

## Nitrate regulation of Fe reduction and transport by Fe-limited *Thalassiosira oceanica*

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### Abstract

Under Fe-limiting conditions, nitrate ( $\text{NO}_3^-$ )-grown marine diatoms have higher intracellular Fe requirements, but divide as fast or faster than ammonium ( $\text{NH}_4^+$ )-grown cells by maintaining faster steady-state Fe uptake rates. Here we report that *Thalassiosira oceanica*, clone 1003, possesses an Fe reductase that reduces Fe(III) bound to a variety of organic ligands, including the siderophore desferrioxamine B (DFB), a high affinity, Fe(III)-specific ligand. Reduction is mediated extracellularly and is induced by Fe deficiency. Cellular rates of Fe(III) reduction are significantly faster in  $\text{NO}_3^-$ - than in  $\text{NH}_4^+$ -grown cultures suggesting a link with N metabolism. At subsaturating Fe concentrations, short- and long-term Fe uptake rates are also significantly faster in  $\text{NO}_3^-$ - than in  $\text{NH}_4^+$ -grown cells. The results suggest that when Fe is limiting, faster rates of reduction of organically bound Fe(III) by phytoplankton promote faster rates of Fe transport and growth. The implications of these findings could be significant for understanding phytoplankton Fe nutrition in oceanic waters where organic complexation dominates the speciation of Fe. We hypothesize that the reductive Fe transport pathway may enable phytoplankton to directly utilize Fe bound to strong organic ligands in the sea.

Iron plays a catalytic role in many biochemical reactions as a cofactor of enzymes and proteins involved in chlorophyll synthesis, detoxification of reactive oxygen species, respiratory and photosynthetic electron transport, and N assimilation. Changes in the activity of these reactions or their replacement by functionally equivalent Fe-deficient pathways can greatly influence cellular Fe requirements of organisms. Because the  $\text{NO}_3^-$  assimilatory pathway is highly Fe dependent, utilization of  $\text{NO}_3^-$  by marine centric diatoms (*Thalassiosira* spp.) imparts a higher metabolic demand for Fe than the use of  $\text{NH}_4^+$ . The demand for Fe is such that the Fe quotas (Fe:C) of  $\text{NO}_3^-$ -grown cells are 1.8 times higher than those of phytoplankton using  $\text{NH}_4^+$  (Maldonado and Price 1996). Despite the higher Fe requirement for  $\text{NO}_3^-$  assimilation, under moderately Fe-limiting conditions (ca.  $0.7\text{--}0.85 \mu\text{M}/\mu\text{M}_{\text{max}}$ ), growth rates of  $\text{NO}_3^-$ -amended cultures are not slower than those of  $\text{NH}_4^+$ -amended ones (Maldonado and Price 1996). Nitrate-grown cells apparently compensate for their extra Fe requirement by sustaining faster steady-state Fe uptake rates (Maldonado and Price 1996). The

mechanism by which the  $\text{NO}_3^-$ -dependent cells maintain these faster rates is presently unknown.

Iron uptake by phytoplankton is largely determined by dissolved inorganic Fe ( $\text{Fe}'$ ) concentration (Anderson and Morel 1982; Hudson and Morel 1990, 1993). In Fe-poor, chelated culture medium,  $\text{Fe}'$  concentration can be extremely low as most of the Fe is organically complexed. The Fe complexes, including FeEDTA (ethylenediaminetetraacetic acid) species, are kinetically less labile than  $\text{Fe}'$ , are impermeable to cell membranes, and are thus not thought to be directly available for uptake (Hudson and Morel 1993). However, photochemical reactions can catalyze the dissociation of Fe from these organic ligands by a reductive reaction, typically by an intramolecular charge transfer in which Fe(III) is reduced to Fe(II) and the ligand is oxidatively degraded (e.g., in EDTA by a decarboxylation reaction; Budac and Wan 1992). The photo-degradation of the ligand substantially contributes to the photo-dissociation of ferric chelates, thus increasing  $[\text{Fe}']$ . This effect may have important biological consequences. Indeed, photoreduction of Fe(III)EDTA increases Fe uptake rates by phytoplankton (Anderson and Morel 1982) and presumably could ameliorate Fe-deficiency.

Biologically mediated reduction of Fe may be an alternative means to increase the chemical reactivity of Fe in organic complexes. Higher plants, yeast, and bacteria employ this strategy to obtain Fe when it is limiting (Guerinot 1994; Guerinot and Yi 1994). Reduction of Fe is primarily mediated by transplasmalemma bound redox enzymes, which are induced under Fe-limitation (Bienfait 1987). Since most organic Fe chelators have a higher affinity for ferric (Fe(III)) than ferrous iron (Fe(II)), reduction of complexed Fe(III) results in a net dissociation of Fe from the ligand, increasing  $[\text{Fe}']$ .

In the present study, we investigated physiological mechanisms by which cells grown with  $\text{NO}_3^-$  maintain faster steady-state Fe uptake rates and thus achieve faster division rates than  $\text{NH}_4^+$ -grown cells under Fe-limiting conditions. In particular, we examined how N metabolism affected Fe transport and reduction by Fe-limited *Thalassiosira oceani-*

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ca. Reduction rates were measured with BPDS, an organic ligand with high affinity for Fe(II), and by direct determination of Fe(II) using an automated, flow injection analysis system with luminol-based chemiluminescence detection (King et al. 1995). Iron uptake rates and growth rates were measured concurrently.

## Methods

*Iron-limiting culture media*—*T. oceanica* (clone 1003), a small 6- $\mu\text{m}$  diameter, centric diatom isolated from the Sargasso Sea, was obtained from the Provasoli-Guillard Center for Culture of Marine Phytoplankton and grown under Fe-limiting conditions in the artificial seawater medium Aquil (Morel et al. 1979; Price et al. 1988/89). The basal medium had a pH of  $8.02 \pm 0.2$ , contained 100  $\mu\text{M}$  EDTA, and was enriched with either 50  $\mu\text{M}$   $\text{NO}_3^-$  or  $\text{NH}_4^+$  (Maldonado and Price 1996). Additions of Cu, Mn, Zn, and Co were adjusted to achieve free-ion concentrations of  $10^{-13.79}$  M,  $10^{-8.27}$  M,  $10^{-10.88}$  M, and  $10^{-10.88}$  M, respectively. Total Mo and Se concentrations were  $10^{-7}$  and  $10^{-8}$  M, respectively. Iron was added as a premixed FeEDTA complex (1:1.05) at a total concentration of 12.5 nM, corresponding to a free ferric ion concentration of  $10^{-21}$  M (pFe = 21, where pFe =  $-\log[\text{Fe}^{3+}]$ ) and an inorganic Fe (Fe') concentration of 25 pM (calculated using MINEQL; Westall et al. 1976). The inorganic Fe concentration was estimated for illuminated media (Sunda and Huntsman 1995), and measured using the sulfoxine method ( $\approx 30$  pM) (Hudson et al. 1992). In some experiments Fe was not added, but the medium contained small quantities of it as a contaminant (Maldonado and Price 1996).

Two other metal complexing ligands were used in experiments: DTPA (diethyltriaminepentaacetic acid), a synthetic chelator, and DFB (desferrioxamine B), a fungal siderophore (a high affinity, Fe(III)-specific ligand that facilitates Fe acquisition by Fe-limited microorganisms). In the DTPA-buffered (5  $\mu\text{M}$ ) media, EDTA was omitted, and the concentrations of Cu, Mn, Zn, and Co were adjusted to achieve the same free-ion concentrations as were obtained with EDTA. Iron was omitted in some experiments and added in others as a FeDTPA complex at a concentration of 7.72 nM (pFe 21, calculated for nonilluminated media using MINEQL, Westall et al. 1976). The DFB-based medium was identical to the EDTA basal medium, except Fe was added as a FeDFB complex (all other trace metals were added bound to 100  $\mu\text{M}$  EDTA). The concentration of DFB was kept constant (129 nM) in all experiments, and Fe varied from 1.29 to 129 nM. Sterile, trace metal-clean techniques were used during all manipulations and the growth media were allowed to equilibrate chemically overnight before use.

*Growth measurements*—*T. oceanica* was grown in acid-washed, 28 ml polycarbonate tubes at 20°C under a continuous saturating photon flux density of 200  $\mu\text{E m}^{-2} \text{s}^{-1}$  supplied by cool white fluorescent lamps. Biomass (in vivo fluorescence or cell density) and absolute (divisions  $\text{d}^{-1}$ ) and specific ( $\text{d}^{-1}$ ) growth rates were determined as previously described (Maldonado and Price 1996). The cultures were continuously maintained in exponential phase by serial di-

lution into fresh medium as required. Phytoplankton were assumed to be acclimated when the growth rates between successive transfers varied by less than 10% (Brand et al. 1981). The rates reported are the mean of at least six transfers. Cell density and volume ( $\text{fl} = \mu\text{m}^3$ ,  $\text{fl} = 10^{-15}$  L) were measured with a Coulter Counter (model ZM) that was calibrated with 2.1  $\mu\text{m}$  diameter polystyrene beads. Because  $\text{NO}_3^-$ -grown cells are smaller than  $\text{NH}_4^+$ -grown cells (Maldonado and Price 1996), all Fe uptake and reduction rates were normalized to cell volume or surface area.

*Long-term Fe uptake rates*—To investigate the effects of pH on growth and Fe acquisition, Fe-limited *T. oceanica* was grown in pFe 21 medium enriched with either  $\text{NO}_3^-$  or  $\text{NH}_4^+$ . Iron was added as  $^{55}\text{Fe}$  (12.9 nM  $^{55}\text{Fe}$ , 25–40 mCi  $\text{mg}^{-1}$ , DuPont) and cells were taken through 2 transfers to achieve isotopic equilibrium. The long-term uptake experiment was initiated by inoculating  $^{55}\text{Fe}$ -labeled cells, in exponential phase, into the experimental flasks containing the same  $^{55}\text{Fe}$ -limiting medium (pFe 21). Intracellular Fe concentrations (Fe quotas) of the cells used as inocula were 1.5 times higher for the  $\text{NO}_3^-$  ( $4.09 \pm 1.54 \times 10^{-20}$  mol Fe  $\text{fl}^{-1}$ ) than the  $\text{NH}_4^+$  cultures ( $2.79 \pm 1.7 \times 10^{-20}$  mol Fe  $\text{fl}^{-1}$ ). Cell densities ( $6.37$  and  $5.9 \times 10^4$  cell  $\text{ml}^{-1}$ ), growth rates ( $1.85$  and  $1.8$  divisions  $\text{d}^{-1}$ ), and pH ( $8.3$  and  $8.23$ ) of the inocula were similar for the  $\text{NO}_3^-$  and  $\text{NH}_4^+$  cultures.

