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A role for manganese in superoxide dismutases and growth of iron-deficient diatoms

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Abstract

We have discovered that coastal and oceanic diatoms require more manganese (Mn) to grow in iron (Fe) -deficient than in Fe-sufficient seawater. At low inorganic concentrations, like those of the open sea, Fe and Mn can thus colimit *Thalassiosira pseudonana* and *T. oceanica* so that maximum rates of cell division are achieved only when both resources are added simultaneously to cultures. Colimited diatoms amended with either Fe or Mn alone show unique physiological responses, which implies that the observed interaction between Fe and Mn is not caused by a substitution of one metal for the other. Iron deficiency increases the Mn quota of *T. pseudonana* by three times compared with controls and enhances the production of reactive oxygen species by 1.7 times in *T. weissflogii*. Both diatoms respond to this oxidative stress by increasing the activities of the antioxidant enzyme superoxide dismutase (SOD). The Mn content of the SODs increases by 1.8 to 2.8 times when Fe is limiting, which suggests that the SODs contain Mn and may account for part of the observed increase in the Mn quota. Such an increased biochemical requirement may elevate the Mn content of low Fe diatoms, and possibly other phytoplankton, resulting in high Mn : Fe ratios in surficial particulate matter in Fe-limited regions of the sea.

Iron greatly influences the ecology and physiology of phytoplankton in some open-ocean regions and upwelling regimes (Coale et al. 1996; Hutchins et al. 1998). Subnanomolar concentrations of dissolved Fe are typical of surface waters and limiting to phytoplankton growth (Price and Morel 1998). Phytoplankton exist in these environments because they are able to reduce their Fe requirements (Sunda and Huntsman 1995) and to acquire what little Fe exists with high-affinity Fe transport systems (Maldonado and Price 1999). Intense demand for limiting Fe nonetheless leads to characteristic changes in phytoplankton physiology and biochemistry.

A large body of literature has examined the strategies of Fe acquisition by plankton. Under limiting conditions, phytoplankton up-regulate Fe transport capacity (Morel 1987), produce siderophores (Wilhelm and Trick 1994), reduce Fe(III) chelates (Maldonado and Price 2001), and even ingest insoluble, particulate forms of Fe (Maranger et al. 1998; Nodwell and Price 2000). These strategies represent adaptations to maintain high cellular Fe quotas (Q_{Fe}) and facilitate normal functioning of cellular metabolism, which is highly dependent on Fe (Raven 1990). When the Fe supply is insufficient to meet cellular demand, the Fe : C ratio drops (Sunda and Huntsman 1995; Maldonado and Price 1996), electron transport chains—such as those involved in photosynthesis—are compromised (Greene et al. 1991), and the growth rate slows.

The replacement of Fe-containing enzymes and proteins with their Fe-free equivalents is one way to use Fe sparingly and to maintain biochemical function. Some organisms have even gone so far as to evolve Fe-independent metabolisms and, thus, to rely on other metals and enzymes for their survival (Archibald 1983; Posey and Gheradini 2000). The induction of the electron transfer protein flavodoxin to replace the Fe-containing ferredoxin (La Roche et al. 1996) is a response of phytoplankton to low Fe (Raven 1990). It is likely to be one of a number of Fe conservation mechanisms of phytoplankton. Other possible ways to economize on Fe include the use of certain metals to take the place of Fe in metabolic pathways, either by catalyzing analogous or identical reactions to those of the Fe dependent enzymes or by replacing Fe directly in apoproteins (Merchant and Bogorad 1986; Silva and Williams 2001). Manganese is of particular interest in this regard, because it is present in relatively high concentrations compared with Fe in surface waters of the sea (Price and Morel 1998; Morgan 2000), has a reduction potential that overlaps with that of Fe (Silva and Williams 2001), and can substitute for Fe(III) in some biologically important reactions.

The subject of phytoplankton metal substitution has focused so far on Cd, Co, and Zn (Price and Morel 1990). Each of these metals serves a number of unique biochemical functions but is also able to activate a particular isoform of carbonic anhydrase (Lane and Morel 2000), an enzyme that is involved in C acquisition by phytoplankton. The concentrations of ρCO_2 and Zn influence the levels of the different carbonic anhydrases and their metal cofactors, thereby affecting the metal requirements of phytoplankton (Cullen et al. 1999).

