better able than other phytoplankton (Sunda and Huntsman 1995) to use low levels of dissolved iron in their environment. However, because the cultures were not axenic, we are uncertain whether they used dissolved iron or whether they consumed the resident bacteria to obtain iron in these media. Significant ingestion of bacteria iron seems unlikely, because the cultures contained only 10^4–10^5 bacteria ml^{-1}. At these densities, we estimate that between 0.05 and 0.5 pM of particulate iron would be present in the bacteria, which is 30 times less than is necessary for slow growth of the flagellates (Fig. 7). We tentatively conclude that dissolved iron must be used for growth but recognize that short-term measurements of iron transport rates are needed to confirm this.

Evidence for colloidal iron use by mixotrophic phytoplankton—The chemical composition of the media must be considered to interpret correctly the results of the colloid bioassay experiments. Small amounts of dissolved iron did not react during synthesis of the colloids and were present in the stock solutions of ferrihydrite, magnetite/maghemite, hematite, and goethite. When the colloids were added to prepare the media, low concentrations of dissolved iron were thus unavoidably introduced. The amount of iron added in this way can be estimated from the growth rates of the diatoms at pFe21, because they are only able to use dissolved species (Rich and Morel 1990; Wells et al. 1991*b;* Kuma and Matsunaga 1995). Under the assumption of a linear relationship between growth and inorganic iron concentration (Fe′), the ferrihydrite medium contained ~18 pM Fe′, the magnetite/maghemite medium 25 pM Fe′, the goethite medium 6 pM Fe′, and the hematite medium 2 pM Fe′. These levels are extraordinarily low, considering that 1 μM of colloidal iron was added, but they were sufficient to allow the diatoms to grow slowly. To reduce Fe′ to even lower levels, so we could test whether the colloids themselves could directly support growth, we added 10 μM DFB, a strong iron-binding siderophore. The iron complex, FeDFB, can be used by *T. oceanica* (Maldonado and Price 2000) but at such low concentrations (equivalent to the Fe′ concentration, under the assumption of 100% complexation) growth was not possible. This result is not surprising, since the half-saturation constant for reduction of FeDFB is 0.68 μM, and high concentrations of DFB effectively compete with surface transport ligands of the diatoms for free Fe′ (Maldonado and Price unpubl. data). DFB was thus effective in reducing the Fe′ concentration and preventing diatom growth due to dissolved iron (Fig. 6). The colloid-amended media were fresh-

ly prepared in each experiment, because DFB was expected to scavenge rapidly Fe′ and dissolve the colloids. Since the flagellates were unable to use FeDFB (Fig. 5), they must have been able to use the colloidal iron directly for growth (Fig. 6). As discussed below, the indigenous bacteria were not likely an indirect source of iron in the colloid treatments.

The possible role of bacteria in colloidal iron acquisition—Bacteria are known to use FeDFB (Granger and Price 1999) and to dissolve iron colloids (Keshtacher-Liebson et al. 1995), so in the colloid-amended media they may be able to concentrate iron and make it available to the flagellates. As argued above, the densities of bacteria were too low for this to be effective, even if their iron content was 100 times higher, as expected when they are iron-replete (Granger and Price 1999). To experimentally rule out this possibility, however, we added high densities of heat-killed bacteria, to reduce ingestion of the resident bacteria by the flagellates. The growth rates and biomass yields were reduced for all the species supplied iron-poor bacteria alone, confirming that iron was limiting (Fig. 7). This was particularly true of *Ochromonas* sp., which failed to grow under these low iron conditions. Addition of ferrihydrite to *Ochromonas* sp., however, resulted in an immediate resumption of growth, suggesting that flagellates used the colloidal iron directly without the intervention of the resident bacteria (Fig. 8). Biomass of *Chrysochromulina* increased at the same rate in the hematite medium, in the presence and absence of heat-killed bacteria (Figs. 6, 8), so it was unlikely that they accessed the colloidal iron indirectly by consuming the metabolically active bacteria in the culture.

