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Copper-dependent iron transport in coastal and oceanic diatoms

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Abstract

We investigated the presence of plasmalemma-bound copper-containing oxidases associated with the inducible iron (Fe) transport system in two diatoms of the genus *Thalassiosira*. Under Fe-limiting conditions, *Thalassiosira oceanica*, an oceanic isolate, was able to enzymatically oxidize inorganic Fe(II) extracellularly. This oxidase activity was dependent on copper (Cu) availability and diminished by exposure to a multi-Cu oxidase (MCO) inhibitor. The rates of Fe uptake from ferrioxamine B by Fe-limited *T. oceanica* were also dependent on Cu availability in the growth media. The effects of Cu limitation on Fe(II) oxidation and Fe uptake from ferrioxamine B were partially reversed after a short exposure to a Cu addition, indicating that the putative oxidases contain Cu. Limited physiological experiments were also performed with the coastal diatom *Thalassiosira pseudonana* and provided some evidence for putative Cu-containing oxidases in the high-affinity Fe transport system of this isolate. To support these preliminary physiological data, we searched the newly available *T. pseudonana* genome for a multi-Cu-containing oxidase gene and, using real-time polymerase chain reaction (PCR), quantified its expression under various Fe and Cu levels. We identified a putative MCO gene with predicted transmembrane domains and found that transcription levels of this gene were significantly elevated in Fe-limited cells relative to Fe-replete cells. These data collectively suggest that putative MCOs are part of the inducible Fe transport system of Fe-limited diatoms, which act to oxidize Fe(II) following reductive dissociation of Fe(III) from strong organic complexes.

To date, most studies of Cu nutrition in marine phytoplankton have focused on its toxicity. Laboratory and field studies have shown that the concentrations of Cu(II) in some coastal regions are high enough to inhibit the growth of certain phytoplankton taxa (Brand et al. 1986) and to affect the composition of phytoplankton assemblages (Sunda et al. 1981; Moffett et al. 1997). In turn, phytoplankton are known to influence the speciation of dissolved Cu in seawater by releasing strong Cu organic ligands in response to Cu toxicity (Moffett and Brand 1996; Croot et al. 2000; Vasconcelos and Leal 2001).

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Less is known about the essentiality of Cu to marine phytoplankton. It has been established that Cu, a redox-active transition metal, acts as a cofactor in enzymes that scavenge reactive oxygen species and catalyze other redox reactions, and is therefore vital for growth. Thus far, Cu has been shown to be involved in the detoxification of superoxide radicals (i.e., Cu-containing superoxide dismutases) (Chadd et al. 1996), the breakdown of organic N sources (Palenik et al. 1988, 1989), and the electron transport chain reactions of photosynthesis (i.e., plastocyanin) (Sandmann et al. 1983) and respiration (cytochrome oxidase) (Stryer 1988).

One of the most intriguing findings about Cu nutrition in microorganisms over the past decade has been the identification of Cu as a critical component in the high-affinity Fe transport system of *Saccharomyces cerevisiae*, a yeast that has been the subject of the vast majority of research on Cu physiology in eukaryotic microorganisms. This Fe transport system, which is induced under Fe-limiting conditions, includes transmembrane ferric reductases, multi-Cu containing ferroxidases, and Fe(III) permeases (reviewed by Van Ho et al. 2002). The ferric reductases mediate the reductive dissolution of insoluble Fe(III) species and/or the reductive dissociation of Fe(III) from strong organic complexes (Eide et al. 1992). The Fe(II) produced by the reductases is reoxidized by a multi-Cu-containing ferroxidase during the membrane transport step (Askwith et al. 1994; Stearman et al. 1996). The

requirement for Cu in this inducible Fe transport system is sufficiently large that the yeast requires more Cu when it is Fe limited, and is unable to acquire Fe in low Fe media in the absence of Cu (Dancis et al. 1994). Closely related high-affinity Fe transport pathways have been shown to operate in the fungi *Schizosaccharomyces pombe* (Askwith and Kaplan 1997), the fungal pathogen *Candida albicans* (Knight et al. 2002), and the freshwater green algae *Chlamydomonas reinhardtii* (Herbik et al. 2002*a*,*b*; La Fontaine et al. 2002).

Marine phytoplankton also possess an inducible Fe acquisition mechanism. This mechanism is required for species inhabiting oceanic waters (Maldonado and Price 1999), because the dissolved Fe is in extremely low concentrations (~0.07 nmol L^{-1}) (Johnson et al. 1997), and the vast majority is bound to strong organic ligands (Gledhill and Van den Berg 1994; Rue and Bruland 1995; Wu and Luther 1995). Iron transporters on phytoplankton cell surfaces react with inorganic Fe(III) species, and organically bound Fe is not directly available for uptake (Hudson and Morel 1990). However, Fe-limited marine diatoms, both in the field and laboratory, exhibit enzymatic activity (Maldonado and Price 1999, 2000, 2001) that allows extracellular reduction of organically complexed Fe(III), thereby promoting the release of Fe(II) from a variety of organic complexes (Maldonado and Price 2000, 2001). Once reduced, Fe(II) is believed to be reoxidized before it reacts with the putative Fe(III) transporter (Anderson and Morel 1982; Maldonado and Price 2001). Whether this oxidation step is abiotic or enzymatic remains to be established (Anderson and Morel 1982; Maldonado and Price 2001). Even though the oxidation of Fe(II) occurs spontaneously and rapidly in oxic seawater (King et al. 1995), a multi-Cu-containing ferroxidase—as observed in *C. reinhardtii* and *S. cerevisiae*—may be important in order to acquire the reduced Fe before it diffuses away from the cell.

We thus hypothesized that multi-Cu oxidases (MCOs) are a major component of the inducible Fe transport system in diatoms, and began a study of Cu nutrition in two species of the genus *Thalassiosira*—a coastal, *Thalassiosira pseudonana*, and an oceanic centric diatom, *Thalassiosira oceanica*. Evidence for the presence of a putative MCO in the inducible Fe transport system of these organisms was obtained by examining the effects of Cu supply on their maximum potential rates of cellular Fe(II) oxidation and on their ability to acquire Fe from ferrioxamine B at subsaturating Fe concentrations. In addition, we identified a putative MCO gene in the newly available *T. pseudonana* genome (Armbrust et al. 2004), and monitored the expression of this putative MCO gene transcript in Fe-limited and Fe-sufficient cultures of *T. pseudonana* using quantitative real-time polymerase chain reaction (PCR).

Materials and methods

Study organisms—Two centric diatom species of the genus *Thalassiosira* were examined; *T. oceanica* (clone 1003), an oceanic isolate from the Sargasso Sea, and *T. pseudonana* (clone 3H), a coastal isolate from Moriches Bay, Long Island, New York. These isolates were obtained from the Provasoli-Guillard Center for Culture of Marine Phytoplankton (Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, Maine). The mean diameter of the cells under optimal trace metal conditions was ~6 and 4 μm for *T. oceanica* and *T. pseudonana*, respectively.

Culture media—Thalassiosira spp. were grown in the artificial seawater medium Aquil (Price et al. 1988, 1989), which consists of a major salt mixture (synthetic ocean water, SOW) at pH 8.2, enriched with standard additions of nitrate (300 μmol L^{-1} NO_3^-), phosphate (10 μmol L^{-1} PO_4^{3-}), and silicate (100 μmol L^{-1} SiO_3^{2-}), as well as vitamins. The trace elements Mn, Zn, Co, Mo, and Se were added bound to ethylenediaminetetra-acetate (EDTA) to attain a final EDTA concentration of 100 μmol L^{-1} in the growth medium (Maldonado and Price 1996). Additions of Mn, Zn, and Co were adjusted to achieve free-ion concentrations of $10^{-8.34}$, $10^{-10.97}$, and $10^{-10.97}$ mol L^{-1}, respectively. Total Mo and Se concentrations were 10^{-7} and 10^{-8} mol L^{-1}, respectively. Iron and Cu were added separately as a premixed FeEDTA or CuEDTA complex (1 : 1.05). Iron was added at a total concentration of 1.37 μmol L^{-1} (pFe 19) or 42 nmol L^{-1} (pFe 20.5) for the Fe-sufficient or Fe-limiting media, respectively (speciation calculated using MINEQL) (Westall et al. 1976). The inorganic Fe concentrations ([Fe′]) in the EDTA-buffered media were determined using the sulfoxine-reactive [Fe′] method (Hudson et al. 1992), and were 3.6 nmol L^{-1} and 107 pmol L^{-1} for the Fe-sufficient and the Fe-limiting media, respectively. Copper was added at total concentrations of 10.2 nmol L^{-1} and 1.96 nmol L^{-1} in the Cu-sufficient (pCu 14) and low-Cu media (pCu 15). In some experiments, Fe and/or Cu were not added (−Fe and/or −Cu media), although Fe and Cu was present as a contaminant at <2 and 1 nmol L^{-1}, respectively (determined by high-resolution inductively coupled plasma mass spectrometry [HR-ICPMS]). Sterile, trace metal–clean techniques were used during all manipulations, and the metal–EDTA reactions in the media were allowed to equilibrate overnight before the media were used.

Growth measurements—Thalassiosira spp. were grown at 20°C under a continuous, saturating photon flux density of 150 μmol quanta m^{-2} s^{-1}, as previously described (Maldonado and Price 2001). Under these conditions, the maximal rates of growth (μ_{max}) ever attained by *T. pseudonana* and *T. oceanica* were 3.8 and 3.0 dd^{-1}, respectively. Cell density and volume were measured with a Coulter Counter (model Z2) that was calibrated with 2.1-μm diameter polystyrene beads. Cellular surface areas were calculated assuming spherical cell shape and used to normalize the rates of all physiological processes.