The results presented here illustrate a new type of phytoplankton metal-metal interaction between Mn and Fe. We show that Fe-limited diatoms produce greater amounts of reactive oxygen species (ROS) and need more Mn to activate the antioxidant enzyme superoxide dismutase (SOD). Low Fe and low Mn may thus increase the oxidative stress of phytoplankton by simultaneously increasing the rates of production and decreasing the rates of consumption of ROS. Field data obtained from published reports show that particulate matter is enriched in Mn in Fe-deficient compared with

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Fe-sufficient waters, which suggests that the Mn : Fe interaction observed here may occur in some parts of the sea.

Materials and methods

Cultures—Three diatom species, *Thalassiosira pseudonana* (clone 3H, CCMP 1335), *T. weissflogii* (clone Actin, CCMP 1336), and *T. oceanica* (CCMP 1005) were obtained from the Provasoli-Guillard Centre for Culture of Marine Phytoplankton and grown in Aquil medium (Price et al. 1988/89) under continuous, saturating light (160 μmol quanta m^{-2} s^{-1} supplied by cool white fluorescent bulbs) at 20°C. The culture volume varied from 23 ml to 2 liters, according to the experiment. $MnCl_2$ and $FeCl_3$ were of the highest purity (ANALAR grade; BDH), because reagent-grade $FeCl_3$ (Aldrich, BDH) contains trace amounts of Mn that masked the interaction reported here. The $FeCl_3$ used in the present study did not contain any significant contaminating Cu or Zn. The background concentration of Mn in Aquil was determined by mass spectrometry to be 65 ± 25 pmol L^{-1}. Iron was added to seawater bound to ethylene diaminetetraacetic acid (EDTA; 1 : 1.05), and Mn was added directly to medium that contained 100 μmol L^{-1} EDTA and other trace metals, as reported elsewhere (Price et al. 1988/89). The concentrations of inorganic Mn (Mn′: Mn^{2+}, $MnCl^+$, and $MnCl_2$) and Fe (Fe′: $Fe(OH)_2^+$, $Fe(OH)_3$, and $Fe(OH)_4^-$) ions were calculated using MINEQL software and as described elsewhere (Sunda and Huntsman 1997). All media and radiolabel additions, with the exception of additions to the transient experiments, were allowed to equilibrate for at least 24 h before being used.

Transient experiment—Cultures of *T. pseudonana* were acclimated for at least 32 generations to either Fe-deficient (81.5 pmol L^{-1} Fe′/14.24 nmol L^{-1} Mn′), Mn-deficient (5.66 nmol L^{-1} Fe′/0.59 nmol L^{-1} Mn′), or Fe- and Mn-deficient (81.5 pmol L^{-1} Fe′/0.59 nmol L^{-1} Mn′) conditions. After acclimation, Fe-deficient cultures were transferred into medium enriched with Mn (32.9 nmol L^{-1} Mn′), Fe (5.66 nmol L^{-1} Fe′), or Fe plus Mn (5.66 nmol L^{-1} Fe′/32.9 nmol L^{-1} Mn′). Control cultures were transferred into the original Fe-deficient medium. Mn-deficient cultures were transferred into medium enriched with Mn (14.24 nmol L^{-1} Mn′), Fe (16.8 nmol L^{-1} Fe′), or Fe plus Mn (16.8 nmol L^{-1} Fe′/14.24 nmol L^{-1} Mn′). Control cultures were transferred into the original Mn-deficient medium. Fe- and Mn-deficient cultures were transferred into medium enriched with Mn (14.24 nmol L^{-1} Mn′), Fe (5.66 nmol L^{-1} Fe′), or Fe plus Mn (5.66 nmol L^{-1} Fe′/14.21 nmol L^{-1} Mn′). Control cultures were transferred into the original Fe- and Mn-deficient media. Acclimated cultures were inoculated into duplicate 500-ml polycarbonate bottles, and biomass was measured using in vivo chlorophyll fluorescence. The maximum quantum yield of photosystem II (PSII), F_v/F_m, was determined using the fluorescence measurement with dichlorophenyldimethyl urea (DCMU) described by Parkhill et al. (2001).