To support these results, we tested the hypothesis that the bacteria in the cultures were able to use the colloids and thus be a source of iron for the flagellates. In these experiments, we chose the oceanic clone of *C. ericina* as a source of bacteria and hematite as the iron colloid. When the bacteria were separated from the flagellates and grown in organically enriched medium, the final biomass was related to the amount of dissolved inorganic iron present (Table 2). Greater amounts of bacteria were produced in the high (pFe18) than in the low (pFe21) iron and no iron (−Fe) treatments. No significant differences were observed in biomass yield if the bacteria were grown with colloids alone, DFB alone, or colloids plus DFB (Table 2), and none of these were distinguishable from the pFe21 treatment (1-way ANOVA, $P < 0.001$). Similar results were obtained by use of a second organic ligand, PWF-L (Granger and Price 1999): the final biomass yields were negligible, well below those in the −Fe and pFe21 media, and were similar in both treatments. We conclude that the bacteria were unable to use the colloidal iron for growth. The observation that the flagellates were unable to grow with FeDFB as the sole iron source suggests that the resident bacteria could not be an indirect source of iron from the complex. Collectively, these results suggest that the resident bacteria were not a significant source of iron to the mixotrophs in the colloid-amended media and that the mixotrophs must directly use colloidal iron for growth without prior dissolution. Although we have no direct proof, ingestion of the colloids by the flagellates is the likely pathway of assimilation.

Mechanism of colloidal iron use by mixotrophic phytoplankton—Particle ingestion is well known in species of *Chrysochromulina* and *Ochromonas* (*see* Jones et al. 1993) and is an important means by which nutrients and energy are acquired. The mechanism of ingestion involves the haptonema, a microtubule-bearing appendage that directs or moves food particles to the nonflagellar pole of the cell (Kawachi et al. 1991). A colorless, slightly granular substance flows out and surrounds the particles to be ingested, which are subsequently channeled into the food vacuole.

Phagotrophic flagellates ingest a number of different types of particles, including inert ones composed of carmine and graphite (Jones et al. 1993). Although some species are selective in what they eat (Bennett et al. 1988), nutritional quality does not appear to be an overriding criterion, so it may not be too surprising that iron colloids are also consumed. Since the sizes of these particles are similar to bacteria and other prey items, ingestion of colloids should not be sterically hindered. Digestion of the colloids is likely to occur in the food vacuole (Jones et al. 1993), as it does with other prey items in other phagotrophs (e.g., Estep et al. 1986), followed by dissolution and assimilation of the iron. Barbeau et al. (1996) concluded that regeneration of colloidal iron by a heterotrophic flagellate occurred in this way, with chemical transformation of the colloids in the low-pH food vacuole. We surmise that a similar ingestion-digestion process existed in the mixotrophs examined in this study, although experimental evidence of this pathway is needed.

Variation in use of colloidal iron by mixotrophic phytoplankton—Variation in response of the flagellates to the different colloids is interesting to note. *Ochromonas* sp., for example, the coastal chrysophyte, was only able to grow in the presence of ferrihydrite, whereas *C. cf ericina* (coastal) and *C. ericina* (oceanic), the prymnesiophyte species, grew well with all the iron colloids except ferrihydrite (Fig. 6). Previous studies have shown that the availability of colloidal iron is related to the thermodynamic stability of the solid (Rich and Morel 1990; Wells et al. 1991*b;* Kuma and Matsunaga 1995), which increases as it becomes more crystalline (Cornell and Schwertmann 1996). According to this view, ferrihydrite, the most labile of the colloids tested, would be predicted to support the best growth and goethite the least. Although this may be an explanation for the response of *Ochromonas* sp., it is incompatible with the results obtained with *C. ericina.* One reason may be that *Chrysochromulina* species exhibit size selectivity when grazing. We note that ferrihydrite subunits are, by far, the smallest of the iron colloids synthesized (5–10 nm; Barbeau et al. 1996), whereas the others were similarly sized (between 0.5 and 3 μm). In an extensive analysis by Jones et al. (1993), feeding selection was observed among 16 species of *Chrysochromulina,* some of which preferred smaller-sized and others of which preferred larger-sized prey. *C. ericina* was shown to ingest both a small green flagellate (3–4 μm) and carmine particles in the range of 1–4 μm, whereas a similarly sized *Chrysochromulina* sp. did not ingest either prey item. Boraas et al. (1988), however, found opposite results—that feeding by *Chrysochromulina* was nonselective. Aside from these two studies, prey selectivity is neither well characterized nor

understood in prymnesiophytes. Our results suggest that *C. ericina* may not ingest the very smallest iron colloids (ferrihydrite). Because we are uncertain of the physical structure of the colloids in association with bacteria in the media, this explanation remains speculative.