After inoculating with  $^{55}\text{Fe}$ -labeled cells, particulate  $^{55}\text{Fe}$  concentrations (dpm  $\text{ml}^{-1}$ ), cell densities, cell volumes, fluorescence, and pH (Accumet pH meter 915<sup>®</sup> Fisher calibrated with NBS buffers) were measured daily throughout algal growth. Samples for particulate  $^{55}\text{Fe}$  were collected on 3  $\mu\text{m}$  Poretics<sup>®</sup> polycarbonate filters and rinsed with Ti(III) citrate EDTA (Hudson and Morel 1989) and 10 ml of synthetic ocean water (SOW). Particulate Fe (mol Fe  $\text{ml}^{-1}$ ) was computed from the  $^{55}\text{Fe}$  on the filters (dpm  $\text{ml}^{-1}$ ), determined by liquid scintillation counting, and the specific activity of  $^{55}\text{Fe}$  in the medium (dpm  $\text{mol}^{-1}$ ), after correcting for quenching and decay. Samples for cell counts were preserved with Lugol's solution (Parsons et al. 1984). Cell densities were determined by microscopy using a Palmer-Maloney chamber to enumerate a minimum of 600 cells per sample. Cell volumes were measured in live phytoplankton as described above. The long-term uptake rates of Fe ( $\rho\text{Fe}^{\text{LT}}$ , mol Fe  $\text{fl}^{-1} \text{h}^{-1}$ ) were computed from the increase in particulate Fe (mol Fe  $\text{ml}^{-1}$ ) and the increase in biovolume (cell  $\text{ml}^{-1} \times \text{fl cell}^{-1}$ ) between sampling periods (h) viz:

$$\rho\text{Fe}^{\text{LT}} = \frac{(\text{particulate Fe } \text{ml}^{-1}_{\text{day 2}} - \text{particulate Fe } \text{ml}^{-1}_{\text{day 1}})}{(\text{cell } \text{ml}^{-1}_{\text{day 2}} - \text{cell } \text{ml}^{-1}_{\text{day 1}}) \times (\text{fl cell}^{-1}) \times (\text{h})}$$

Steady state uptake rates ( $\rho\text{Fe}^{\text{ss}}$ , mol Fe  $\text{fl}^{-1} \text{h}^{-1}$ ) were calculated from the product of Fe quota ( $Q_{\text{Fe}}$ , mol Fe  $\text{fl}^{-1}$ ) and specific growth rate ( $\mu$ ,  $\text{d}^{-1}$ ) viz:

$$\rho\text{Fe}^{\text{ss}} = Q_{\text{Fe}} \times \mu$$

*Short-term Fe uptake rates*—Short-term Fe uptake rates of Fe-limited *T. oceanica* were measured using  $^{55}\text{FeCl}_3$  (specific activity 25–40 mCi  $\text{mg}^{-1}$ , DuPont). All the experiments were conducted in the dark to avoid photoreduction of the organically bound Fe complexes (Anderson and Morel 1982). During mid-exponential phase, *T. oceanica* was har-

vested by gentle filtration (<100 mm Hg) onto acid-washed, 3  $\mu\text{m}$  polycarbonate Poretics® filters and immediately resuspended in fresh SOW (with  $\text{PO}_4^{3-}$  and  $\text{SiO}_3^{2-}$ ), containing organically bound  $^{55}\text{Fe}$ . In the FeEDTA uptake experiments, the concentration of EDTA was kept constant (100  $\mu\text{M}$ ) and four different Fe concentrations were added: 1, 10, 50, and 1,000 nM. These Fe additions resulted in  $[\text{Fe}']$  of 5.3, 24.9, 120, and 2,280 pM, respectively (calculated using MINEQL; Westall et al. 1976). In one uptake experiment, Fe was added as an FeDFB complex (12.9 nM Fe : 129 nM DFB), prepared as previously described (Maldonado and Price 1999). The  $^{55}\text{Fe}$  and the organic ligands were premixed (for 2–8 h) and then added to the media 24 h prior to starting the experiments. The pH of the uptake media was identical to that of the basal growth media (pH 8.02), and did not change during the experiments. Duplicate samples for particulate  $^{55}\text{Fe}$  were taken from each flask every hour for 4 hours as outlined above. Volumetric uptake rates ( $\text{mol Fe ml}^{-1} \text{ h}^{-1}$ ) were calculated from the linear regression of particulate Fe concentration as a function of incubation time (h). These rates were normalized to cell biomass, using cell density ( $\text{mol Fe cell}^{-1} \text{ h}^{-1}$ ) and/or biovolume ( $\text{mol Fe fl}^{-1} \text{ h}^{-1}$ ). The effect of N on short-term Fe uptake rates by *T. oceanica* was examined, using subsaturating Fe concentrations (10 nM Fe and 100  $\mu\text{M}$  EDTA), in the presence of 50  $\mu\text{M}$   $\text{NO}_3^-$  or  $\text{NH}_4^+$ .

*Fe(III) reduction rate measurements*—Iron(III) reduction was measured spectrophotometrically using the Fe(II) specific ligand BPDS (batho-phenanthroline-di-sulphonate, Sigma Chemical, extinction coefficient @ 535 nm = 22,140  $\text{M}^{-1} \text{ cm}^{-1}$ ; Blair and Diehl 1961). The reduction assay solution consisted of N-free SOW with standard additions of  $\text{PO}_4^{3-}$  and  $\text{SiO}_3^{2-}$  (10  $\mu\text{M}$  and 100  $\mu\text{M}$ , respectively), 100  $\mu\text{M}$  BPDS, and 6.5 mM HEPPS [N-(2-hydroxyethyl) piperazine-N'-(3-propanesulfonic acid), pH 8.2]. During initial trials,  $\text{PO}_4^{3-}$  (0.8 mM, pH 8) was used to buffer the reduction assay solution, but it titrated most of the Fe, and resulted in fast abiotic Fe reduction rates.

Cultures of *T. oceanica* were grown under Fe-limiting conditions (pFe 21) in 1 liter polycarbonate bottles. Cells were harvested in exponential phase by gentle filtration onto an acid-washed, 3  $\mu\text{m}$  polycarbonate filter and immediately resuspended in 6 ml of the reduction buffer. Densities and cell volumes were determined in subsamples of the resuspended cells. The Fe reduction assay was initiated by adding FeEDTA in a 1 : 10 molar ratio (10  $\mu\text{M}$  Fe : 100  $\mu\text{M}$  EDTA). It was technically necessary to add a high concentration of Fe(III) because of the low detection limit for Fe(II)BPDS<sub>3</sub>. Formation of the Fe(II)BPDS<sub>3</sub> complex was measured by absorbance at 535 nm ( $A_{535}$ ) of the entire cell suspension (continuous method) or of filtered subsamples (manual method).

In the continuous method, cells ( $1\text{--}10 \times 10^6$  cells  $\text{ml}^{-1}$ ) were kept in suspension by mixing with Teflon-coated, magnetic stir bars, and absorbance of each cuvette was measured every minute for 2–6 h using a dual beam Cary 1E spectrophotometer (Varian) equipped with a multi-cell holder. These assays were thus conducted in the dark, except when the samples were exposed to the light from the spectrophotometer ( $535 \pm 0.04$  nm) for 0.033 s each minute while the

absorbance was measured. Absorbance of the experimental cuvette was determined relative to a reference cuvette, which contained cells in the reduction buffer with BPDS, but no FeEDTA. Cuvettes and stir bars were acid-washed and sterilized with 70% ethanol before use. Control experiments were run initially to establish the stability of the reference samples during the experiments. These samples consisted of cells in reduction buffer with additions of either FeEDTA (preequilibrated 10  $\mu\text{M}$  Fe and 100  $\mu\text{M}$  EDTA) or BPDS (100  $\mu\text{M}$ ). Chemical and photochemical reduction of Fe(III)EDTA with BPDS was negligible in samples of sterile SOW, containing all reagents but no cells. Fe(III)DTPA and Fe(III)DFB reduction rates were also determined spectrophotometrically using BPDS as outlined above. In these experiments, Fe was added either as a DTPA complex (10  $\mu\text{M}$  Fe : 100  $\mu\text{M}$  DTPA) or as a DFB complex (1  $\mu\text{M}$  Fe : 10  $\mu\text{M}$  DFB). These Fe complexes were prepared as previously described (Maldonado and Price 1999).

In the manual method, *T. oceanica* was resuspended in 150 ml of reduction buffer with BPDS and kept in the dark at 20°C. Experiments were initiated by adding FeEDTA. Cell densities varied between  $1\text{--}10 \times 10^5$  cells  $\text{ml}^{-1}$ . Every 15–30 min, two 10 ml subsamples were removed, filtered through a Whatman GF/F filter, and  $A_{535}$  was measured relative to a reference sample containing SOW.

*Direct determination of biologically produced Fe(II)*—Extracellular reduction of Fe(III) was also determined by measuring Fe(II) production directly by chemiluminescence (King et al. 1995). The detection limit of the Fe(II) assay was 0.2 nM. To prevent photoreduction of Fe(III), all analyses were conducted in a dark room. A stock solution of 1  $\mu\text{M}$  Fe(II) in 6 mM HCl was prepared weekly using  $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$  and diluted daily in SOW (pH 6.6) to make standards. Chemicals were at least analytical reagent grade and were dissolved in 0.2  $\mu\text{m}$  filtered, 18.2 M $\Omega$  MilliQ-water. Except for the standards that were made daily, all solutions and reagents used in the analyses were aged for at least 2 days to allow the low levels of Fe contaminants to adsorb onto the walls of the reagent bottles.