Short-term Fe uptake rates from organically bound Fe (ρ_{FeDFB})—Rates of Fe uptake from the siderophore ferrioxamine B (FeDFB; Novartis Pharma) were used as a proxy for the activity of the inducible Fe uptake mechanism of diatoms. Previous work has shown that only

Fe-limited diatoms can acquire Fe from FeDFB at significant rates and that the ability of cells to utilize FeDFB increases with increasing cellular Fe deficiency (Maldonado and Price 1999, 2001). Short-term Fe uptake rates of acclimated *Thalassiosira* spp. were determined using 100 nmol L^{-1} $^{55}FeCl_3$ (specific activity, ~1.85 MBq μg^{-1}; PerkinElmer) mixed with 1 μmol L^{-1} DFB. At this Fe : DFB concentration (100 nmol L^{-1} Fe : 1 μmol L^{-1} DFB, corresponding to 3.2 amol L^{-1} [Fe′]) the Fe uptake system of *T. oceanica* is undersaturated (*see* fig. 1 in Maldonado and Price 2001). The Fe uptake medium was prepared as previously described (Maldonado and Price 2001). Cultures were grown in EDTA-buffered media and various Fe and Cu concentrations (consult legends for specific conditions). During midexponential phase, cells were harvested by gentle filtration onto an acid-washed, 2-μm polycarbonate Poretics filter and immediately resuspended in fresh chelexed, pH 8.2 SOW (with PO_4^{3-}, SiO_3^{2-}, but no NO_3^- added), containing 100 nmol L^{-1} ^{55}Fe : 1 μmol L^{-1} DFB. Each resuspended culture was placed in the dark and was sampled in duplicate every hour for 4–6 h—*Thalassiosira* spp. do not require light to acquire Fe (Anderson and Morel 1982; Hudson and Morel 1990). From each sample, cells were filtered onto 2-μm polycarbonate filters and rinsed with 0.2-μm filtered-Ti(III)EDTA-citrate reagent to remove extracellularly absorbed Fe (Hudson and Morel 1989). Unless otherwise stated, the rate of Fe uptake (mol Fe $cell^{-1}$ h^{-1}) for each culture was calculated from linear regression of accumulated particulate/cellular Fe as a function of incubation time (in hours), after correcting for Fe uptake by killed cells. The error in rate measurements was <10% (for examples, *see Figs. 1, 4*). Each cell-specific rate was normalized to cellular surface area (μm^2 $cell^{-1}$).

Short-term Fe uptake rates of inorganic Fe(III) and Fe(II) by T. oceanica—Short-term Fe uptake rates of inorganic Fe(III) and Fe(II) by *T. oceanica* were determined in the presence of 70 nmol L^{-1} inorganic ^{55}Fe(II) or ^{55}Fe(III) at pH 6.6. The ^{55}Fe(II) addition was prepared with sulfur dioxide gas (SO_2) as the reducing agent according to Hudson et al. (1992), using 0.1 mol L^{-1} instead of 1 mol L^{-1} NaCl for the $^{55}Fe(III)Cl_3$ stock solution. By using SO_2 as the reducing agent to generate ^{55}Fe(II) from ^{55}Fe(III), the reductant was removed from the ^{55}Fe(II) stock solution—by bubbling with N_2—before ^{55}Fe(II) was added to the uptake medium. Inorganic ^{55}Fe(II) was added to slightly acidified SOW (pH 6.6) in order to prevent rapid abiotic Fe(II) oxidation during the uptake experiment. At this pH, the cells are not physiologically stressed (Anderson and Morel 1982; Maldonado and Price 2000). Preliminary experiments on the rate of abiotic Fe(II) oxidation at pH 6.6 in SOW demonstrated that 4.4% ± 4.7% of the Fe(II) was oxidized 3 h after the initial addition, yielding a half-life of Fe(II) in our experiments of ~15 h (pH 6.6), which is in good agreement with the 13-h half-life determined previously (pH 6.3) (Anderson and Morel 1982). Thus, these Fe uptake experiments were shortened to 3 h. An additional uptake treatment consisted of an Fe(II) addition in the presence of 1 mmol L^{-1} ascorbate, a ferric reductant. The final pH of this treatment was also pH 6.6. Beside the additions mentioned above to slightly acidified seawater, these experiments were performed as described above for Fe uptake from organically bound Fe.

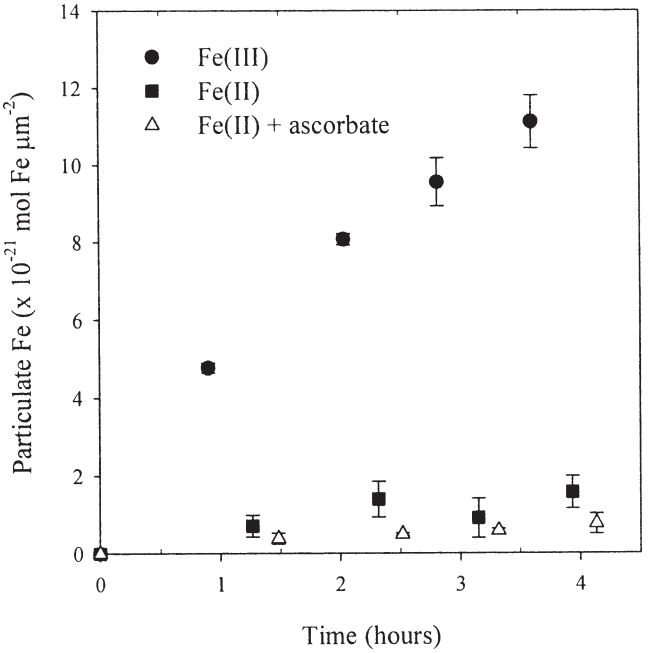

Fig. 1. Time course of accumulation of particulate ^{55}Fe in the dark by Fe-limited (pFe 20.5, pCu 14) *T. oceanica* at pH 6.6 in the presence of 70 nmol L^{-1} inorganic ^{55}Fe(II) or ^{55}Fe(III), with or without additions of 1 mmol L^{-1} ascorbate. Points represent the mean ± range of duplicate analyses of a single culture. The relative growth rate (μ/μ_{max}) of the culture was 0.62. The rates of inorganic Fe uptake were calculated by least-squares regression and were 29.8 (r^2 = 0.95), 3.51 (r^2 = 0.78), and 1.67 × 10^{-21} mol Fe μm^{-2} h^{-1} (r^2 = 0.97) for the Fe(III), Fe(II), and Fe(II) + ascorbate treatments, respectively.

Fe(III) reduction rates by Thalassiosira *spp.*—The cellular rates of Fe(III) reduction [$RED_{Fe(III)EDTA}$] were measured at saturating Fe concentrations (10 μmol L^{-1} Fe : 100 μmol L^{-1} EDTA) (*see* fig. 2 in Maldonado and Price 2001) and determined spectrophotometrically as previously described (manual method) (Maldonado and Price 2000). Cultures of *T. oceanica* and *T. pseudonana* were grown in EDTA-buffered media with Fe-limiting (pFe 20.5 or no Fe added) and Cu-sufficient concentrations (pCu 14).

Fe(II) oxidation rates by Thalassiosira *spp.*—To examine the cellular rates of Fe(II) oxidation [$OX_{Fe(II)}$], the rate of disappearance of 10 μmol L^{-1} Fe(II) over the course of 2–3 h in the presence and absence of the cells was measured spectrophotometrically using the Fe(II) specific ligand ferrozine (3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine; Sigma Chemical) (Stookey 1970). Due to the relatively low sensitivity of this spectrophotometric Fe(II) determination method, it was necessary to perform the oxidation experiments with high concentrations of Fe(II) (10 μmol L^{-1}). The measured rates of cellular Fe(II)

oxidation thus represent maximal potential values. We believe that this Fe(II) concentration is likely to saturate $OX_{Fe(II)}$ by *Thalassiosira* spp., although we do not currently have firm experimental confirmation for this. One of the caveats of these Fe(II) oxidation experiments is the inability to determine whether the disappearance of Fe(II) results from Fe(II) oxidation and subsequent Fe(III) uptake, or from direct Fe(II) uptake. Thus, to ensure that the disappearance of Fe(II) in the presence of the cells did not result from direct Fe(II) internalization, we measured the rates of ^{55}Fe uptake in the presence of $^{55}Fe(II)$ or $^{55}Fe(III)$ at the pH of the oxidation assay (pH 6.6) (*see* Results). The results of these experiments provided evidence that Fe(II) must be oxidized before transport. Thus, the rates of Fe(II) disappearance measured in the presence of the cells are interpreted as $OX_{Fe(II)}$ and subsequent internalization.

The oxidation rate assays were performed in the dark and initiated in a manner identical to the reduction assay, except that the cells were resuspended in 50 mL of the Fe(II) oxidation assay (pH 6.6 SOW containing 10 μmol L^{-1} Fe(II) addition as ferrous ammonium sulfate, $(Fe(NH_4)_2(SO_4)_2$; Sigma Chemical). Every 15–30 min, two 5-mL subsamples were removed and filtered through an acid-washed, 2-μm polycarbonate Poretics filter. The filtrate was then spiked with 100 μmol L^{-1} ferrozine and the absorbance was determined at 562 nm relative to a reference sample cuvette containing the assay solution without cells. The cell densities in the resuspended samples ranged from 1–10 $\times$ 10^5 cells mL^{-1}.

Cu additions to cells grown with low Cu prior to $OX_{Fe(II)}$ and ρ_{FeDFB} determinations—Cultures of Fe-limited (pFe 20.5) *Thalassiosira* spp. grown with low Cu (pCu 15 or no Cu added) were tested for their ability to restore the activity of putative multi-Cu oxidases upon a Cu addition. During midexponential phase, the cultures were harvested gently (*see* above), immediately resuspended in pH 8.2 SOW containing 2 nmol L^{-1} Cu : 2.1 nmol L^{-1} EDTA, and placed in the dark. After 15–30 min, the cells were harvested gently, and rapidly resuspended in either the FeDFB uptake or the Fe(II) oxidation assay medium.