Steady-state growth and elemental composition—The intrinsic rate of growth, μ (d^{-1}), was estimated during exponential phase from fully acclimated, semicontinuous batch cultures (Maldonado and Price 1996). Cell densities and volumes were determined by microscopy using a Palmer-Maloney chamber, under the assumption of a cylindrical shape (Hillebrand et al. 1999). Phytoplankton were harvested by gentle filtration (<10 mm Hg) onto precombusted GF/F filters for carbon analysis. Carbon content was measured on a Carlo Erba EA 1108 analyzer. Metal quotas reported as mol L^{-1} cell volume (Q_{Mn} and Q_{Fe}) were measured by adding trace amounts of $^{54}MnCl_2$ (20.6 mCi mg^{-1}) and $^{59}FeCl_3$ (33.89 mCi mg^{-1}) to the media. Cultures were filtered onto 2-μm, 25-mm diameter polycarbonate filters. Surface-adsorbed metals were removed using the Ti-citrate protocol of Hudson and Morel (1989). In dual-label experiments (^{59}Fe and ^{54}Mn), the filters were subsequently placed in sealed petri dishes and counted in a Canberra-Packard GC-2020 Li-Ge crystal γ-counter. In single-label experiments, radioactivity was measured in a Canberra-Packard Tri-Carb 2100-TR liquid scintillation counter.

Short-term ^{54}Mn uptake rates—Midexponential phase cultures of *T. pseudonana* were gently filtered onto acid-cleaned 2-μm, 25-mm diameter polycarbonate filters. The cells were resuspended in Aquil medium that contained no trace metals or EDTA. The suspension was then aliquoted into medium identical to that used for growth except that it contained 1.42 nmol L^{-1} ^{54}Mn′ (carrier free) with either 0, 0.0815, or 5.66 nmol L^{-1} Fe′. The Fe in these additions was complexed to EDTA. Uptake was measured for 2–3 h, and cells were harvested and rinsed as described above. Time zero filters were subtracted from the final samples to correct for any nonspecific binding of Mn. Cell densities and dimensions were measured as described above, and uptakes were normalized to cell surface area.

SOD—Midexponential phase cultures of *T. pseudonana* and *T. weissflogii* were harvested on 2-μm, 45-mm polycarbonate filters and immediately frozen in liquid nitrogen. These samples were then stored at −30°C for ≤3 weeks before analysis. Cells were resuspended in 0.05 mol L^{-1} phosphate buffer (pH 7.8) with 0.1 mmol L^{-1} EDTA and 0.1 % Triton X-100 and disrupted for a total of 2 min on a 50% duty cycle (Branson immersion sonicator with a tapered microtip) and then centrifuged for 5 min at 16,000 × *g*. Protein concentrations of this crude homogenate ranged 0.3–1.4 mg ml^{-1}. Enzyme activities were measured in crude homogenates according to the methods of McCord and Fridovich (1969). In these assays, one unit of activity represents a 50% reduction in the oxidation rate of 10 μmol L^{-1} cytochrome *c* by a xanthine/xanthine oxidase (XOD) couple (50 μmol L^{-1} xanthine, 0.2 U of XOD in 3 ml).

To quantify the relative change in the Mn content of SOD as a function of the Fe nutritional state, 2-liter cultures were grown under Fe-deficient (81.5 pmol L^{-1} Fe′) and Fe-sufficient (5.66 nmol L^{-1} Fe′) conditions with 14.24 nmol L^{-1} Mn′ added as ^{54}Mn. Cells were harvested and handled as described above. Equal amounts of protein (as estimated by the Bradford method; Berges et al. 1993) were loaded on 12% polyacrylamide gels and run for 1 h at 160 V (*T. weissflogii*) or for 2 h at 140 V (*T. pseudonana*). SODs were localized on the gel using the method of Beauchamp and