In mid-exponential phase, 500 ml of Fe-limited *T. oceanica* (pFe 21) were concentrated by gentle filtration onto an acid-washed, 3  $\mu\text{m}$  polycarbonate filter housed in a 25 mm Sartorius® filter holder. The filter holder was then connected to the sample line and the filter (and cells) continuously flushed with SOW (pH 6.6) containing  $\text{PO}_4^{3-}$  and  $\text{SiO}_3^{2-}$ . In the reduction experiments, FeEDTA (10  $\mu\text{M}$  Fe : 100  $\mu\text{M}$  EDTA) was added to the SOW. Subsamples from the sample line were then automatically injected into the carrier solution (18.2 M $\Omega$  MilliQ-water), mixed with the luminol reagent [0.1 mM 5-amino-2,3-dihydro-1,4-phthalazinedione (Luminol, Sigma Chemical) in  $\text{NH}_3$  buffer solution ( $\approx 1$  M, pH 11.8)], and introduced into the luminescence flow cell every min for up to 4 h. Cellular reduction rates were calculated from the steady-state Fe(II) concentration, the flow rate in the sample line, and the number of cells on the filter (e.g., Parslow et al. 1985).

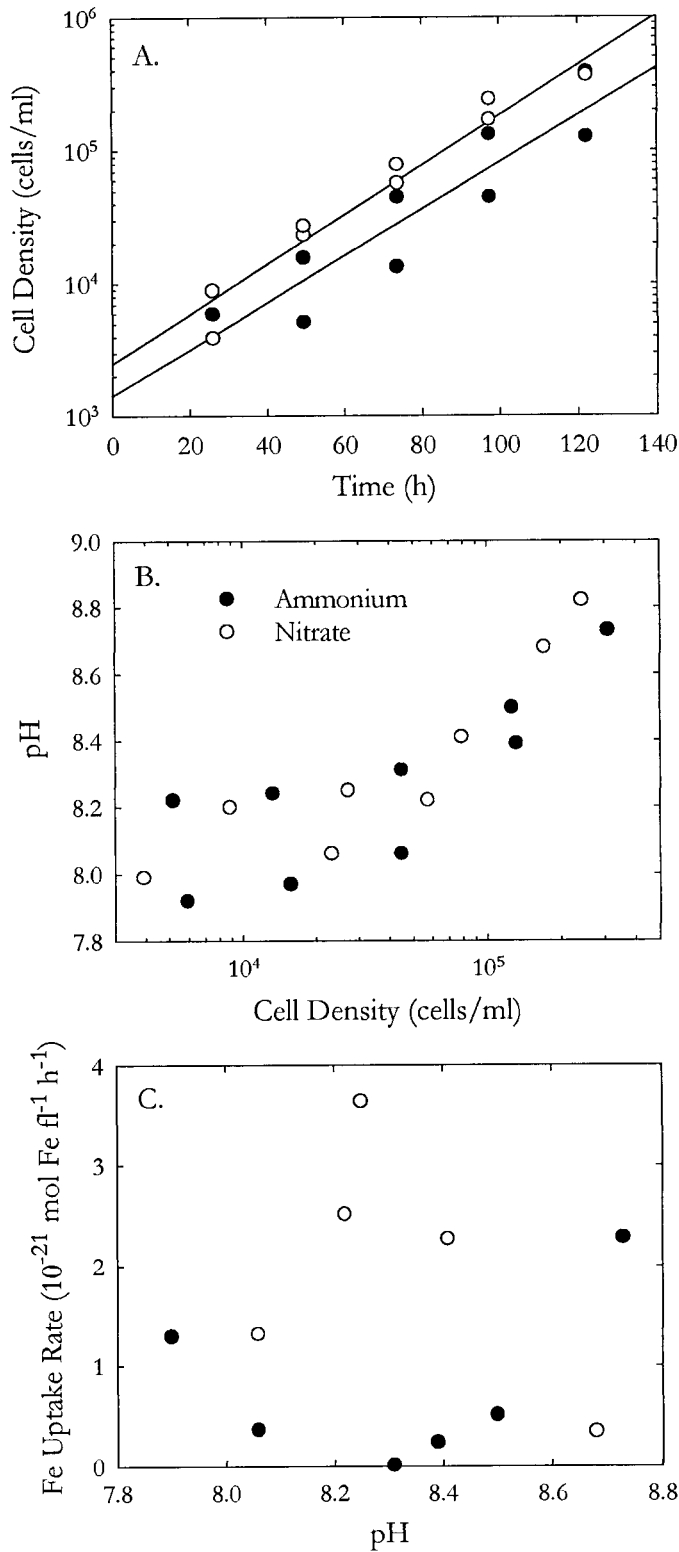


Fig. 1. Growth of *T. oceanica* in Fe-limiting media amended with either 50  $\mu\text{M}$   $\text{NO}_3^-$  or 50  $\mu\text{M}$   $\text{NH}_4^+$ . (A) Cell density (cell  $\text{ml}^{-1}$ ) as a function of time (h); (B) pH of the culture media as a function of cell density (cell  $\text{ml}^{-1}$ ); (C) Long-term Fe uptake rates ( $10^{-21}$  mol Fe  $\text{fl}^{-1}$   $\text{h}^{-1}$ ) as a function of pH of the growth media. The initial pH of the media was 7.9. The plotted lines in (A) were

## Results

*Effects of N source on long-term Fe uptake rate, intracellular Fe concentration, and pH—T. oceanica* grew as fast or faster, on average, in  $\text{NO}_3^-$  than in  $\text{NH}_4^+$ -amended medium, under Fe-limiting conditions (Maldonado and Price 1996). In the experiment reported here (Fig. 1), cells in the  $\text{NO}_3^-$ -amended culture grew at  $1.10$   $\text{d}^{-1}$  and contained  $3.97 \pm 1.67 \times 10^{-20}$  mol Fe  $\text{fl}^{-1}$ , and cells in the  $\text{NH}_4^+$ -amended culture grew at  $1.05$   $\text{d}^{-1}$  and contained  $1.73 \pm 1.08 \times 10^{-20}$  mol Fe  $\text{fl}^{-1}$ . The calculated steady-state Fe uptake rates ( $\rho\text{Fe}^{\text{ss}}$ ) were thus  $1.82 \pm 0.77$  and  $0.76 \pm 0.47 \times 10^{-21}$  mol Fe  $\text{fl}^{-1}$   $\text{h}^{-1}$  for  $\text{NO}_3^-$  and  $\text{NH}_4^+$ -grown cells, respectively. These rates agreed well with those measured in the replicate cultures that were used to inoculate the cultures used in this experiment:  $\rho\text{Fe}^{\text{ss}}(\text{NO}_3^-) = 2.18 \pm 0.82 \times 10^{-21}$  mol Fe  $\text{fl}^{-1}$   $\text{h}^{-1}$ ;  $\rho\text{Fe}^{\text{ss}}(\text{NH}_4^+) = 1.45 \pm 0.88 \times 10^{-21}$  mol Fe  $\text{fl}^{-1}$   $\text{h}^{-1}$ .

Nitrate-grown cells maintained both higher intracellular Fe concentrations and faster long-term rates of Fe uptake than  $\text{NH}_4^+$ -grown cells during the growth period when the pH was identical in both media (Fig. 1; Table 1). Mean long-term Fe uptake rates were 2.4–2.6 times faster in the  $\text{NO}_3^-$  ( $2.03 \times 10^{-21}$  mol Fe  $\text{fl}^{-1}$   $\text{h}^{-1}$  or  $2.29 \times 10^{-21}$  mol Fe  $\mu\text{m}^{-2}$   $\text{h}^{-1}$ ) than in the  $\text{NH}_4^+$ -amended cultures ( $0.79 \times 10^{-21}$  mol Fe  $\text{fl}^{-1}$   $\text{h}^{-1}$  or  $0.97 \times 10^{-21}$  mol Fe  $\mu\text{m}^{-2}$   $\text{h}^{-1}$ ). The long-term Fe uptake rates that we measured were remarkably similar to the steady-state rates reported above. Although the pH was generally higher at the end of exponential phase in  $\text{NO}_3^-$  (pH =  $8.6 \pm 0.2$ ,  $n = 30$ ) than in  $\text{NH}_4^+$  (pH =  $8.4 \pm 0.2$ ,  $n = 27$ ) cultures, mid-exponential pH differed little between N sources ( $<0.05$ ) and varied mainly with cell density (Fig. 1).

*Short-term Fe uptake rates*—The presence (50  $\mu\text{M}$ ) or absence of N in the uptake media had little effect on the short-term Fe uptake rates of the  $\text{NO}_3^-$ - or  $\text{NH}_4^+$ -grown cells (Table 2), so subsequent experiments were conducted in N-free media. We observed, however, that in one experiment, Fe uptake rates of  $\text{NO}_3^-$ -grown cells were inhibited by nitrate (5 vs.  $1.79 \times 10^{-21}$  mol Fe  $\text{fl}^{-1}$   $\text{h}^{-1}$ , for control vs. N addition,  $P < 0.01$ ;  $4.58 \times 10^4$  cell  $\text{ml}^{-1}$ ). An examination of this culture revealed that *T. oceanica* was undergoing sexual reproduction and growing remarkably slowly ( $1.25$  divisions  $\text{d}^{-1}$ ). The results of this experiment were thus not included with the data reported in Table 2.

Short-term rates were directly proportional (1:1) to total Fe at low concentrations and approached an asymptote at 1  $\mu\text{M}$ , the highest concentration tested (Table 3). Regardless of N source used for growth, saturated rates were indistinguishable ( $P > 0.05$ , Table 3) and were greatly enhanced (by 30 times) compared to the steady-state uptake rates ( $\rho\text{Fe}^{\text{ss}}$ ) at 12.5 nM Fe. Iron uptake rates of  $\text{NO}_3^-$ -grown cells were significantly faster than those of  $\text{NH}_4^+$ -grown cells

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obtained by least-squares regression ( $y = 3.36 + 0.019x$ ,  $r^2 = 0.96$  and  $y = 3.16 + 0.018x$ ,  $r^2 = 0.84$  for  $\text{NO}_3^-$  and  $\text{NH}_4^+$ -grown cultures, respectively).

Table 1. Long-term Fe uptake rates determined during daily intervals of growth of *T. oceanica* in  $\text{NO}_3^-$  and  $\text{NH}_4^+$ -amended media. The initial pH of the culture media was 7.9. Replicate cultures had identical growth rates ( $\text{NH}_4^+$  [A and B]:  $1.05 \pm 0.05 \text{ d}^{-1}$  and  $\text{NO}_3^-$  [C and D]:  $1.10 \pm 0.05 \text{ d}^{-1}$ ) so the data were pooled. Mean cell volumes and surface areas were 164 and 204  $\text{fl cell}^{-1}$  and 145 and 167  $\mu\text{m}^2 \text{ cell}^{-1}$  for the  $\text{NO}_3^-$  and the  $\text{NH}_4^+$ -cultures, respectively. Mean uptake rates are reported  $\pm$  SD.