Effects of TTM additions on $OX_{Fe(II)}$ and Fe uptake by T. oceanica—To investigate the role of putative MCOs in Fe uptake and Fe(II) oxidation, the MCOs were inhibited with TTM (ammonium tetrathiomolybdate, $(NH_4)_2MoS_4$; Alfa Aesar) (Chidambaram et al. 1984; Bissig et al. 2001). Tetrathiomolybdate selectively inhibits a variety of cuprooxidases, including the multi-Cu ferroxidase, ceruloplasmin (Chidambaram et al. 1984). It is believed that TTM reversible inhibition of Cu-oxidases results from complexation of Cu by TTM, and is most likely mediated by the formation of a tertiary TTM−Cu-oxidase complex (Bissig et al. 2001). During midexponential phase, *T. oceanica* grown in Fe-limiting media (pFe 20.5, pCu 14) was exposed to 25 μmol L^{-1} TTM for 3 h. After this time, the cells were harvested gently, rinsed with SOW, and rapidly resuspended in the Fe(II) oxidation assay buffer. In one experiment, $^{55}Fe(II)$ was added as a tracer, in order to determine the rates of Fe uptake along with the $OX_{Fe(II)}$. In addition, the rates of ^{14}C uptake by control and TTM-treated cells were determined to verify that other cellular metabolic functions were not hampered by TTM.

Statistical analysis—Our preliminary statistical analysis consisted of Student's *t*-test for differences between treatment means within each group. Since the rates of Fe uptake and oxidation were determined using repeated measurements (independent cultures were sampled every hour for 2–6 h), we also used a mixed-effects model to distinguish the two sources of error in the data set; within and between individual culture measurements. The mixed-effects model (PROC MIXED in SAS, version 9.1) was used to model the covariance structure of the data (blockwise diagonal matrix) prior to testing for group/treatment effects using the fitted coefficients. In the model, the response variable was Fe uptake or Fe(II) oxidation, the predictor variable was time, and, depending on the data set, the covariate variables were pCu level (pCu 14 vs. pCu 15), organism (*T. oceanica* vs. *T. pseudonana*), and/or Cu addition (no Cu vs. plus Cu).

Extraction of ribonucleic acid (RNA) from T. pseudonana—During midexponential phase, *T. pseudonana* cultures were harvested gently by filtering between 750 and 950 mL onto an acid-washed 47-mm 2-μm polycarbonate Poretics filter inside a laminar flow hood. Immediately after harvesting, the samples were frozen by immersion in liquid N_2 and stored at −80°C.

Total RNA was extracted from the filtered cells using an Ambion RNAqueous-4PCR kit (Ambion). Just prior to extraction, the filters were inserted into 15-mL Falcon tubes, submerged in liquid N_2, and ground into small pieces. The filter pieces were then suspended in 750 μL of lysis/binding solution and vortexed for 1–2 min. Extractions then proceeded following the instructions of the manufacturer. The RNA samples were eluted in 60 μL of elution buffer. After extraction, samples were treated with 2.5 U of DNase I at 37°C for 1 h. The RNA extracts were quantified and inspected qualitatively using an Agilent 2100 bioanalyzer with the RNA 6000 Nano assay (Agilent Technologies).

Complementary deoxyribonucleic acid (cDNA) synthesis and real-time reverse transcription (RT)-PCR for T. pseudonana—The PCR primers for *T. pseudonana* cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and putative MCO genes were designed based on the gene models newV2.0.genewise.11.619.1 and grail.4.304.1, respectively (http://genome.jgi-psf.org/thaps1/thaps1.home.html). The primers GAPDH forward primer, TCCTCGACCTCGACTATGCT, and GAPDH reverse primer, AACCGATCCCCAAGGAATAG, amplify a 179-bp amplicon. The MCO forward primer, GTCGTCGAGCAAGTTTCGTT, and MCO reverse primer, GGGAGCAACGTGGATAACAT, amplify a 148-bp amplicon.

Incidentally, the MCO gene (gene model grail.4.304.1) does not have expressed sequence tag (EST) support in the

publicly available *T. pseudonana* EST database. However, it is important to note that this database was constructed from mRNA extracted from Fe-sufficient cells, which express this putative membrane targeted multi Cu-oxidase gene at extremely low levels (*see* below). We have previously observed that GAPDH expression in *T. pseudonana* does not change significantly across various light and nutrient conditions (unpubl. data). In this study, expression of GAPDH transcripts remained constant from treatment to treatment (data not shown). Therefore, we believe that GAPDH is a reliable endogenous control.

Total cDNA was synthesized using a SuperScript First-Strand System for RT-PCR following the instructions of the manufacturer (Invitrogen). Briefly, 500 ng of total RNA were converted into first-strand cDNA using 50 μmol L^{-1} oligo$(dT)_{20}$. A volume of 2.5 μL of cDNA was included in 20-μL real-time PCR reactions with 0.4 μmol L^{-1} of each primer using a QuantiTect SYBR Green PCR kit (Qiagen). Real-time PCR reactions were performed in LightCycler glass capillaries on a LightCycler (Roche Diagnostics). The following LightCycler experimental run protocol was used: denaturation (95°C for 10 min), amplification and quantification repeated 45 times (95°C for 15 s, 55°C for 10 s, 72°C for 30 s with a single fluorescence measurement), melting curve program (60–95°C with a heating rate of 0.1°C per second and a continuous fluorescence measurement), and finally a cooling step to 40°C.

Five-point standard curves for GAPDH and MCO were constructed in duplicate from a dilution series of cDNA template. The RNA samples were evaluated for contaminating DNA by including 2.5 μL of RNA in control "no RT" PCR reactions, as well as no template negative controls. The curves were used to evaluate the real-time PCR efficiency for the different primer sets. For quantification, the unknown reactions were run in triplicate. The average and standard deviation of the threshold cycle (C_T) were determined. The C_T value indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold or rises appreciably above the background fluorescence. There is an inverse relationship between C_T and the abundance of target in the reaction. The C_T values were determined using the "Second Derivative Maximum Method," in which quantification is done at the most efficient point of the real-time PCR when the second derivative is at its maximum (LightCycler software 3.3; Roche Diagnostics). Real-time PCR efficiency was calculated by plotting C_T versus various cDNA dilutions. The slope of this line was then used to calculate efficiency according to the equation $E = 10^{(-1/\text{slope})}$. A slope of -3.2 corresponds to an optimal real-time PCR efficiency of $E = 2$. Standard curves of a dilution series of cDNA template for the putative Cu-oxidase and GAPDH always yielded a slope between 3.1 and 3.4. Under these conditions, the $2^{-\Delta\Delta C_T}$ method provides a reliable calculation of fold change in transcript abundance between two samples (Pfaffl 2001). Therefore, the data were analyzed according to the following equation: $\Delta\Delta C_T = (C_{T,\ \text{Target}} - C_{T,\ \text{GAPDH}})_{\text{treatment1}} - (C_{T,\ \text{Target}} - C_{T,\ \text{GAPDH}})_{\text{treatment2}}$. Treatment 2 is any treatment and treatment 1 represents the 1 × expression of the target normalized to GAPDH. The $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001) was then used to calculate the fold change in gene expression in the Fe-limited, and Fe- and Cu-deficient cultures normalized to GAPDH relative to the Fe- and Cu-sufficient cultures.

Phylogenetic and Domain Fishing analysis of putative T. pseudonana *MCO gene*—The predicted protein was evaluated with Domain Fishing, which is a sensitive interactive global sequence alignment procedure that queries the Protein Data Bank (PDB), the Protein FAMilies database (PFAM), and the Structural Classification of Proteins (SCOP), while taking into account predicted secondary structure information for the query sequence (Contreras-Moreira and Bates 2002). This combination of sequence profiles and secondary structure information yields more reliable alignments than using only sequence data (Elofsson 2002).

For phylogenetic analysis, the amino acid sequences of homologous ferroxidase, laccase, and ascorbate oxidase proteins were aligned with the putative MCO from *T. pseudonana*. Sequences were aligned using the ClustalX multiple sequence alignment algorithm. Alignments were edited using the Bioedit software package. A multiple sequence alignment of 120 amino acids, primarily from predicted MCO domains, without gaps was generated. Phylogenetic trees were inferred and drawn using the TREECON software package (version 1.3b) (Van de Peer and De Wachter 1997). In addition, the Kimura two-parameter model for inferring evolutionary distances/Bootstrap estimates (1,000 replicates) of confidence intervals was also completed using the algorithms available in the TREECON package.

Results

In this study, *T. oceanica* was used as a model diatom for the physiological experiments. Much information is available on the inducible Fe acquisition mechanism of this oceanic diatom (Maldonado and Price 2000, 2001). Limited experiments were also performed with *T. pseudonana*, a coastal diatom whose genome was recently sequenced (Armbrust et al. 2004). *T. pseudonana* is discussed separately in Results but jointly with *T. oceanica* in Discussion.

Fe acquisition by T. oceanica—In the presence of strong organic ligands, Fe-limited *T. oceanica* enzymatically reduces Fe(III) to release Fe from the organic complexes (Maldonado and Price 2000, 2001). Once reduced, Fe(II) may be oxidized before uptake or directly internalized as Fe(II). To determine whether the putative Fe transporters react with Fe(III) or Fe(II), we examined the rates of Fe uptake in the presence of inorganic Fe(III) or Fe(II) at identical pH. Iron-limited *T. oceanica* transported inorganic Fe(III) at a rate 8.5 times faster than Fe(II) (Fig. 1), and in the presence of the ferric reductant ascorbate, Fe(II) uptake was inhibited by 50% (Fig. 1). These results provide indirect evidence that putative Fe permeases react with Fe(III), and reducing conditions at