Inorganic colloidal iron in the ocean—The principal colloidal phases in soils and natural waters are ferrihydrite, hematite, maghemite, goethite, and lepidocrocite (γ-FeOOH). Ferrihydrite, a precursor or immature phase of oxyhydroxides (goethite) and oxides (hematite), is the first to form during iron precipitation and is an initial hydrolysis product found in estuaries and other coastal regions subject to high iron inputs. Sediment upwelling and rivers directly supply colloidal iron to the sea, as may hydrothermal vents in certain areas, but the majority is thought to originate from atmospheric dust (Moore et al. 1984; Duce and Tindale 1991; Kuma et al. 1992). Aerosol samples obtained over the Gulf of Maine contained goethite, hematite, and aluminosilicates (Moore et al. 1984; Carder et al. 1986), suggesting that these forms are likely to exist in surface waters of the sea and to be encountered by phytoplankton. Similar types of colloids, including lepidocrocite and magnetite, were also detected in airborne particles collected over Europe and Asia (Kopcewicz and Dzienis 1971; Fukasawa et al. 1980; Dedik et al. 1992). The pure colloids used in our experiments thus appear to have strong similarities with particulate iron forms found in nature.

Vertical profiles in the western North Atlantic Ocean show a roughly constant concentration of 0.15–0.25 nM colloidal iron (in the 0.2–0.4 μm fraction) with depth (Wu and Luther 1994), and particulate concentrations in the Pacific Ocean are often in great excess of the dissolved species (Johnson et al. 1997). Kuma et al. (1998) determined that colloidal iron concentrations in the 0.025–0.45 μm fraction in coastal and oceanic waters of the eastern North Pacific was 4–7 and $<$0.1 nM, respectively. Since the amount of iron $>$0.45 μm was not determined, the total colloidal iron concentrations are likely underestimated. In excess of 10 nM, particulate iron was found in the sea surface near the coast of Japan (Kuma et al. 1998). Although the composition of colloidal iron in the sea is largely inferred from other environments, the concentration of these phases appears to be high and their distribution widespread.

Potential of colloidal iron use in the ocean—Few examples exist in nature of the use of colloidal iron for growth. Many bacteria have been shown to reduce ferric oxides (e.g., Straub et al. 1998; Urrutia et al. 1999); however, they represent the only group with this widespread ability. Our observation that some marine mixotrophs may directly use colloidal ferric oxides for growth provides, as far as we are aware, the first report of colloidal iron use by eukaryotic species.

Mixotrophy is considered to be an advantageous mode of nutrition during nutrient limitation (Bennett et al. 1988; Nygaard and Tobiesen 1993; Maranger et al. 1998), and so in iron-limited waters it may be abundant. The ability to acquire colloidal iron for growth means that a large fraction of the iron in the sea could potentially be available to phagotrophic species of photosynthetic algae. Mixotroph species occur abundantly from tropical to polar ocean systems (Thomsen et al. 1994; Andersen et al. 1996) and are thought to make up ~50% of the phytoplankton biomass (Havskum and Riemann 1996). Pigmented flagellates may account for $>$50% of primary productivity in the ocean (Estep et al. 1984 and references therein). Perhaps these phytoplankton play such an important role in ocean ecology because of their ability to acquire different forms of iron. Dissolved iron is present in pM concentrations as organic complexes (Rue and Bruland 1995) of unknown biological availability, so assimilation of other forms of iron may be advantageous. We speculate that colloidal and particulate iron may be important for the survival of mixotrophic phytoplankton in low-iron waters. Ingestion of iron particles could be an important mechanism of iron acquisition by phytoplankton and a critical part of the iron cycle in the surface ocean.

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