Nitrogen substrate	Replicate	pH of medium	Particulate Fe produced (fmol $\text{Fe ml}^{-1}$ )	Cells produced ( $10^3 \text{ cells ml}^{-1}$ )	Particulate Fe produced per biovolume ( $10^{-21} \text{ mol Fe fl}^{-1}$ )	Elapsed time (h)	Fe uptake rate per biovolume ( $10^{-21} \text{ mol Fe fl}^{-1} \text{ h}^{-1}$ )	
								mean = $0.79 \pm 0.85$
Ammonium	B	7.97	61	9.7	30.8	23.55	1.31	
	B	8.06	53	29.1	8.9	24.20	0.37	
	A	8.31	2.0	31.5	0.3	23.80	0.01	
	B	8.39	102	87.2	5.8	23.80	0.24	
	A	8.5	215	82.2	2.8	24.84	0.52	
	A	8.73	1,890	182.0	50.9	22.33	2.28	
							mean = $0.79 \pm 0.85$	
	Nitrate	C	8.06	98	19.18	31.0	23.55	1.37
		C	8.22	343	34.3	61.0	24.20	2.52
		D	8.25	256	18.2	85.6	23.55	3.64
D		8.41	466	51.7	55.0	24.20	2.27	
C		8.68	155	113.6	8.3	23.80	0.35	
							mean = $2.03 \pm 1.24$	

Table 2. Short-term Fe uptake rates ( $10^{-21} \text{ mol Fe fl}^{-1} \text{ h}^{-1}$ ) of Fe-limited *T. oceanica* grown in medium containing 50  $\mu\text{M NO}_3^-$  or  $\text{NH}_4^+$ . Rates were determined at subsaturating Fe concentrations (10 nM Fe: 100  $\mu\text{M EDTA}$ , pH 8.02) in the absence or the presence of 50  $\mu\text{M NO}_3^-$  or  $\text{NH}_4^+$ , corresponding to the preconditioning N source used for growth. The mean cell densities were  $6.1 \pm 3.4$  (range 1.3–11.0) and  $9.1 \pm 6.6 \times 10^4 \text{ cells ml}^{-1}$  (range 2.1–15.0) for  $\text{NO}_3^-$ - and  $\text{NH}_4^+$ -grown cells, respectively. The uptake rates are means  $\pm$  SE.

Preconditioning N substrate	Short-term Fe uptake rate ( $10^{-21} \text{ mol Fe fl}^{-1} \text{ h}^{-1}$ )		Number of replicates
	-Nitrogen	+Nitrogen	
$\text{NO}_3^-$	$2.66 \pm 0.33$	$2.87 \pm 0.35$	7
$\text{NH}_4^+$	$0.73 \pm 0.05$	$0.90 \pm 0.20$	3

when Fe concentrations were subsaturating ( $P < 0.05$ , Table 3;  $P < 0.01$ , Table 2). An analysis of all uptake data showed that the discrepancy between the rates of  $\text{NO}_3^-$ - and  $\text{NH}_4^+$ -grown cells ranged from 1.4 to 3.6 times (normalized to cell volume).

**Iron(III) reduction: Fe(III)EDTA**—In the reduction experiments, cells were collected on filters and quickly resuspended in assay buffer. To determine whether such treatment adversely affected the phytoplankton, carbon fixation rates were measured in nonresuspended and resuspended cells using 0.2  $\mu\text{Ci}$  of  $^{14}\text{C-HCO}_3^-$  (specific activity 8.4 mCi  $\text{mmol}^{-1}$ ). Samples for particulate organic  $^{14}\text{C}$  were withdrawn every 5 min and added to scintillation vials containing 0.5 ml 1 N HCl to remove inorganic  $^{14}\text{C}$ . No significant difference was observed between treatments (15.6 fmol C  $\text{cell}^{-1} \text{ h}^{-1}$ , control; 15 fmol C  $\text{cell}^{-1} \text{ h}^{-1}$ , resuspended cells) indicating that the cells were not physiologically stressed by filtration and resuspension, and that BPDS in the reduction buffer was not toxic.

Iron (III) reduction by Fe-limited *T. oceanica* was constant over the duration of the experiments, and did not re-

Table 3. Short-term Fe uptake rates of Fe-limited *T. oceanica* grown in medium containing  $\text{NO}_3^-$  or  $\text{NH}_4^+$  as a function of total concentration of Fe  $[\text{Fe}]_T$  and the inorganic Fe concentration  $[\text{Fe}']$  in the medium. Rates were measured in N-free SOW (pH 8.02) with 100  $\mu\text{M EDTA}$ . During the uptake experiment, cell densities of the  $\text{NO}_3^-$  and  $\text{NH}_4^+$  preconditioned cultures were  $5.64 \pm 1.57 \times 10^4 \text{ cells ml}^{-1}$  and  $14.9 \pm 0.6 \times 10^4 \text{ cells ml}^{-1}$ , respectively. \*\* Significantly different ( $t$ -test,  $P < 0.005$ ); \* Significantly different ( $t$ -test,  $P < 0.05$ ) ( $n = 3$ ,  $\pm$  SD).

$[\text{Fe}]_T$ (nM)	$[\text{Fe}']$ (pM)	Short-term Fe uptake rate ( $10^{-21} \text{ mol Fe fl}^{-1} \text{ h}^{-1}$ )	
		$\text{NH}_4^+$ -preconditioned cells	$\text{NO}_3^-$ -preconditioned cells
1	5.3	$0.12 \pm .019$	$0.17 \pm 0.011^{**}$
10	24.9	$1.25 \pm .22$	$1.71 \pm 0.26^*$
50	120	$3.95 \pm .66$	$5.84 \pm 0.16^{**}$
1,000	2,280	$73.9 \pm 11.8$	$75.7 \pm 13.6$

Table 4. Reduction rates of Fe(III) bound to EDTA (10  $\mu\text{M}$  Fe: 100  $\mu\text{M}$  EDTA), DTPA (10  $\mu\text{M}$  Fe: 100  $\mu\text{M}$  DTPA) and DFB (1  $\mu\text{M}$  Fe: 10  $\mu\text{M}$  DFB) by Fe-limited *T. oceanica* grown in media enriched with either  $\text{NH}_4^+$  or  $\text{NO}_3^-$ . Assays were conducted in N-free BPDS reduction assay buffer (pH 8.2). Stability constants (Log  $K_{\text{Fe(III)L}}$ ) of Fe(III) organic ligand complexes ( $I = 0.5$ ) are reported. Reduction rates are means  $\pm$  SD and the numbers of independent samples are in parentheses. \*\* Significantly different (paired *t*-test,  $P < 0.05$ ).

Ligand	Log $K_{\text{Fe(III)L}}$	Fe(III) reduction rate ( $10^{-18}$ mol Fe $\text{fl}^{-1}$ $\text{h}^{-1}$ )	
		$\text{NH}_4^+$ - preconditioned cells	$\text{NO}_3^-$ - preconditioned cells
EDTA	24.0 $\dagger$	1.61 $\pm$ 0.2 (18)	3.23 $\pm$ 0.2 (18)**
DTPA	26.2 $\dagger$	—	2.77 $\pm$ 0.6 (2)
DFB	31 $\ddagger$	0.63 $\pm$ 0.3 (7)	2.0 $\pm$ 0.3 (7)**

$\dagger$  Ringbom (1963).

$\ddagger$  Anderson and Morel (1982).

quire illumination (Fig. 2). No reduction occurred in the control cuvette without cells, demonstrating that abiotic reduction of Fe(III) was insignificant during the BPDS assay. Both  $\text{NO}_3^-$ - and  $\text{NH}_4^+$ -grown cells reduced Fe bound to EDTA (10  $\mu\text{M}$  Fe: 100  $\mu\text{M}$  EDTA), but reduction rates of  $\text{NO}_3^-$ -grown cells were  $2.0 \pm 0.1$  times faster (Table 4). The mean Fe(III) reduction rates of phytoplankton in the  $\text{NO}_3^-$ - and  $\text{NH}_4^+$ -amended cultures were  $3.23 \pm 0.2$  ( $n = 18$ ) and  $1.61 \pm 0.2$  ( $n = 18$ )  $\text{amol Fe fl}^{-1} \text{h}^{-1}$ , respectively (paired *t*-test,  $P < 0.05$ ;  $\text{amol} = 10^{-18}$  mol). Because of the high concentration of Fe(III)EDTA that was added, some ferric hydroxides could have precipitated (Sunda and Huntsman 1995) and been reduced by the phytoplankton.

Low levels of Fe(II) were detected in the SOW by the chemiluminescence assay (King et al. 1995), but decreased once the cells on the filter were connected to the sample line, most likely because of uptake (Fig. 3). When FeEDTA (10  $\mu\text{M}$  Fe: 100  $\mu\text{M}$  EDTA) was added to the SOW, the Fe(II) concentration increased slightly, indicating the presence of Fe(II) in the Fe(III)EDTA stock solution. The Fe(II) concentration increased dramatically, from below the detection limit to 12 nM, in the presence of the phytoplankton. Iron(II) production due to cellular excretion or abiotic reduction of Fe(III) was below the detection limit of the system (0.2 nM). Direct measurement of Fe(II) thus provided unequivocal evidence of Fe(III) reduction by *T. oceanica*. Using this method, the mean Fe reduction rate by Fe-limited  $\text{NO}_3^-$ -grown cells was  $0.2 \pm 0.08$   $\text{amol Fe fl}^{-1} \text{h}^{-1}$  ( $n = 27$ ). This net rate was roughly 15 times slower than that determined using BPDS (Table 4), possibly because differences between the two assays in pH, cell density, flowing compared to stagnant buffer, or the amount of reduced Fe that was taken up by the cells.