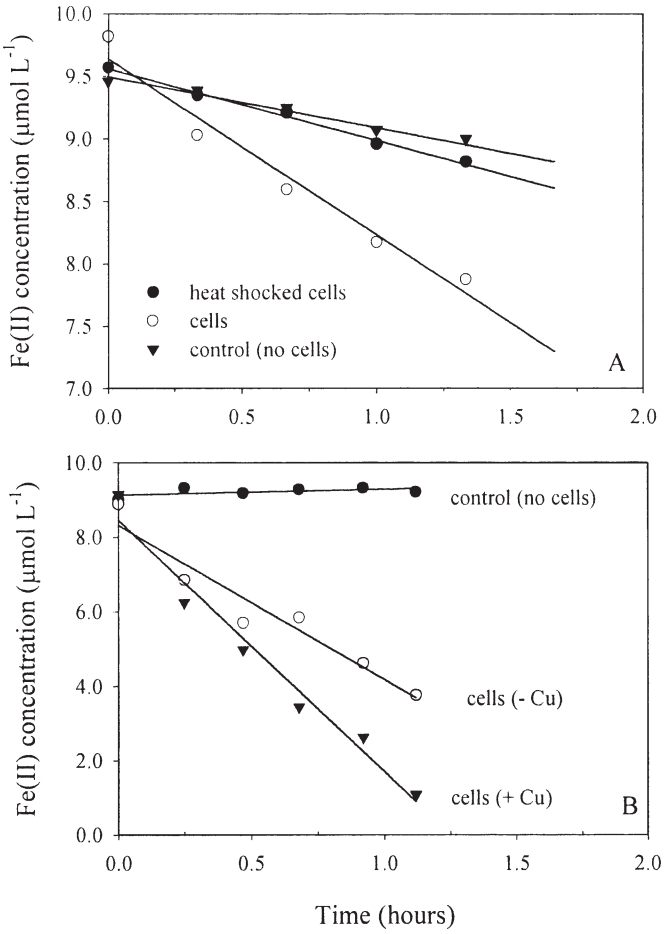

Fig. 2. Time course of the disappearance of Fe(II) in SOW (pH 6.6) in the dark in the absence and in the presence of Fe-limited *T. oceanica* cells. (A) The cells were grown in Fe-limiting medium (pFe 20.5_pCu 14), harvested in midexponential media, and resuspended in oxidation assay buffer (5×10^5 cells mL^{-1}). At time zero, 10 μmol L^{-1} Fe(II) was added to a subsample of the cells suspension (cells). Another subsample of the cell suspension was heated in a 60°C water bath for 10 min and then cooled for 5 min on ice to 20°C before the assay was initiated (heat-shocked cells). These cells remained intact following this treatment. The control sample was the same as the experimental treatment but contained no cells. Subsamples of 5 mL were removed from the treatments every 20 min, filtered through a swinex filter containing a glass-fiber filter, and then assayed at A_{564} for Fe(II) concentrations using 100 μmol L^{-1} ferrozine. (B) The cells were grown in Fe-limiting and low-Cu medium (pFe20.5_pCu 15), harvested in midexponential phase, and resuspended in either the oxidation assay buffer or SOW (pH 8.2). The final cell densities of these cell resuspensions were identical, 10.9×10^6 cells mL^{-1}. The cells resuspended in the SOW (pH 8.2) were then spiked with 2 nmol L^{-1} Cu : 2.1 nmol L^{-1} EDTA. After 15 min, these cells were filtered, rinsed with SOW, and resuspended in the oxidation buffer. At time zero, 10 μmol L^{-1} Fe(II) was added to both cell treatments (−Cu and +Cu), and the control sample. Subsamples of 5 mL were removed from the treatments every 20 min, filtered through a swinex filter containing a glass-fiber filter, and then assayed at A_{564} for Fe(II) concentrations using 100 μmol L^{-1} ferrozine.

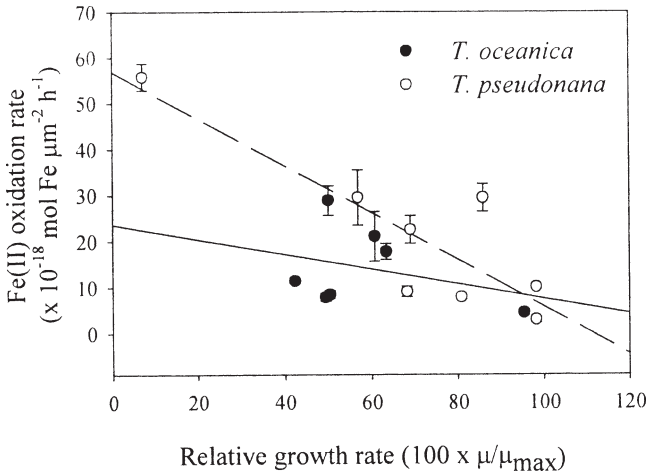

Fig. 3. Oxidation rates of inorganic Fe(II) by *T. oceanica* and *T. pseudonana* cultures as a function of Fe-limited relative growth rate ($100 \times \mu/\mu_{max}$). Each mean (±range) corresponds to the average rate of duplicate samples from the same culture. The plotted line was obtained by least-squares regression. For *T. oceanica* (solid line): oxidation rate ($\times 10^{-18}$ mol Fe μm^{-2} h^{-1}) = 23.68 – 0.16 × relative growth rate; $r^2 = 0.106$; $F = 0.59$; $p = 0.475$; residual df = 5). For *T. pseudonana* (dashed line): oxidation rate ($\times 10^{-18}$ mol Fe μm^{-2} h^{-1}) = 56.89 – 0.51 × relative growth rate; $r^2 = 0.748$; $F = 17.8$; $p = 0.005$; residual df = 6).

the cell surface hamper the oxidation of Fe(II) and subsequent uptake. As previously suggested (Anderson and Morel 1982; Maldonado and Price 2001), these data indicate that oxidation of Fe(II) is an obligate step before Fe internalization.

To establish the relative rates of abiotic and cellular Fe(II) oxidation in seawater, we determined rates of Fe(II) oxidation in the presence and absence of the cells. Maximal, potential rates of cellular Fe(II) oxidation [$OX_{Fe(II)}$] were significant compared to those of abiotic Fe(II) oxidation (Fig. 2). Even though oxidation of Fe(II) occurred spontaneously in seawater at the pH of the assay buffer (pH 6.6) (*see* Materials and methods), the apparent $OX_{Fe(II)}$ were always significantly faster than the abiotic rates (Fig. 2). The heat-shocked treatment, which denatures the proteins, completely inhibited the $OX_{Fe(II)}$, suggesting that the $OX_{Fe(II)}$ was indeed enzymatically mediated (Fig. 2A). The $OX_{Fe(II)}$ by Fe-limited *T. oceanica* varied by approximately one order of magnitude (Fig. 3) and were slowest for an Fe-sufficient culture ($\mu/\mu_{max} = 0.95$).

The $OX_{Fe(II)}$ were also affected by the level of Cu in the growth media (Table 1), indicating Cu dependence of oxidase activity. The $OX_{Fe(II)}$ by Fe-limited cultures grown in Cu-sufficient media were ~3.5 times faster than those grown in low-Cu media (pCu 14 vs. pCu 15; Table 1). Preliminary statistical analysis by *t*-test indicated that this Cu effect was significant (Table 1, *t*-test, $p < 0.001$). This analysis was supported by a more refined mixed-effects model analysis, which also yielded statistical significance (Table 1, mixed-effects model, $p < 0.0001$). Slow $OX_{Fe(II)}$ in the Cu-limited cells could be restored by Cu additions (increased by approximately twofold; Fig. 2B; *t*-test, $p <$

Table 1. Oxidation rates of inorganic Fe(II) by Fe-limited (pFe 20.5) *T. oceanica* cultures grown in sufficient Cu (pCu 14) or low Cu (pCu 15) media. The experiments were performed at pH 6.6 in the presence of 10 μmol L^{-1} Fe(II). Experiments identified with the same letter were performed with cells from the same culture. In these experiments, the mean relative growth rates ($\mu/\mu_{max} \pm$ SD) of the *T. oceanica* cultures were 0.66 ± 0.03 and 0.59 ± 0.05 for pCu 14 and pCu 15, respectively. Cultures grown with low Cu concentrations (pCu 15) were split in two before the experiments. Half of the culture (pCu 15 +Cu) was exposed to 2 nmol L^{-1} Cu : 2.1 nmol L^{-1} EDTA for 15 min prior to the Fe(II) oxidation experiment. The other half of the culture received no additions. The *p* value between two columns represents that of a one-tailed unpaired (assuming equal variance) or paired *t*-test analysis between the pCu 14 and pCu 15 or the pCu 15 and pCu 15 +Cu treatment means, respectively. The results of a linear mixed-effects model statistical analysis are also presented (*see* Materials and methods).

	T. oceanica, Fe(II) oxidation rates ($\times 10^{-18}$ mol Fe μm^{-2} h^{-1})		
	pCu 14	pCu 15	pCu 15 +Cu
	22.08	3.29	
	33.17	4.42[a]	8.79[a]
	25.79	5.21[b]	8.88[b]
		12.17[c]	20.20[c]
		14.38[d]	14.30[d]
Mean	27.01	7.89	13.04
Standard error	3.26	2.24	2.71
t-test *p* value		0.001 0.048	
Mixed-effects model *p* value		0.0001 0.049	

Table 2. Effects of TTM on the rates of inorganic Fe(II) oxidation and Fe uptake by *T. oceanica* cultures grown in Cu-sufficient and either Fe-limiting or Fe-deficient media (pFe 20.5 or −Fe, respectively). Half of the culture (+TTM) was exposed to 25 μmol L^{-1} TTM (ammonium tetrathiomolybdate, $(NH_4)_2MoS_4$) for 3 h prior to the experiments. The experiments were performed at pH 6.6 in the presence of 10 μmol L^{-1} Fe(II).

Growth media pFe (μ/μ_{max})	*T. oceanica*, rates ($\times 10^{-18}$ mol Fe μm^{-2} h^{-1})			
	Fe(II) oxidation		Fe uptake	
	−TTM	+TTM	−TTM	+TTM
20.5 (0.53)	9.61	2.31		
20.5 (0.63)	2.24	0.86	3.08	0.12
−Fe (0.3)	14.4	9.07		

0.048, and mixed-effects model, $p < 0.049$, Table 1), suggesting that Cu has an explicit role in Fe(II) oxidation. The restored rates, however, were still slower than those of Cu-sufficient cells (pCu 14; Table 1), implying that the oxidase activity was only partially restored during the span of the Fe(II) oxidation experiment (~2 h).

A potent inhibitor of multi-Cu-containing oxidases, tetrathiomolybdate (TTM; MoS_4^{2-}), was used in some experiments. The addition of TTM led to a ~60% decrease in the Fe(II) oxidase activity by the cells (Table 2). In one TTM experiment, ^{55}Fe was added as a tracer to determine the rates of Fe uptake along with the $OX_{Fe(II)}$. A concomitant inhibition (by 97%) of the rates of Fe uptake was observed for the cells incubated with TTM prior to the experiment (Table 2). In contrast, rates of C uptake were only slightly affected by TTM addition (decreased by 10%), indicating that in the short term, TTM did not have significant, nonspecific effects on cell metabolism. These experiments support the hypothesis that Fe(II) oxidation by Fe-limited diatoms is mediated by putative MCOs.