A number of experiments were designed to investigate the mechanism of Fe(III) reduction. For most of these we used the simpler BPDS assay. Iron reduction was apparently not mediated by reducing metabolites that were actively excreted by the cells (Fig. 4) because no activity was detected in the sample filtrate after resuspended cells were allowed to stand

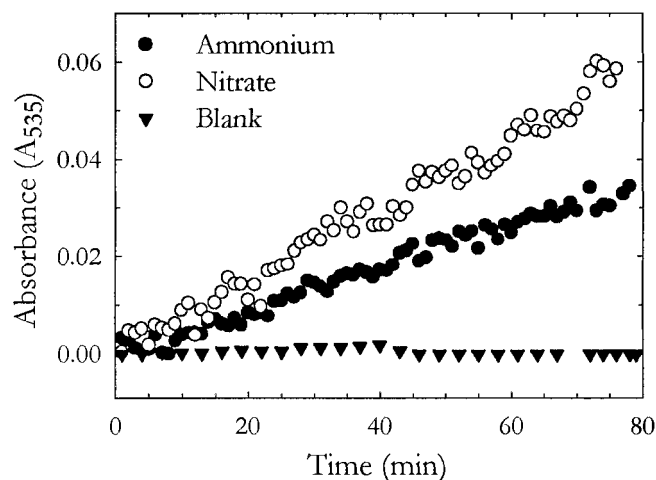


Fig. 2. Kinetics of Fe(III) reduction by Fe-limited *T. oceanica*. Formation of Fe(II)BPDS<sub>3</sub> was measured by absorbance at 535 nm ( $A_{535}$ ) following additions of Fe(III)EDTA (10  $\mu\text{M}$ : 100  $\mu\text{M}$ ) at time zero. Phytoplankton were grown with  $\text{NO}_3^-$  and  $\text{NH}_4^+$  and resuspended in N-free reduction assay buffer with BPDS (100  $\mu\text{M}$ ). The blank contained no cells. The phytoplankton cell density in the  $\text{NO}_3^-$  treatment was  $1.4 \times 10^7$  cells  $\text{ml}^{-1}$ , and in the  $\text{NH}_4^+$  treatment was  $1.9 \times 10^7$  cells  $\text{ml}^{-1}$ .

in reduction buffer for 4 h. Similar results were obtained with the chemiluminescence assay, which showed that no Fe(II) was detected if FeEDTA was introduced to the sample line immediately “downstream” of the cells. Iron reduction (measured with BPDS) by homogenized cells occurred to some degree, but at a much slower rate than was observed for intact cells (Fig. 4). Measurements made for a longer duration established that Fe(III) reduction by the cell homogenate was a short-lived response which subsided after ca. 90 min. In contrast, intact cells continued to reduce Fe at a constant rate for 6 h. The heat-shock treatment completely inhibited Fe(III) reduction (Fig. 4).

Although bacteria comprised a negligible fraction of the biomass in the cultures, they could potentially contribute to Fe(III) reduction during the assays if they were retained by 3  $\mu\text{m}$  filters. Their importance in Fe reduction was established by size fractionation experiments where they were separated from the phytoplankton using filters. The results showed that bacteria were not important as they reduced Fe at a significantly slower rate than was observed for phytoplankton (Fig. 5). However, the bacterial Fe reduction rate was barely distinguishable from rates measured in controls that were run in other experiments, suggesting that it was probably equivalent to a blank. Indeed, the rates of Fe(III) reduction by the phytoplankton alone ( $>3 \mu\text{m}$ ) and by the unfractionated samples containing phytoplankton and bacteria ( $>0.4 \mu\text{m}$ ) were indistinguishable ( $111$  vs.  $98 \times 10^{-12}$  mol Fe  $\text{ml}^{-1} \text{h}^{-1}$ ).

Iron reduction rates were not only affected by the N source used for growth, but also by the degree of Fe-limitation experienced by the cells. These rates increased significantly in cells that were grown under more severe Fe-deficiency, but only in those that were grown in the presence

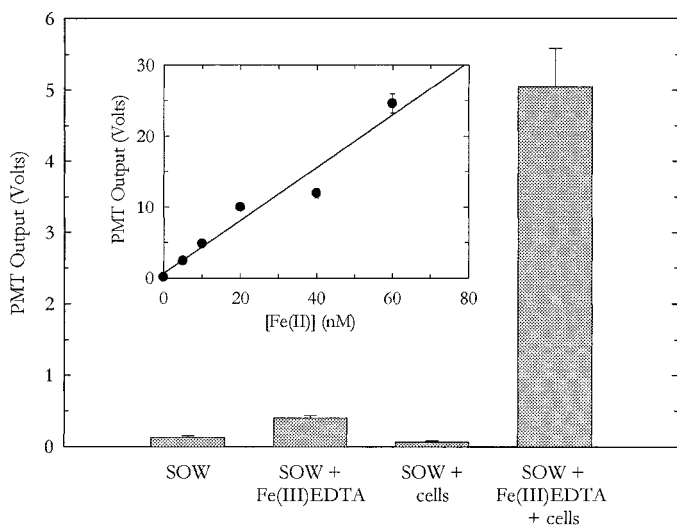


Fig. 3. Chemiluminescence detection of Fe(II) in SOW, and SOW + Fe(III)EDTA in the presence and absence of *T. oceanica*. The insert shows a typical relationship between voltage output (Volts) from the photomultiplier tube (PMT) detector and the Fe(II) concentration of the standard solution.  $\text{PMT}(\text{Volts}) = 0.384 \times [\text{Fe}(\text{II})]_{\text{nM}} - 123$ ,  $r^2 = 0.95$ . Sample flow rate was  $0.3 \text{ ml min}^{-1}$  and  $8.3 \times 10^7$  cells were on the in-line filter. Production of this amount of Fe(II), corresponded to a cellular Fe(III) reduction rate of  $2.2 \text{ amol cell}^{-1} \text{ h}^{-1}$  ( $\text{amol} = 10^{-18} \text{ mol}$ ).

of  $\text{NO}_3^-$  (Fig. 6). No such increase was evident in those cells that were cultured with  $\text{NH}_4^+$  (data not shown).

**Reduction of Fe(III) bound to DTPA and DFB**—The chemical specificity of the cellular Fe reduction pathway was investigated using two other strong Fe binding ligands: DTPA, a synthetic chelator, and DFB, a fungal siderophore. Both of these compounds have considerably higher stability constants of their Fe complexes than EDTA:  $\log K = 26.2$  and  $\log K = 31$ , respectively, compared to 24.2 for EDTA ( $I = 0.05$ , Anderson and Morel 1982; Ringbom 1963) (Table 4). Reduction rates of Fe(III) in these complexes were slower than those determined for FeEDTA (FeEDTA  $3.32 \pm 0.2$ , FeDTPA  $2.77 \pm 0.2$ , FeDFB  $2.0 \pm 0.3 \text{ amol Fe fl}^{-1} \text{ h}^{-1}$ ). The slower rate of Fe reduction for FeDFB was most likely the result of its 10-fold lower concentration ( $1 \mu\text{M Fe}$ :  $10 \mu\text{M DFB}$ ) compared to FeEDTA and FeDTPA ( $10 \mu\text{M Fe}$ :  $100 \mu\text{M EDTA}$  or DTPA). Nitrate-grown cells reduced Fe(III) in FeDFB more than three times faster ( $2.0 \pm 0.3 \text{ amol Fe fl}^{-1} \text{ h}^{-1}$ ) than  $\text{NH}_4^+$ -grown cells ( $0.63 \pm 0.3 \text{ amol Fe fl}^{-1} \text{ h}^{-1}$ ,  $P < 0.05$ ) (Table 4). These faster reduction rates corresponded with faster subsaturating Fe uptake rates ( $12.9 \text{ nM Fe}$  and  $129 \text{ nM DFB}$ ) of  $\text{NO}_3^-$ - than  $\text{NH}_4^+$ -grown cells ( $3.44 \pm 0.1$  vs.  $2.06 \pm 0.2 \times 10^{-21} \text{ mol Fe fl}^{-1} \text{ h}^{-1}$ , respectively,  $P < 0.001$ ).

**Fe-limited growth rates**—*T. oceanica* was able to grow with nanomolar concentrations of Fe ( $1.29$ – $129 \text{ nM}$ ) bound to the fungal siderophore, DFB ( $129 \text{ nM}$ ) (Fig. 7). As Fe became more limiting at lower Fe:DFB ratios, growth rates of *T. oceanica* decreased concomitantly, from  $1.98$  to  $0.84$  divisions  $\text{d}^{-1}$  in the  $\text{NO}_3^-$ -amended cultures, and from  $1.77$

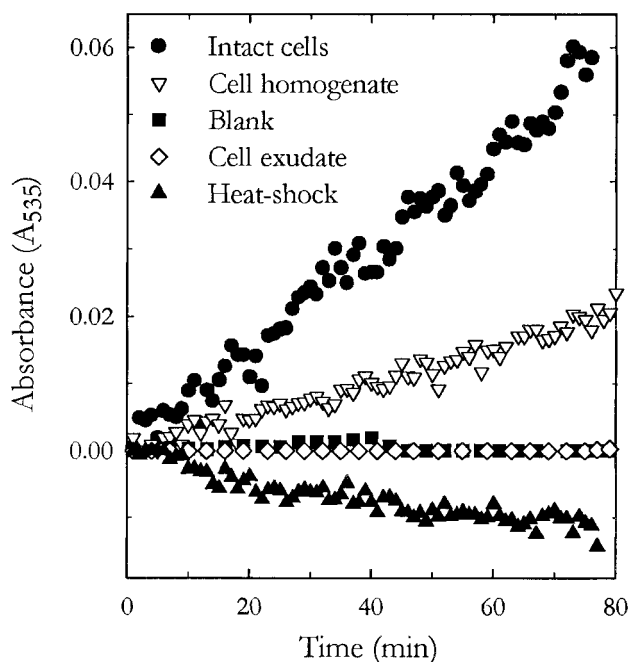


Fig. 4. Kinetics of Fe(III) reduction determined from Fe(II)BPDS<sub>3</sub> formation ( $A_{535}$ ). Iron-limited *T. oceanica* was grown in  $\text{NO}_3^-$ -amended medium, harvested, and resuspended in reduction assay buffer with BPDS ( $100 \mu\text{M}$ ). At time zero, Fe(III)EDTA ( $10 \mu\text{M}$ :  $100 \mu\text{M}$ ) was added to a subsample of the cells ( $1.4 \times 10^7$  cells  $\text{ml}^{-1}$ ) and then assayed for Fe(II) production (intact cells). The blank contained no cells. Another subsample was heated in a  $50^\circ\text{C}$  water bath for 10 min and then cooled on ice to  $20^\circ\text{C}$  before the assay was initiated (heat-shocked). These cells remained intact following this treatment. A third subsample was ground on ice for 2 min with a Teflon tissue homogenizer to disrupt all cells (cell homogenate). Some intact cells from the same culture were resuspended in reduction buffer and incubated for 4 h in darkness (cell exudate). The sample was subsequently filtered through a  $0.45 \mu\text{m}$  acid-washed, polycarbonate filter, augmented with BPDS and assayed for Fe(III) reduction activity. Reference samples were the same as the experimental treatments, but contained no Fe(III)EDTA. Absorbance of the reference sample was subtracted from the absorbance of the experimental treatment.

to  $0.66$  divisions  $\text{d}^{-1}$  in the  $\text{NH}_4^+$ -amended cultures. The relative difference in rates ( $1.2 \pm 0.03$  times) between the two N cultures remained constant over the range of Fe concentrations tested: in all cases those receiving  $\text{NO}_3^-$  grew significantly faster ( $P < 0.05$ ).

Growth rates of *T. oceanica* were also measured under low Fe conditions (pFe 21) with the two synthetic organic ligands and with  $\text{NO}_3^-$  or  $\text{NH}_4^+$  as N source (Fig. 8). In both the EDTA- and DTPA-based media, rates were less than maximum and significantly faster for cells in the presence of  $\text{NO}_3^-$  compared to  $\text{NH}_4^+$  ( $P < 0.05$  or  $P < 0.01$ , respectively). Growth rates decreased further when Fe was not added to the medium, but under these conditions  $\text{NH}_4^+$ -grown cells divided significantly faster than  $\text{NO}_3^-$ -grown ones (Fig. 8).

## Discussion

A number of lines of evidence point to a role for  $\text{NO}_3^-$  metabolism in Fe uptake by *T. oceanica*. Under Fe-limiting

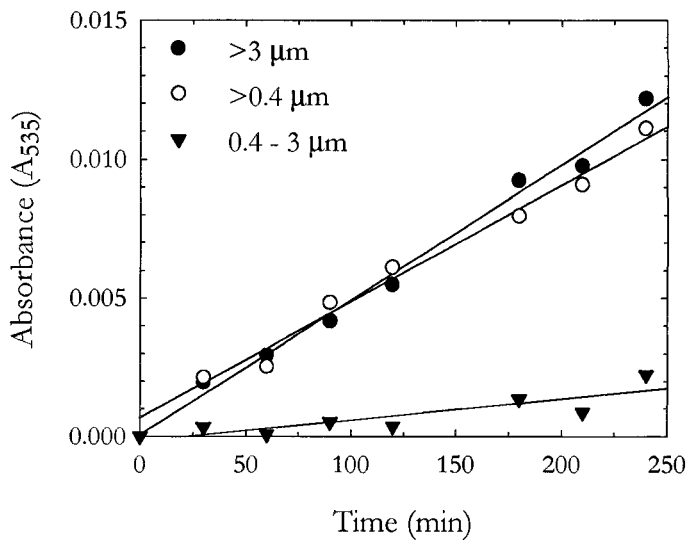


Fig. 5. Kinetics of Fe(III) reduction following addition of Fe(III)EDTA to samples of an Fe-limited culture of *T. oceanica* collected on filters of different porosity. Cells from a  $\text{NO}_3^-$ -amended culture were harvested onto a  $0.4 \mu\text{m}$  ( $>0.4 \mu\text{m}$ ) and a  $3 \mu\text{m}$  ( $>3 \mu\text{m}$ ) filters and the filtrate that passed through the  $3 \mu\text{m}$  filter was then refiltered through a  $0.4 \mu\text{m}$  filter ( $0.4\text{--}3 \mu\text{m}$ ). The filters were placed in reduction assay buffer, shaken to resuspend the cells (*T. oceanica*  $7.76 \times 10^5$  cells  $\text{ml}^{-1}$ ), and the formation of Fe(II)BPDS<sub>3</sub> was measured ( $A_{535}$ ) as described.

conditions,  $\text{NO}_3^-$ -grown cells contain higher intracellular Fe concentrations and maintain faster long- and short-term Fe uptake rates than cells grown with  $\text{NH}_4^+$ . The uptake rate measurements are in good agreement with one another and corroborate the faster steady-state Fe uptake rates calculated for  $\text{NO}_3^-$ -dependent cells. Collectively the results demonstrate that at moderately low Fe concentrations *T. oceanica* accumulates Fe more efficiently when  $\text{NO}_3^-$  is present. As discussed below, phytoplankton that assimilate  $\text{NO}_3^-$  may be able to acquire Fe at a faster rate because they possess higher levels of ferric chelate reductase activity. Physiological adaptations such as smaller cell size and more efficient cell surface Fe transporters may also be important.

**Cell size**—Cell size reduction is an adaptive strategy for nutrient acquisition by phytoplankton because it increases the rate at which resources such as Fe are transported relative to their cellular requirements for growth (Hudson and Morel 1990, 1993; Sunda and Huntsman 1995; Maldonado and Price 1996). *T. oceanica* and other diatoms are typically smaller when growing on  $\text{NO}_3^-$  compared to  $\text{NH}_4^+$  so they may have a greater competitive ability to obtain Fe when it is a limiting resource. As discussed previously (Maldonado and Price 1996), the surface area to volume ratio of  $\text{NO}_3^-$ -grown cells was only 1.1 times greater than that of the  $\text{NH}_4^+$ -grown cells and thus cannot entirely account for their larger quotas or faster volumetric uptake rates. Expressed per unit of surface area ( $\text{mol Fe } \mu\text{m}^{-2} \text{ h}^{-1}$ ), the results clearly show that at limiting Fe concentrations  $\text{NO}_3^-$ -dependent phytoplankton possess 1.3–3.3 times faster transport rates than  $\text{NH}_4^+$ -grown cells.

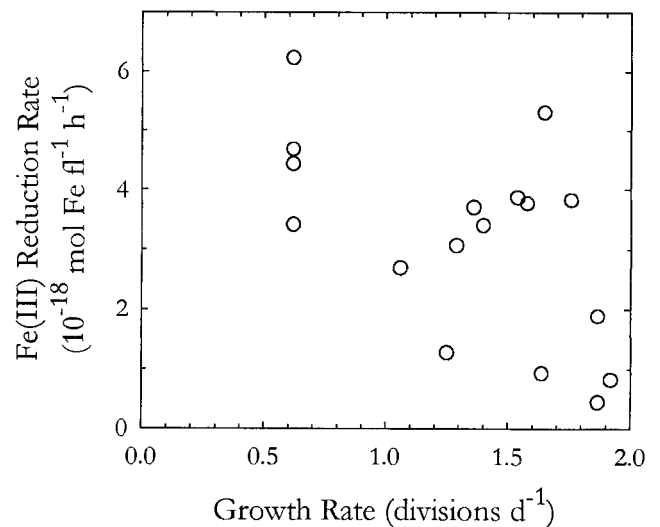


Fig. 6. Iron (III) reduction rate ( $10^{-18}$  mol  $\text{Fe fl}^{-1} \text{ h}^{-1}$ ) of *T. oceanica*, as a function of Fe-limited growth rate (divisions  $\text{d}^{-1}$ ) in  $\text{NO}_3^-$ -amended media. Maximum, Fe-sufficient growth rate was 1.9 divisions  $\text{d}^{-1}$ . Reduction rate increases significantly as Fe-limited growth rate decreases ( $P < 0.01$ ,  $r^2 = 0.40$ , linear regression;  $P < 0.05$ ,  $r^2 = 0.46$ , 2nd order polynomial regression).

**Fe transport ligand density**—The dependence of Fe uptake on cellular Fe nutritional state is well documented and described, but its modulation by N is not. Low Fe concentrations limit phytoplankton growth rates and lead to an increase in Fe transport capacity (Harrison and Morel 1986). Under such conditions, maximum Fe uptake rates are considerably faster than those of Fe-replete cells because Fe-limited phytoplankton possess a greater number of transport ligands (Hudson and Morel 1990). Judging from the similarity in the maximum specific Fe uptake rates ( $V_{\text{max}} \text{NO}_3^- = 0.8 \text{ h}^{-1}$ ,  $V_{\text{max}} \text{NH}_4^+ = 1.0 \text{ h}^{-1}$ ; where  $V_{\text{max}} = \rho^{\text{max}}/Q_{\text{Fe}}$ ,  $\rho^{\text{max}} = 75.68$  and  $73.86 \times 10^{-21}$  mol  $\text{Fe fl}^{-1} \text{ h}^{-1}$  for  $\text{NO}_3^-$  and  $\text{NH}_4^+$  pFe 21 grown cultures, respectively [Table 3], and  $Q_{\text{Fe}} = 10$  and  $7.3 \times 10^{-20}$  mol  $\text{Fe fl}^{-1}$  for  $\text{NO}_3^-$  and  $\text{NH}_4^+$  pFe 21-grown cultures, respectively; Maldonado and Price 1996), our data seem to rule out differences in Fe nutritional state as an explanation for the effects of N source. For this comparison we were careful to select only Fe-limited cultures growing at similar rates with  $\text{NO}_3^-$  and  $\text{NH}_4^+$ . Equal maximum saturated uptake rates ( $\rho^{\text{max}}$ , Table 3) suggest that transport ligand density and internalization rate constant are identical for  $\text{NO}_3^-$ - and  $\text{NH}_4^+$ -cultured cells. However, a combination of different internalization rate constants and transport ligand densities may also account for the similarity in the rates. Since Fe-limited phytoplankton have the greatest number of Fe transporters that can fit in their plasma membrane (e.g., *T. weissflogii*, Hudson and Morel 1990), it seems reasonable that Fe-limited  $\text{NO}_3^-$ - and  $\text{NH}_4^+$ -grown cells would have similar Fe ligand densities.