The $RED_{Fe(III)EDTA}$ and $OX_{Fe(II)}$ were determined simultaneously at identical Fe concentrations for numerous cultures to investigate the relative $RED_{Fe(III)EDTA}$ and $OX_{Fe(II)}$ prior to Fe uptake (Table 3). In general, the $OX_{Fe(II)}$ were similar to the $RED_{Fe(III)EDTA}$, and the means were not significantly different.

In addition to modulating Fe(II) oxidation, Cu also appeared to affect Fe uptake from the FeDFB complex. Growth in low-Cu media (pCu 15) greatly reduced the surface area–specific rates of Fe uptake from FeDFB (ρ_{FeDFB}) by Fe-limited *T. oceanica* (Fig. 4; Table 4). The ρ_{FeDFB} were on average three times faster for Cu-sufficient than Cu-limited cells (Fig. 4; Student's *t*-test, $p < 0.009$, Table 4; mixed-effects model, $p < 0.0002$, Table 6). In two experiments, cells grown with various levels of Cu and Fe (pFe 19_−Cu, pFe 20.5_pCu 15) were spiked with Cu (2 nmol L^{-1}) prior to the ρ_{FeDFB} determinations (Table 5). As seen in the Fe(II) oxidation experiments (Table 1), the Cu spike restored ρ_{FeDFB} to higher values (154% and 35%; Table 5). These data suggest that the inducible mechanism for Fe acquisition from strong organic complexes in *T. oceanica* is a Cu-dependent process. Surprisingly, the greatest enhancement in ρ_{FeDFB} was observed for the *T. oceanica* culture grown under Fe sufficiency (pFe 19_−Cu; Table 5). Since Fe uptake from Fe(III)DFB only occurs when cells are Fe limited (Maldonado and Price 2001), this experiment indicates that Cu limitation of Fe-sufficient cells may essentially result in Fe limitation.

Fe acquisition by T. pseudonana—A limited set of physiological experiments was performed with *T. pseudonana* to investigate whether it exhibited physiological processes of the inducible Fe uptake system similar to those observed in *T. oceanica*. In particular, we measured $OX_{Fe(II)}$ and $RED_{Fe(III)EDTA}$ by cells grown with various Fe levels, ρ_{FeDFB} of Fe-limited cells grown in pCu 14 or pCu 15, and the effect of Cu addition on ρ_{FeDFB} for cultures grown in the absence of Cu.

On average, *T. pseudonana* exhibited $OX_{Fe(II)}$ slightly faster than those by *T. oceanica* (Table 3; Fig. 3). The $OX_{Fe(II)}$ by severely Fe-stressed *T. pseudonana* was significantly ($p < 0.005$) faster than by cells Fe sufficient or only mildly Fe limited (Fig. 3). A similar trend was observed with *T. oceanica* but was not significant (Fig. 3). Even though the significant relationship between $OX_{Fe(II)}$ and growth rates for *T. pseudonana* is driven by a single point, these physiological data are strongly supported by our data on the effect of Fe availability on the expression of a putative MCO gene (*see* following). The $RED_{Fe(III)EDTA}$ and $OX_{Fe(II)}$ by *T. pseudonana* were very similar (Table 3).

Table 3. Simultaneous rates of inorganic Fe(II) oxidation (10 μmol L^{-1} Fe) and organically bound Fe(III) (10 μmol L^{-1} Fe : 100 μmol L^{-1} EDTA) reduction by cultures of *Thalassiosira* spp. ($\times 10^{-18}$ mol Fe μm^{-2} h^{-1}) grown under various Fe-limiting conditions. Each mean (±range) corresponds to the average rate of duplicate samples from the same culture. The relative Fe-limited growth rate of each culture is given (μ/μ_{max}). Maximal growth rate is that obtained by cultures grown in Fe-sufficient (pFe 19) and Cu-sufficient (pCu 14) media (*T. oceanica*, μ_{max} = 3.0 dd^{-1}; *T. pseudonana*, μ_{max} = 3.8 dd^{-1}).

Rates ($\times 10^{-18}$ mol Fe μm^{-2} h^{-1})					
	T. oceanica			*T. pseudonana*	
μ/μ_{max}	Oxidation	Reduction	μ/μ_{max}	Oxidation	Reduction
0.61	21.1±5.4	9.9±0.9	0.98	10.1±0.5	2.3±0.6
0.49	7.8±0.9	4.4±0.9	0.98	2.9±0.6	2.6±0.2
0.63	17.8±1.8	53.7±7.2	0.86	29.5±3	7.9±0.5
0.95	4.5±0.6	13.0±1.1	0.81	7.9±0.4	2.7±0.1
0.50	8.4±0.8		0.68	9.1±1.1	80.1±11
0.42	11.4±0.5	1.5±0.5	0.69	22.5±3	2.5±0.8
0.50	28.9±3.2	3.7±1.5	0.57	29.5±6	29.7±1.7
			0.7	56±2.9	7.1±2.3
Mean	14.3	14.4		20.9	16.9
Standard error	3.1	7.1		6.2	9.7

As in *T. oceanica*, the ρ_{FeDFB} of Fe-limited *T. pseudonana* were dependent on Cu availability in the growth media. The ρ_{FeDFB} were on average 3.5 times faster for pCu 14 than pCu 15 cultures (Student's *t*-test, $p < 0.03$, Table 4; mixed-effects model, $p < 0.0002$, Table 6).

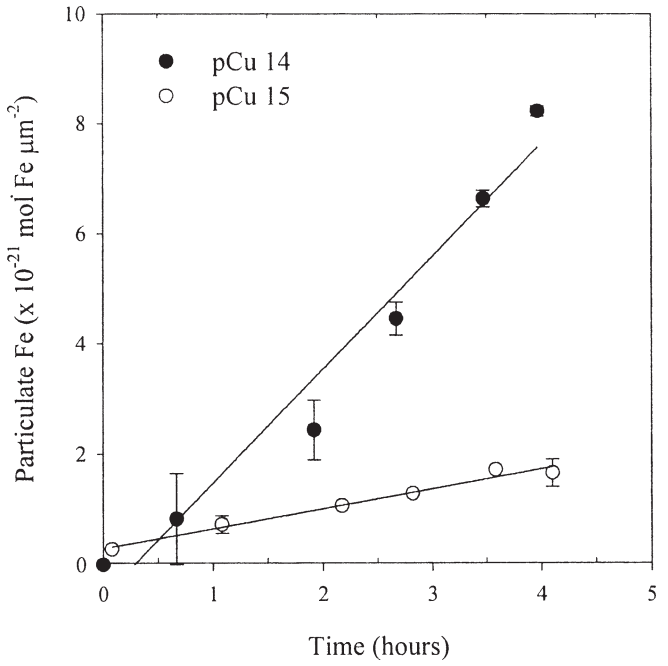

Fig. 4. Time course of accumulation of particulate ^{55}Fe in the dark by Fe-limited (pFe 20.5) *T. oceanica* grown in sufficient Cu (pCu 14) or low Cu (pCu 15) media. The Fe was added bound to DFB in a 100 nmol L^{-1} Fe : 1 μmol L^{-1} DFB ratio. Points represent the mean ± range of duplicate analyses of a single culture. The relative growth rates (μ/μ_{max}) of the sufficient Cu and low-Cu cultures were 0.57 and 0.45, respectively. The plotted lines were obtained by least-squares regression: for the pCu 14 culture (r^2 = 0.97; slope of the regression = 2.05 × 10^{-21} mol Fe μm^{-2} h^{-1}) and the pCu 15 culture (r^2 = 0.98; slope of the regression = 0.36 × 10^{-21} mol Fe μm^{-2} h^{-1}).

Moreover, the mixed-effects model analysis also indicated that Fe-limited (pFe 20.5) *T. pseudonana* grown with Cu (pCu 14 or pCu 15) had significantly slower ρ_{FeDFB} than *T. oceanica* ($p < 0.03$; Table 6).

As observed for *T. oceanica*, the ρ_{FeDFB} of *T. pseudonana* cultures grown in the absence of Cu were also enhanced by a Cu spike (Table 5). Despite the fact that these experiments had few replicates, some differences between *T. oceanica* and *T. pseudonana* are evident. For example, the response to the Cu spike was higher by *T. oceanica* (35% and 154%) than *T. pseudonana* cultures (10–20%). Interestingly, the fastest ρ_{FeDFB} measured was for a very slow-growing culture of Fe-limited *T. pseudonana* in

Table 4. Short-term Fe uptake rates of Fe bound to DFB (100 nmol L^{-1} Fe : 1 μmol L^{-1} DFB) by Fe-limited (pFe 20.5) *Thalassiosira* spp. cultures grown in sufficient (pCu 14) or low-Cu (pCu 15) media. Cell densities range from 0.9–6 × 10^5 cells mL^{-1}. The error associated with these measurements is <10% (for examples, *see Figs. 1, 4*). The *p* value between two columns represents that of a one-tailed unpaired *t*-test analysis (assuming unequal variance) between the pCu 14 and pCu 15 treatment means. The mean relative growth rates (μ/μ_{max} ± SD) of these *T. oceanica* cultures were 0.65 ± 0.23 and 0.57 ± 0.10 for pCu 14 and pCu 15, respectively, and those of *T. pseudonana* were 0.62 ± 0.13 and 0.54 ± 0.13 for pCu 14 and pCu 15, respectively.