**Increasing the pool of bioavailable Fe**—In Aquil culture media, high concentrations of EDTA are added to buffer trace metal concentrations and maintain chemically well-defined conditions (Price et al. 1988/89). Most of the metals

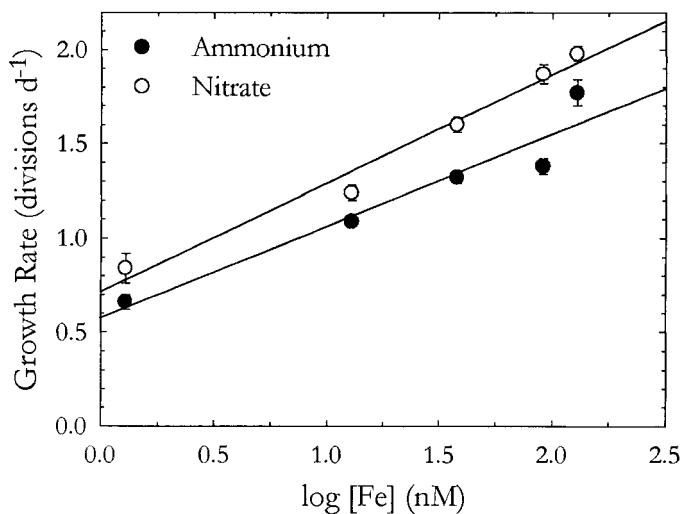


Fig. 7. Iron-limited growth rates (divisions  $d^{-1}$ ) of *T. oceanica* as a function of Fe-siderophore (FeDFB) concentration, in media containing 129 nM DFB and a range of Fe(III) concentrations (1.29, 12.9, 38.7, 90.3, and 129 nM), with either  $NO_3^-$  or  $NH_4^+$  as N source. Error bars represent the standard error of the mean ( $n = 6$ ). The line describes a least-squares linear regression fit to the data.

are bound to EDTA, leaving only small amounts present as dissolved inorganic species ( $Fe'$ ). The organic complexes react slowly with transport ligands (Hudson and Morel 1990) and are impermeable to biological membranes (Hudson and Morel 1993; Price et al. unpubl.), so they are not thought to be biologically important. In contrast, inorganic Fe species are bioavailable and their concentration determines the rate of Fe uptake (Anderson and Morel 1982; Hudson and Morel 1990, 1993). Thus, if phytoplankton are able to catalyze the dissociation of organic Fe complexes in the media, then the supply of  $Fe'$  from Fe chelates would increase and Fe could be more readily accessible. Two biologically mediated mechanisms may enhance the dissociation of organic Fe complexes: changes in pH associated with N assimilation, and enzymatic reduction of Fe bound to organic complexes.

Changes in pH associated with N uptake could potentially increase the lability of some Fe complexes (Hudson et al. 1992), and  $NO_3^-$ - and  $NH_4^+$ -grown cells undoubtedly differ in this regard (Raven and Smith 1976). Cells that metabolize  $NO_3^-$  excrete  $OH^-$  to maintain charge balance, so the pH of culture media would be expected to rise to a greater extent when  $NO_3^-$  opposed to  $NH_4^+$  is the N source for growth (Raven and Smith 1974). Higher pH may increase the dissociation of FeEDTA by favoring the formation of biologically labile Fe hydroxide species (Hudson et al. 1992). These inorganic Fe species ( $Fe'$ ) are believed to control Fe uptake in diatoms (Hudson and Morel 1990; 1993), so that  $NO_3^-$ -induced increase in external pH during growth might enhance long-term cellular Fe uptake rates, and thus Fe:C quotas.

The results of the long-term exponential growth experiments seem to rule out this potential artifact. Although pH was indeed higher for  $NO_3^-$  (pH  $8.6 \pm 0.2$ ) than  $NH_4^+$  (pH  $8.4 \pm 0.2$ )-grown cultures at the end of exponential growth, during mid-exponential phase the pH was indistinguishable

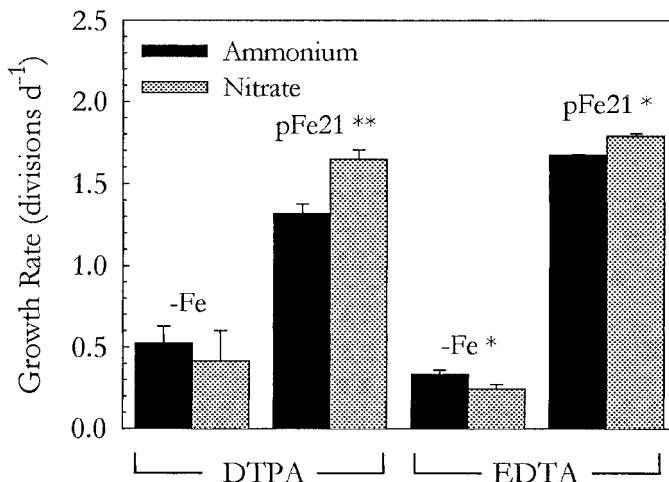


Fig. 8. Iron-limited growth rates (divisions  $d^{-1}$ ) of *T. oceanica* in EDTA- and DTPA-buffered media containing no added Fe (-Fe) or Fe concentration adjusted to pFe 21, with  $NO_3^-$  or  $NH_4^+$  as N source. Growth rate data for EDTA are from Maldonado and Price (1996). Error bars represent the standard error of the mean. \* Significantly different (paired  $t$ -test,  $P < 0.05$ ); \*\* Significantly different (paired  $t$ -test,  $P < 0.01$ ).

between N sources. Furthermore, at identical pH ( $8.02 \leq pH \leq 8.7$ ),  $NO_3^-$ -grown cells maintained higher intracellular Fe concentrations and transported Fe at a significantly faster rate than  $NH_4^+$  cells. Thus, differences in culture pH that may arise from N metabolism do not seem to account for the higher intracellular Fe concentrations or the faster long-term Fe uptake rates of the  $NO_3^-$ -grown cells under our experimental conditions. Furthermore, in all the short-term Fe uptake experiments,  $NO_3^-$ - and  $NH_4^+$ -grown cells were resuspended in the same uptake medium, at a pH of 8.02. This medium was not enriched with N, to avoid pH changes associated with N assimilation, and measurements established that pH did not vary during the course of the uptake. At subsaturating Fe concentrations, cells grown in  $NO_3^-$ -amended media always took up Fe at a significantly faster rate than the cells grown with  $NH_4^+$ .

The influence of N most likely exists because of physiological and biochemical differences associated with transport and assimilation of the two different N forms. One obvious difference between  $NO_3^-$ - and  $NH_4^+$ -grown phytoplankton is the presence of nitrate (NR) and nitrite (NiR) reductases that are involved in  $NO_3^-$  assimilation (although unique membrane transporters for  $NO_3^-$  and  $NH_4^+$  are also likely). Low levels of NR are detected in the plasmalemma of cells using  $NH_4^+$  (Tischner et al. 1989; Corzo et al. 1991), but its activity is greatly increased in cells that use  $NO_3^-$  for growth (Tischner et al. 1989; Stöhr et al. 1993). This difference has potentially important ramifications for Fe acquisition because of the substrate specificity of NR. Ostensibly, NR reduces  $NO_3^-$  to  $NO_2^-$ , using electrons derived from NAD(P)H (Taiz and Zeiger 1991). In higher plants and algae, both soluble NR (Solomonson and Vennesland 1972; Redinbaugh and Campbell 1983; Castignetti and Smarrelli 1984) and plasmalemma bound NR (Jones et al. 1987; Jones and Morel 1988; Corzo et al. 1991; Stöhr et al. 1993), can

catalyze the reduction of Fe bound to organic complexes. In heterotrophic bacteria, mutants lacking NR are deficient in the Fe reductase pathway (Ottow 1968, 1970). Thus,  $\text{NO}_3^-$ -grown phytoplankton may be able to increase the concentration of Fe' in the medium by extracellular enzymatic reduction of Fe bound to organic complexes.

*Fe reduction*—Although prevailing ideas regarding the availability of metals to phytoplankton emphasize the importance of the free ions and the inorganic species (Anderson and Morel 1982; Hudson and Morel 1990, 1993), the biological redox lability of metal organic complexes has been known for sometime. Phytoplankton reduce Fe and Cu bound to organic compounds, including EDTA (Anderson and Morel 1980; Jones et al. 1987), by extracellular redox enzymes that chemically resemble NAD(P)H cytochrome c reductases (Jones et al. 1987; Jones and Morel 1988). Our results suggest that the Fe reductase of *T. oceanica* may also be a plasmalemma bound form of NR (or a reductase that is regulated by N metabolic pathways), similar to that proposed for Cu reductase of *T. weissflogii* (Jones and Morel 1988). As outlined below, support for this model comes from our observations of greater Fe reductase activity in  $\text{NO}_3^-$ -dependent cells.

Our initial experiments established that intact, living cells were required for rapid, continuous Fe reduction, a reaction that was apparently enzymatic as heat-shock completely inhibited its activity. Reduced substances excreted from the cells were not important, although their rapid inactivation by oxidizing substances prior to the reduction assay cannot be ruled out. In the homogenized extracts, the transient reduction by intracellular metabolites demonstrated their finite capacity to reduce Fe and their slower reaction rate compared to whole cell samples. Since EDTA and BPDS complexes are membrane impermeable (Anderson and Morel 1980; 1982; Price et al. unpubl.), reduction must occur extracellularly. The average rates of Fe reduction we measured with BPDS ( $2.1 \text{ amol Fe } \mu\text{m}^{-2} \text{ h}^{-1}$ ) are in good agreement with those reported for *T. weissflogii* with FeEDTA ( $1.6 \text{ amol Fe } \mu\text{m}^{-2} \text{ h}^{-1}$ , Anderson and Morel 1980) and cytochrome *c* ( $2.7 \text{ amol Fe } \mu\text{m}^{-2} \text{ h}^{-1}$ , Jones et al. 1987) as substrate. Like those of higher plants (Bienfait et al. 1983; Bienfait 1987), the ferric reductase lacks substrate specificity, as cells reduced Fe(III) bound to a variety of organic ligands (Table 4). Indeed, *T. oceanica* was able to reduce Fe bound to the fungal siderophore DFB at fast rates. Reductase activity is considerably higher in  $\text{NO}_3^-$  than  $\text{NH}_4^+$ -grown cells, a result that was observed regardless of the organic Fe complex tested. The organic Fe complexes themselves appear to be substrates for the reaction, not Fe', because the latter varied in concentration by more than  $10^6$ -fold, but the reduction rates were virtually identical.