	Fe uptake rates ($\times 10^{-21}$ mol Fe μm^{-2} h^{-1})			
	T. oceanica		*T. pseudonana*	
	pCu 14	pCu 15	pCu 14	pCu 15
	0.791	0.123	0.154	0.064
	0.088	0.180	0.530	0.041
	0.608	0.127	0.248	0.164
	0.726	0.334	0.320	
	0.811	0.203		
	0.419	0.219		
Mean	0.574	0.197	0.313	0.09
Standard error	0.113	0.031	0.08	0.04
t-test *p* value	0.009		0.03	

Table 5. Short-term Fe uptake rates of Fe bound to DFB (100 nmol L^{-1} Fe : 1 μmol L^{-1} DFB) by cultures of *Thalassiosira* spp. grown in low-Cu (pCu15) or Cu-deficient media (no Cu added, −Cu) and various levels of Fe (Fe-sufficient, pFe 19; Fe-limiting, pFe 20.5; and severe Fe limitation, no Fe added, −Fe). The cultures were split in two before the uptake experiments. Half of the culture was spiked to 2 nmol L^{-1} Cu : 2.1 nmol L^{-1} EDTA for 15 min prior to the experiment (+Cu). The other half of the culture received no Cu additions (−Cu). The error associated with these measurements is <10% (for examples, *see Figs. 1, 4*). Cell densities range from 0.5–5 × 10^5 cells mL^{-1}. The *p* value between the rates of Fe uptake by the −Cu and +Cu cultures represents that of a paired *t*-test analysis between the two treatment means. The mixed-effects model statistical analysis could not be applied to this data set due to unequal sampling times during the experiments.

	Fe uptake rates ($\times 10^{-21}$ mol Fe μm^{-2} h^{-1})		
Growth media	(μ/μ_{max})	−Cu	+Cu
T. pseudonana			
pFe 19_−Cu	(0.81)	0.163	0.196
pFe 20.5_−Cu	(0.57)	0.364	0.404
−Fe _−Cu	(0.07)	1.36	1.64
T. oceanica			
pFe19_−Cu	(0.49)	0.255	0.647
pFe 20.5_pCu15	(0.70)	0.220	0.296
Mean		0.472	0.637
Standard error		0.224	0.262
t-test *p* value		0.043	

medium without Cu (−Fe _−Cu; Table 5). Surprisingly, a ρ_{FeDFB} for a ιFe 20.5_−Cu culture measured in these experiments (Table 5) was faster than those measured previously for pFe 20.5 cultures grown with higher Cu levels (pCu 14 and 15; Table 4).

Although the physiological data showing Cu-dependent Fe transport are less robust for *T. pseudonana* than for *T. oceanica* (due in part to the smaller number of experiments that were conducted), the results do provide some evidence for putative Cu-containing oxidases in the high-affinity Fe transport system of *T. pseudonana.* To support these preliminary physiological data, we searched the *T. pseudonana* genome for a multi-Cu-containing oxidase gene and, using real-time PCR, quantified its expression under various Fe and Cu conditions.

We identified two gene models in the *T. pseudonana* genome with InterPro Cu-oxidase domains. One of the gene models is homologous to bacterial secreted metalloproteases. The other gene model (grail.4.304.1) is predicted to contain a Cu-oxidase domain and is homologous to the *S. cerevisiae* FET5 and FET3, as well as the fungal laccase and the vascular plant ascorbate oxidase. Each of these proteins contains an MCO domain with a recognizable amino acid signature (Messerschmidt and Huber 1990; Ouzounis and Sander 1991). The protein also contains several predicted transmembrane domains near the carboxyl-terminal region, which suggests that it could function as a cell surface oxidase.

The predicted MCO protein in *T. pseudonana* was evaluated with Domain Fishing analysis and revealed a significant score (E-value of 6×10^{-41}) for the presence of a Multicopper oxidase domain (Pfamily PF00394) homologous to those found in laccase and ascorbate oxidase proteins for which the crystal structure is known. Structural protein predictions indicate that the putative *T. pseudonana* MCO protein is most similar to the fungal Cu-containing laccase from *Melanocarpus albomyces* and the Cu-depleted laccase from *Coprinus cinereus*. Phylogenetically, the putative *T. pseudonana* MCO most closely resembles fungal laccases, followed by yeast FET family of ferroxidases, and lastly plant ascorbate oxidases (Fig. 5). A variety of fungal laccase genes along with the putative *T. pseudonana* MCO form a very well-supported clade that contains a subclade comprised of yeast ferroxidases. Vascular plant ascorbate oxidase genes form a separate and distinct clade from that of fungal laccases and yeast ferroxidases (Fig. 5).

Table 6. Results of linear mixed-effects model statistical analysis of the Fe uptake–rates data presented in Table 4 (*see* Materials and methods).

Parameter	Treatment	Significance level
Fe uptake	pCu 14 vs. pCu 15	$p<0.0002$
	T. oceanica vs. *T. pseudonana*	$p<0.03$

We targeted the putative Cu-containing oxidase gene, model grail.4.304.1, in *T. pseudonana* for expression analysis under different Fe and Cu levels. The putative membrane-targeted Cu-oxidase (MCO) gene in *T. pseudonana* was expressed in Fe-sufficient cells, but at a level 65 times lower than that of the GAPDH gene, the endogenous control (ΔC_T values for pFe19 cultures, ~ 5.94; Table 7). In contrast, under Fe-limiting conditions, the expression of the putative MCO gene was always detected at a level similar to that of the GAPDH gene (ΔC_T values for pFe20.5 cultures, ~0; Table 7).

The putative MCO gene in *T. pseudonana* is highly expressed under conditions of Fe limitation. In two separate Fe-limited cultures, the putative MCO expression, normalized to GAPDH expression, was increased 57- and 63-fold relative to the Fe-replete cells (Table 7). These results suggest that the expression of the putative MCO gene (gene model grail.4.304.1) of *T. pseudonana* is regulated by intracellular Fe levels. In addition, when Fe-limited cells were grown in the absence of Cu, the MCO expression was upregulated further, increasing 90-fold (Table 7). These data represent highly significant increases in levels of gene expression of the putative MCO for Fe-limited *T. pseudonana* (analysis of variance [ANOVA], $p < 0.0001$) and an additional significant increase as a result of a decrease in Cu availability (ANOVA, $p < 0.01$) relative to Fe-sufficient *T. pseudonana*.

Discussion

It is well established that Cu, a redox-active trace element, is essential for phytoplankton growth, and is required in various metabolic functions. This study, however, is the first to investigate the effect of Cu limitation

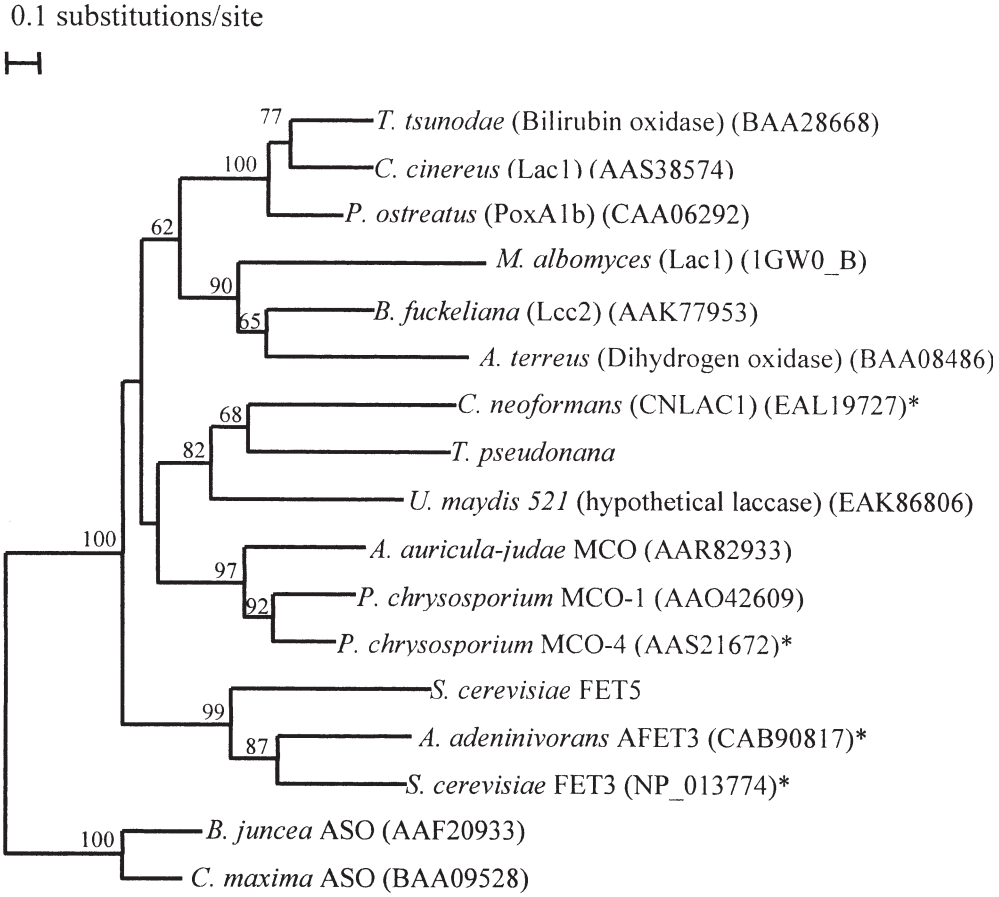

Fig. 5. Inferred phylogenetic relationships of the MCO domain of selected laccases, ferroxidases, and ascorbate oxidases amino acid sequences from yeast (*S. cerevisiae* and *Arxula adeninivorans*), fungi (*Trachyderma tsunodae*, *C. cinereus*, *Pleurotus ostreatus*, *M. albomyces*, *Botryotinia fuckeliana*, *Aspergillus terreus*, *C. neoformans*, *Ustilago maydis*, *Auricularia auricula-judae*, and *P. chrysosporium*) and vascular plants (*Brassica juncea* and *Cucurbita maxima*). The MCO domain of the *C. reinhardtii* ferroxidase Fox1 gene was found to be too distant to the depicted sequences for meaningful phylogenetic resolution. Sequences are labeled according to how they have been annotated in the literature or in GenBank. Sequences marked by an asterisk have been shown to encode proteins that exhibit ferroxidase activity. Bootstrap values greater than 50 (out of 100) are shown at the nodes. Vascular plant ascorbate oxidase sequences form a natural out-group in relation to the fungal laccases and ferroxidases, and the phylogenetic associations are rooted through the internode that defines the clades. GenBank accession numbers are given next to their designated sequences.

Table 7. Gene expression of a putative, membrane-targeted Cu-oxidase (MCO) in cultures of *T. pseudonana* grown under various Fe and Cu conditions (*see* Materials and methods). The $\Delta\Delta C_T$ and $2^{-\Delta\Delta C_T}$ values were calculated based on the C_T, the PCR cycle number at which the amount of amplified target reaches a fixed threshold or rises appreciably above the background (*see* Materials and methods for the determination of C_T). The $2^{-\Delta\Delta C_T}$ values (right column) represent fold changes in gene expression in each sample normalized to the endogenous reference gene GAPDH, relative to the Fe- and Cu-sufficient control sample (pFe 19_pCu 14). Data are shown separately for experiments performed on duplicate pFe 20.5_pCu 14 cultures. *T. pseudonana* MCO and GAPDH amplification were performed in triplicate in separate wells and the C_T values were averaged. The standard deviation, estimated from the replicate C_T values, is carried through to the final calculation using standard propagation of error. The asymmetric error distribution relative to the average $2^{-\Delta\Delta C_T}$ value is a consequence of converting the results of an exponential process into a linear comparison of amounts.

Sample culture conditions	ΔC_T (Avg. MCO C_T – Avg. GAPDH C_T)	$\Delta\Delta C_T$ (Avg. ΔC_T – Avg. ΔC_T, pFe 19_pCu 14)	$2^{-\Delta\Delta C_T}$ relative to pFe19_pCu 14
pFe 19_pCu 14	−5.94±0.16	0.00±0.22	0.00 (0.86–1.16)
pFe 20.5_−Cu	−0.55±0.11	−6.49±0.19	90.20 (78.98–103.01)
pFe 20.5_pCu 14	−0.04±0.41	−5.99±0.44	63.05 (56.20–70.73)
pFe 20.5_pCu 14	−0.10±0.01	−5.84±0.16	57.38 (51.5–63.93)

on the inducible Fe transport system of Fe-limited diatoms. The physiological, phylogenetic, and genomic data presented here indicate that Cu plays a role in the inducible Fe transport system of these organisms as part of a putative MCO. We note that our measured physiological rates cannot be extrapolated directly to the oceans because the experiments were conducted with Fe concentrations significantly higher than those found in Fe-limited regions. For this reason, the measured rate processes represent physiological capabilities of the Fe-limited cells rather than rates under in situ or steady-state conditions. Nevertheless, our findings suggest that Cu may be an important nutrient for diatoms in Fe-limited oceanic regions.

Iron acquisition by Fe-limited T. oceanica—The oceanic diatom *T. oceanica* is able to grow in low-Fe media containing the siderophore Fe(III)DFB as the sole Fe source (Maldonado and Price 2001). This organism possesses an enzymatic, cell-surface reductive mechanism that is induced under Fe-limiting conditions, allowing the release of Fe(II) from the siderophore complex (Maldonado and Price 2000, 2001). Indeed, a new kinetic model for Fe uptake by marine diatoms supports these findings and predicts that extracellular reduction of all Fe species is a necessary step for iron acquisition (Shaked et al. 2005). Previous data suggest that the Fe(II) liberated by the reductases must be reoxidized before reacting with the putative Fe(III) permeases (Anderson and Morel 1982; Maldonado and Price 2001). Several lines of evidence presented here support this conclusion. The rates of inorganic Fe uptake by *T. oceanica* in the presence of Fe(II) were always slower (two to nine times) than those in the presence of Fe(III) at identical concentration and pH (Fig. 1). Moreover, the ferric reductant ascorbate decreased the rates of Fe(II) uptake by 50% (Fig. 1), suggesting that oxidation of Fe(II) is essential for transport. The combined results of this study and others (Anderson and Morel 1982; Maldonado and Price 2001) indicate that in marine diatoms Fe(III) reacts with the permeases and that Fe(II) oxidation is presumably an obligate step before internalization of Fe(III).

Even though the abiotic oxidation of Fe(II) in seawater is fast (King et al. 1995), the presence of a plasmalemma-bound oxidase may ensure acquisition of the reduced Fe before it diffuses away from the cell. The results of our Fe(II) oxidation assay experiments established that the extracellular Fe(II) oxidation in *T. oceanica* was indeed mediated by an enzyme (Fig. 2). In addition, Cu seems to be involved in Fe(II) oxidation, because the $OX_{Fe(II)}$ by Fe-limited *T. oceanica* were significantly slower for Cu-limited than Cu-sufficient cells (Table 1). This diminished oxidase activity of cells grown with low Cu was partially restored (approximately two times enhanced; Table 1) with a 15-min Cu addition prior to the oxidation experiment. Moreover, when Fe-limited cells are treated with TTM, an inhibitor of Cu-containing oxidases, the $OX_{Fe(II)}$ and Fe(II) uptake are greatly diminished (Table 2). These data indicate that Cu is essential for the Fe(II) oxidation step in Fe uptake, and more specifically, that MCOs may be mediating this reaction.

Having established the presence of putative Fe(II) oxidases, and an apparent role for Cu in controlling the activity of these enzymes, we hypothesized that *T. oceanica* possessed a high-affinity Fe uptake system with a Cu-dependent Fe(II) oxidation step, as observed in freshwater eukaryotic phytoplankton (Herbik et al. 2002*a*,*b*; La Fontaine et al. 2002). We thus conducted experiments with FeDFB as the sole Fe to ensure negligible concentrations of inorganic Fe(III) in the uptake experiments. Under these conditions, cells must access siderophore-bound Fe using plasmalemma-bound reductases and the suspected oxidases. Thus, ρ_{FeDFB} was used as a proxy for the activity of the inducible Fe uptake system. In general, the ρ_{FeDFB} by *T. oceanica* measured here (0.574×10^{-21} mol Fe μm^{-2} h^{-1}, corresponding to ~33 nmol Fe mol C^{-1} h^{-1}; Table 4) are similar to those in the Fe-limited regions of the subarctic Pacific (7–75 nmol Fe mol C^{-1} h^{-1}) (Maldonado and Price 1999) and the Southern Ocean (~25 nmol Fe mol C^{-1} h^{-1}) (Maldonado et al. 2001), and those of cultured cells (Maldonado and Price 2001).

Our initial experiments showed that the ability of the Fe-limited cells to acquire Fe from FeDFB was dependent on Cu availability during growth (Tables 4, 6). When Fe-limited *T. oceanica* was grown with low Cu, the ρ_{FeDFB} were reduced relative to those of cultures grown with sufficient Cu (Tables 4, 6). Furthermore, as observed for the $OX_{Fe(II)}$, Cu additions to cultures grown without Cu enhanced their ρ_{FeDFB} (range, 11–153%; Table 5). In phytoplankton, de novo synthesis of proteins is expected to require at least a few hours (S. Merchant pers. comm.). Therefore, the immediate enhancement on the $OX_{Fe(II)}$ and ρ_{FeDFB} following a short Cu addition, suggests that—as observed in yeast (Askwith et al. 1994)—Fe-limited diatoms synthesize a limited quantity of the apoprotein of the putative MCO under low Cu that are activated quickly upon a Cu addition. These data suggest that Cu is linked to the inducible Fe transport system of Fe-limited *T. oceanica*. Surprisingly, the greatest enhancement in V_{FeDFB} (152%) after a Cu addition to cultures grown with low Cu was observed for a *T. oceanica* culture grown under Fe sufficiency (pFe 19_−Cu; Table 5). Thus, Cu-limitation of Fe-sufficient *T. oceanica* may essentially result in Fe limitation.

Iron acquisition by Fe-limited T. pseudonana—Preliminary physiological experiments with *T. pseudonana* indicate that this coastal diatom possesses a high-affinity transport system similar, but not identical, to that of *T. oceanica*. This coastal organism is able to reduce organically bound Fe(III), as well as oxidize Fe(II) at rates comparable to those measured for *T. oceanica* (Table 3). Iron deficiency significantly increases the potential rates of $OX_{Fe(II)}$ by *T. pseudonana* (Fig. 3). Although *T. pseudonana* is also able to acquire Fe from FeDFB (Tables 4, 5) under identical Fe-limiting conditions (pFe 20.5), its ρ_{FeDFB} are significantly slower than those of *T. oceanica* ($p < 0.03$; Table 6). At this time, we are unable to fully explain the superior competence of oceanic diatoms to acquire Fe from the FeDFB complex. As observed with *T. oceanica*, ρ_{FeDFB} by Fe-limited *T. pseudonana* grown with low Cu (pCu 15) are

slower than those by cells grown with high Cu levels (pCu 14) (Tables 4, 6), and a short addition of Cu to Fe-limited cells grown in −Cu medium enhanced the ρ_{FeDFB} (by 11–20%; Table 5). However, the magnitude of the enhancement was marginal compared to that observed for *T. oceanica* (35–154%). In general, these preliminary data suggest that this coastal diatom also has an inducible Fe transport system that allows acquisition of Fe from strong organic ligands, which includes putative Fe(III) reductases and Fe(II) oxidases. Even though Cu seems to be involved in the operation of this system in *T. pseudonana*, presumably as part of a putative MCO, the Cu effect is less pronounced than that observed for *T. oceanica*.

In particular, two observations would appear to contradict a role of Cu in the high-affinity Fe transport system of *T. pseudonana*. In one experiment, iron-limited *T. pseudonana* grown without added Cu (pFe 20.5_−Cu; Table 5) achieved faster ρ_{FeDFB} than cultures grown under identical Fe levels but with Cu additions (Table 4). Moreover, we measured the fastest ρ_{FeDFB} in an experiment where the cells were grown without Fe and Cu. These two results might suggest that Cu is, in fact, not required for the high-affinity Fe uptake system of *T. pseudonana*. However, these results may likely be explained by the presence of background Cu contamination in the −Cu culture medium. We have previously measured Cu levels of 0.5–1 nmol L^{-1} in our basal medium (*see* Materials and methods). According to our calculations, $\sim 6 \times 10^{-18}$ mol Cu per cell is required for the putative Cu component of the Fe transport system of these organisms (*see* below). Thus, a Cu contamination of 0.6 nmol L^{-1} in the −Cu media could be sufficient to provide enough Cu for the high-affinity Fe transport system of 10^5 cells mL^{-1}, a typical cell density in our cultures. In addition, preliminary experiments in our laboratory indicate that *T. pseudonana* has significantly lower Cu quotas than *T. oceanica* (approximately fourfold) (A. Annett pers. comm.), and its growth rate is not as affected by low Cu. Previous work also suggests that *T. pseudonana* can transport Cu twice as fast as *T. oceanica* (Hudson 1998). We thus believe that Cu contamination in these specific −Cu growth media was sufficient to allow *T. pseudonana* to partially fulfill its Cu requirement for the high-affinity Fe transport system. In contrast, under these low-Cu levels, *T. oceanica*, having significantly higher Cu requirements (A. Annett pers. comm.)—presumably associated with its photosynthetic apparatus—and slower Cu uptake rates than *T. pseudonana* (Hudson 1998), is unable to acquire sufficient Cu for Fe uptake and fast growth.