Reductase activity appears to be under control of cellular Fe status, but such control is only expressed in cells that metabolize  $\text{NO}_3^-$ . In  $\text{NO}_3^-$ -dependent cells, increased Fe-deficiency enhances the rate of Fe reduction, but no such enhancement is evident in  $\text{NH}_4^+$ -grown cells. At present we are unable to fully explain this result. If NR indeed mediates the reduction, then our observation that the activity of total cellular NR increases under mild Fe deficiency in  $\text{NO}_3^-$ -

dependent *T. weissflogii* (Price unpubl.) may be relevant. We note that ferric chelate reductases from yeast and *Arabidopsis* have a high degree of homology with members of the ferredoxin-NADP<sup>+</sup> reductase family of reductases, which include NR (Roman et al. 1993; Segal and Abo 1993; Robinson et al. 1999). The ability of  $\text{NH}_4^+$ -grown *T. oceanica* to reduce Fe, although at a slower rate, may imply that they too have low levels of a NR-like redox enzyme in the plasmalemma. Consistent with this hypothesis are observations of the insensitivity to  $\text{NH}_4^+$  of basal plasmalemma NR activity of  $\text{NH}_4^+$ -grown cells (Tischner et al. 1989; Corzo et al. 1991; Jones and Morel 1988).

*Fe-limited growth with synthetic chelators*—Severely Fe-limited *T. oceanica* achieved similar or significantly slower division rates in  $\text{NO}_3^-$  compared to  $\text{NH}_4^+$ -based medium with no supplemental Fe additions and either DTPA or EDTA as chelators (Fig. 8). Growth was supported by a low Fe concentration present as a contaminant ( $\sim 1.5 \text{ nM}$ ), and Fe:C ratios of the cells were the same in both cultures (Maldonado and Price 1996). The results thus provide support for the colimitation hypothesis (Maldonado and Price 1996; Price and Morel 1991; Price et al. 1991, 1994), in demonstrating that the high Fe requirements for  $\text{NO}_3^-$  metabolism reduce fitness at very low Fe concentrations. We surmise that the reductase pathway was ineffective under these conditions and that the  $\text{NO}_3^-$ -grown cells were unable to obtain the extra Fe needed for growth. At first glance, this result may seem to contradict our data concerning Fe reduction, because ferric reductase activity increased as Fe became more limiting (Fig. 6). However, the reductase rates we measured were maximum potential rates determined with high concentrations of FeEDTA ( $10 \mu\text{M}$ ), orders of magnitude greater than those realized in the cultures with only  $1.5 \text{ nM}$  Fe. Although faster rates of Fe transport were observed for  $\text{NO}_3^-$  than  $\text{NH}_4^+$  preconditioned *T. oceanica* at a similar substrate concentrations ( $1 \text{ nM}$  FeEDTA), these cells had been maintained at pFe 21 prior to the experiment and were thus not severely Fe-limited (Table 3). During these uptake experiments, pFe 21  $\text{NO}_3^-$ -cultured cells had sufficient reductase activity to increase the concentration of the dissolved Fe pool available for transport. At the lowest Fe concentration tested for growth, *T. oceanica* had the same Fe quota with both N sources but its division rate was slower with  $\text{NO}_3^-$ . If the reductase contains haem Fe as it does in yeast (Finegold et al. 1996), it may not be sufficiently active to confer an advantage to  $\text{NO}_3^-$  dependent phytoplankton during prolonged growth at very low Fe concentrations.

*Iron-siderophore complex (FeDFB) as a source of Fe*—As previously demonstrated with other phytoplankton (Bailey and Taub 1980; Soria-Dengg and Horstmann 1995), *T. oceanica* was able to grow in the presence of Fe-siderophores (FeDFB). However, in contrast to growth with synthetic organic ligands (DTPA and EDTA), *T. oceanica* always grew significantly faster with  $\text{NO}_3^-$  than with  $\text{NH}_4^+$  even at  $1.29 \text{ nM}$  FeDFB. Uptake and reduction of Fe(III) bound to DFB have been linked in iron-stressed *Chlorella vulgaris* (Allnutt and Bonner 1987a,b), suggesting that a re-

ductive mechanism might be involved in Fe uptake from FeDFB.

The most compelling evidence for the importance of biological reduction in Fe acquisition comes from uptake experiments in the dark with nanomolar additions of FeDFB ([Fe] = 12.9 nM, and [DFB] = 129 nM). If the small amount of Fe' in equilibrium with FeDFB is substrate for the Fe transport reaction, then its rate of supply should equal or exceed the measured rate of Fe transport. However, using the rate constant of FeDFB dissociation ( $k'_d = 63.25 \times 10^{-12} \text{ s}^{-1}$ , calculated with  $K'_{\text{FeL}} = k_r/k_d$ , where  $K' = 10^{16.5}$  and  $k'_r = 2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ , Hudson et al. 1992) the supply of inorganic Fe is estimated to be at most  $0.003 \text{ pM h}^{-1}$  ( $12.9 \text{ nM} \times 63.25 \times 10^{-12} \text{ s}^{-1} \times 3,600 \text{ s h}^{-1}$ ). This rate is five orders of magnitude slower than the measured Fe uptake rate of  $13.42 \text{ pM h}^{-1}$  ( $3.44 \times 10^{-21} \text{ mol Fe fl}^{-1} \text{ h}^{-1}$ ,  $65 \text{ fl cell}^{-1}$ , and  $6 \times 10^7 \text{ cells L}^{-1}$ ), so thermal dissociation of FeDFB is unlikely to be important. Direct acquisition of Fe bound to DFB may be a mechanism to account for the fast rates of Fe uptake and our results suggest that a reductive step is involved in this process. If the rate of Fe reduction is directly proportional to [FeDFB], then it roughly corresponds to  $100 \text{ pM h}^{-1}$ , suggesting that the ferric chelate reductase could provide all of the necessary Fe for uptake. Faster Fe uptake rates by  $\text{NO}_3^-$  compared to  $\text{NH}_4^+$  grown cells supported faster rates of growth at all FeDFB concentrations. Collectively, the results suggest that phytoplankton have an inducible uptake system for Fe bound to siderophores that is linked to Fe nutritional state and  $\text{NO}_3^-$  metabolism.

*Oceanographic relevance*—Recent measurements demonstrate that almost all dissolved Fe in oceanic waters is bound to strong organic ligands (Gledhill and van den Berg 1994; Rue and Bruland 1995; Wu and Luther 1995). If inorganic Fe (Fe') is the only form available for biological uptake, then its concentration (estimated to be 0.01 pM, Rue and Bruland 1995) would be well below the diffusion-limited barrier (4 pM) for adequate Fe uptake even by the picoplankton. Phytoplankton in the open ocean must therefore acquire Fe from organic complexes via photochemical or biological reduction. As illustrated below both processes are likely to be important.

Photochemical reactions alter Fe speciation and could increase Fe' concentration in surface waters, particularly in the presence of organic chromophores that greatly enhance ferric reduction (Finden et al. 1984; Waite and Morel 1984; Wells and Mayer 1991; Hudson et al. 1992, Kuma et al. 1992). In coastal seawater, supplemented with natural humic material, the rate constant of the reaction is  $6.3 \times 10^{-4} \text{ s}^{-1}$  (Miller et al. 1995). To estimate the photoreduction rate of Fe(III) in the open sea we can assume that virtually all dissolved Fe (70 pM, Johnson et al. 1997) is complexed to organic ligands and that these are as photolabile as Fe humic complexes. Accordingly, the rate of Fe(II) production is  $1.3 \text{ nM Fe(II) d}^{-1}$  with 8 h of sunlight. This rate is fast and could significantly increase the concentration of bioavailable Fe. However, the ligands that bind Fe in the sea ( $K'_{\text{LI/Fe}'} = 1.2 \times 10^{13} \text{ M}^{-1}$ , Rue and Bruland 1995) have a higher affinity for Fe than humic substances ( $K'_{\text{HA/Fe}'} = 2.4 \times 10^6 \text{ M}^{-1}$ , calculated from  $K'_{\text{HA/Fe}'} = 9 \times 10^6 \text{ L kg}^{-1}$ , Hudson et al. 1992;

and assuming  $M.W._{\text{HA}} = 800$  daltons, Aiken et al. 1989), so the actual rate of Fe reduction in the field could be considerably slower.

Extracellular biological reduction may be another mechanism by which phytoplankton directly obtain organic Fe. Our results demonstrate that Fe complexed by DFB, a fungal siderophore with high affinity for Fe ( $K'_{\text{DFB/Fe}'} = 10^{16.5} \text{ M}^{-1}$ , Hudson et al. 1992) compared to seawater ligands ( $K'_{\text{LI/Fe}'} = 1.2 \times 10^{13} \text{ M}^{-1}$ , Rue and Bruland 1995), was indeed reducible at low concentrations (1.29 nM FeDFB), similar to those present in oceanic waters (e.g., 0.24 nM, Rue and Bruland 1995). The rate of biological Fe(III) reduction in situ can be estimated using the FeDFB reduction rate ( $4.8 \times 10^{-15} \text{ mol Fe cell}^{-1} \text{ d}^{-1}$ ), if we assume that the rate saturates at  $0.1 \text{ }\mu\text{M}$  and is directly proportional to organic Fe concentration. With 70 pM of dissolved organic Fe, the rate varies between 3.5 and  $350 \text{ pM d}^{-1}$  when cell densities are  $10^6 - 10^8 \text{ L}^{-1}$ . This calculation is likely to underestimate the in situ Fe(III) reduction rate, because phytoplankton in the open ocean are generally smaller (e.g.,  $1.5 \text{ }\mu\text{m}$  in diameter) than *T. oceanica* ( $6 \text{ }\mu\text{m}$ ) and the rate per unit biovolume should increase inversely with cell diameter. Accordingly, the in situ Fe(III) reduction rate is predicted to range from  $0.014 - 1.4 \text{ nM d}^{-1}$  and is comparable to the rate of phytoplankton demand for Fe (e.g.,  $2 \text{ pM d}^{-1}$  in the subarctic Pacific, Maldonado and Price 1999;  $4.8 \text{ pM d}^{-1}$  in the equatorial Pacific, Johnson et al. 1994) and to the rate of biological Fe(III) reduction measured in the subarctic Pacific (Maldonado and Price 1999). Biological reduction of organic Fe could potentially supply a large fraction of the Fe required for growth of phytoplankton in the sea. We note, however, that although the ferric chelate reductase may allow phytoplankton to obtain Fe bound in organic complexes, it represents only a partial solution to the problem of Fe nutrition, because phytoplankton communities continue to be under Fe-stress judging from their response to Fe additions (e.g., Price et al. 1994).

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