High-affinity Fe transport by eukaryotic microorganisms—The mechanisms of Fe acquisition by eukaryotic organisms have been intensely studied for the last decade. In the common yeast, *S. cereviciae*, two Fe uptake systems have been identified: a low and a high affinity. The low-affinity Fe transport system ($K_s \sim 30$ μmol L^{-1}) (Dix et al. 1994) operates at high inorganic Fe concentrations, and allows acquisition of Fe by a nonspecific Fe(II) transporter (Hassett et al. 2000). The high-affinity Fe uptake system ($K_s \sim 0.15$ μmol L^{-1}) is induced in response to Fe deficiency (Eide et al. 1992). This system includes the activity of transmembrane reductases (FRE1 and FRE2) (Dancis et al. 1990; Georgatsou and Alexandraki 1994) to reduce Fe(III) to Fe(II), followed by multi-Cu-containing ferroxidases (FET3) (Askwith et al. 1994) that oxidize Fe(II) to Fe(III) during the membrane transport step (FTR1p) (Stearman et al. 1996). Given that the reductases are not specific for Fe and reduce other metals (i.e., Cu(II)) (Georgatsou et al. 1997), the multi-Cu ferroxidase has been hypothesized to impart specificity and selectivity to the high-affinity Fe transport system (Askwith and Kaplan 1998). A similar high-affinity Fe transport system operates in the freshwater green algae *C. reinhardtii* (Herbik et al. 2002*a*,*b*; La Fontaine et al. 2002).

As in yeast and freshwater green algae, Fe-limited marine diatoms also possess a high-affinity Fe transport system with cell surface reductases that allows them to access Fe bound within strong organic complexes (Soria-Dengg and Horstmann 1995; Maldonado and Price 2000, 2001). The data presented here suggest that, in addition to Fe(III) permeases and these putative Fe(III) reductases, the inducible Fe transport system of diatoms also includes putative Fe(II) oxidases. Although low levels of Fe(III) reductase and Fe(II) oxidase activity are detected in Fe-sufficient cells, the activity of these redox enzymes is greatly enhanced in Fe-limited cells (Fig. 3; *see* fig. 6 in Maldonado and Price 2000). Several independent lines of evidence suggest that putative Fe(II) oxidases in phytoplankton are MCOs and that Cu plays a key role in the inducible Fe transport system. The potential ability of Fe-limited cells to oxidize Fe(II) and take up Fe from FeDFB is diminished under low Cu, but oxidase activity is partially restored after a short exposure to Cu. When Fe-limited cells are treated with TTM, an inhibitor of Cu-containing oxidases, the $OX_{Fe(II)}$ and Fe uptake are greatly diminished. A gene containing a MCO InterPro domain and transmembrane domains in *T. pseudonana* is highly responsive to low Fe availability, as well as Cu. These data collectively suggest that putative MCOs are an integral part of the inducible Fe transport system of diatoms.

Previous work has shown that the ferric reductases in Fe-limited phytoplankton are able to reduce organically bound Fe at a rate that is sufficient to supply the Fe needed for uptake (Maldonado and Price 1999, 2001). In the present study, simultaneous measurements of $RED_{Fe(III)EDTA}$ and $OX_{Fe(II)}$—at identical Fe concentrations (10 μmol L^{-1} Fe)—by Fe-limited *Thalassiosira* spp. cultures indicated that the rates are comparable. The coupling between the $RED_{Fe(III)EDTA}$ and $OX_{Fe(II)}$ may ensure rapid oxidation of Fe(II) by the putative MCO before Fe(II) diffuses into the surrounding seawater. In the absence of field measurements of biological Fe oxidation, it is unclear whether this model ($OX_{Fe(II)} \sim RED_{Fe(III)L}$) applies to phytoplankton in Fe-limited regions. The proximity of these putative reductases and oxidases at the cell surface of phytoplankton may also contribute to an efficient cascade of redox reactions. Close proximity of the reductases and oxidases may also permit the formation of a ternary complex, ferric reductase–Fe(III)DFB–putative Fe(II) oxidase, which may facilitate the reductive dissociation of Fe from the siderophore

complex followed by ligand exchange prior to internalization of Fe (reviewed by Boukhalfa and Crumbliss 2002).

At present, we are also unable to establish whether oxidation of Fe(II) occurs during or prior to Fe internalization. In yeast, the multi-Cu-containing ferroxidase and the Fe permease form a single complex at the plasma membrane (Stearman et al. 1996). The activity of the oxidase is believed to impart a conformational change in the permease, such that Fe(III) is immediately internalized following Fe(II) oxidation. Future physiological and molecular research will elucidate whether a similar series of events occur at the plasma membrane of phytoplankton.

In addition to the physiological data for *T. oceanica* and *T. pseudonana* discussed above, genomic and phylogenetic data from *T. pseudonana* support the hypothesis that Fe-limited centric diatoms possess an inducible Fe transport system composed of putative Fe(III) reductases, putative multi-Cu-containing Fe(II) oxidases, and Fe(III) permeases. The three genes encoding for the putative components of this inducible Fe transport system were found within the genome of *T. pseudonana* (Armbrust et al. 2004). In our own analysis, we have identified a gene containing a MCO InterPro domain and transmembrane domains in *T. pseudonana*, and we demonstrated an up-regulation of gene expression of a membrane-bound ferric reductase (data not shown) and the putative, membrane-targeted MCO (Table 7) in Fe-limited *T. pseudonana*. Moreover, it appears that low Cu availability results in even higher levels of the putative MCO transcription (Table 7), suggesting that low Cu may impart an additional level of stress to Fe-limited *T. pseudonana*. Whether this putative multi-Cu-containing Fe(II) oxidase is like the ferroxidase of the high-affinity Fe transport system in *S. cerevisiae* (ScFET3 and ScFET5) (Askwith et al. 1994) and *C. reinhardtii* (CrFOX1) (LaFontaine et al. 2002) or like the fungal laccase (CNLAC1) (Zhu and Williamson 2004) or the vascular plant ascorbate oxidase (Messerschmidt et al. 1992) remains to be determined.

Phylogenetically, the putative *T. pseudonana* MCO most closely resembles fungal laccases. Interestingly, several fungal laccases have been shown to exhibit ferroxidase activity. In particular, the putative *T. pseudonana* MCO shares a well-supported cluster with the laccase sequence from the human fungal pathogen *Cryptococcus neoformans*. Laccase activity in *C. neoformans* is induced by Fe (Zhu and Williamson 2004). It is believed that cryptococcal laccase–iron oxidase activity may protect the fungus from macrophage infections by maintaining Fe in an oxidized form, thereby decreasing the production of antifungal hydroxyl radicals via Fenton reactions (Liu et al. 1999). Most recently, the genome of *Phanerochaete chrysosporium* was searched for laccase-encoding sequences. Four clustered MCO-encoding sequences (MCO1 to MCO4) were identified, but none corresponded to a sequence encoding a conventional laccase. Structural analysis and heterologous expression of MCO1 support that this is a new branch in the MCO family distinct from fungal laccases (Larrondo et al. 2003). The *P. chrysosporium* MCO1 has been identified as an extracellular MCO with strong ferroxidase activity (Larrondo et al. 2003). *P. chrysosporium* MCO1 gene sequences also cluster in phylogenetic proximity to the putative *T. pseudonana* MCO. Future work is needed to confirm the hypothesis that these diatoms have a high-affinity Fe uptake system (consisting of ferric reductases, multi-Cu-containing ferroxidases, and Fe permeases) like that of *S. cereviciae* and *C. reinhardtii*.

Oceanographic implications—Our results suggest that the cellular Cu requirements of Fe-limited phytoplankton will be elevated relative to those of Fe-sufficient cells due to the involvement of putative multi-Cu-containing oxidases (four Cu atoms per protein) (De Silva et al. 1997) in the inducible Fe transport system. Using *T. oceanica* as a model, we estimated a Cu quota of 5.7 μmol Cu per mol C (or 5.76×10^{-18} mol Cu per cell) for an Fe-limited cell. This estimate was based on previous measurements of Fe-transporters density of Fe-limited diatoms ($\sim 3.2 \times 10^{-20}$ mol μm^{-2}) (Hudson and Morel 1990), and the assumption that there is a MCO associated with each Fe transporter, as shown for yeast. Indeed, results from our laboratory indicate that the intracellular Cu levels of Fe-limited *Thalassiosira* spp. are similar to this theoretical amount, and are more than five times higher than those of Fe-sufficient cells (A. Annett pers. comm.). These theoretical and experimental estimates of Cu quotas in Fe-limited diatoms suggest that Fe-limited cells need comparable or higher intracellular levels of Cu (~ 6 μmol Cu per mol C) relative to Fe (~ 3 to 6 μmol Fe per mol C) (Maldonado and Price 1996). These results highlight the importance of Cu nutrition for Fe-limited diatoms.

The findings of this study have significant implications for our understanding of the biogeochemical cycles of Fe and Cu in the sea. Phytoplankton may directly influence the speciation of Fe and Cu in open ocean surface waters through the extracellular activity of redox-active enzymes. The degree of Fe limitation experienced by phytoplankton in the ocean may determine their Cu demand and thus influence the distribution of particulate and dissolved Cu in surface waters of the sea. Future investigations on the Cu nutrition and the interaction between Fe and Cu metabolism in phytoplankton will be essential for a better understanding of how Fe availability and speciation control primary productivity in the surface waters of the ocean.

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