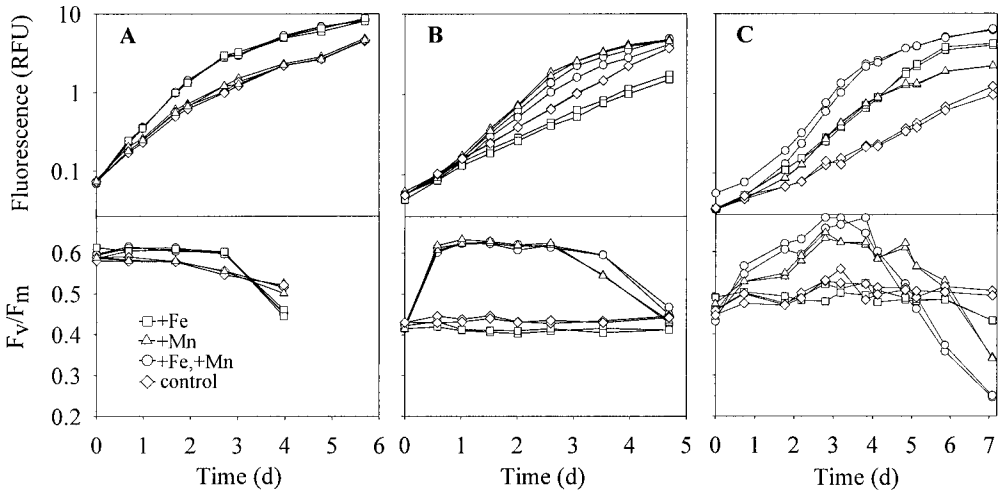

Fig. 1. Growth and photosynthetic efficiency of *T. pseudonana* during transient metal enrichment. *T. pseudonana* was acclimated to different metal conditions and inoculated into medium that contained additional Mn, additional Fe, additional Fe plus Mn, and medium identical to that used in acclimation (see ''Materials and methods'' for metal concentrations). Growth was measured by in vivo fluorescence (RFU) in duplicate cultures of each type of medium, and photosynthetic efficiency (F_v/F_m) was determined coincidently. (A) *T. pseudonana* acclimated to Fe-deficient conditions (81.5 pmol L^{-1}Fe′/14.24 nmol L^{-1}Mn′). (B) *T. pseudonana* acclimated to Mn-deficient conditions (5.66 nmol L^{-1}Fe′/0.59 nmol L^{-1}Mn′). (C) *T. pseudonana* acclimated to Fe- and Mn-deficient conditions (81.5 pmol L^{-1}Fe′/0.59 nmol L^{-1}Mn′).

Fridovich (1971). The gel was soaked in a freshly made solution of 0.028 mmol L^{-1} riboflavin, 25 mmol L^{-1} nitroblue tetrazolium, and 0.028 mol L^{-1} N,N,N′,N′-tetramethylethylenediamene (TEMED) in 36 mmol L^{-1} potassium phosphate buffer (pH 7.8) in the dark for 30 min. It was then quickly rinsed in potassium phosphate buffer (pH 7.8) and exposed to light (~125 μmol quanta m^{-2} s^{-1}) for 10 min. SOD activity appeared as light-colored bands on a uniform blue-violet background. The gel was dried and exposed to BIOMAX-XAR film for 2 weeks before development or exposed overnight to a phosphorimager screen (Storm). The intensity of enzyme activity was quantified by densitometry (ScionImage) using images of gels scanned before drying. The relative amount of ^{54}Mn associated with SODs was estimated from densitometry of digital images of either x-ray film or phosphorimager output.

ROS—Intracellular reactive oxygen species were detected using the method of Maxwell et al. (1999) with dichlorofluorescein diacetate (H_2DCF-DA; Sigma). H_2DCF-DA was added at a final concentration of 5 μmol L^{-1} to 3 ml of fully acclimated phytoplankton culture and incubated in the light under normal culturing conditions. Cell cultures treated similarly, but incubated in the dark, were used as controls. The assay then measured only photogenerated ROS. Cells were collected at 15,000 × *g* for 3 min; then the supernatant was diluted fourfold and measured in a spectrofluorometer with excitation and emission wavelengths of 488 and 520 nm, respectively. Values are reported as relative fluorescent units normalized to cell number.

Results

Transition experiments—To illustrate the response of *T. pseudonana* to single resource limitation, cultures were first grown under Fe-limiting conditions in low Fe (81.5 pmol L^{-1} Fe′) and high Mn (14.24 nmol L^{-1} Mn′) medium. The fully acclimated culture that was used as the inoculum for these experiments was deficient in Fe (μ = 1.1 d^{-1}), and its photosynthetic efficiency of PSII (F_v/F_m = 0.6) was near the theoretical maximum (~0.65, Parkhill et al. 2001). The addition of Fe with or without Mn significantly stimulated growth, as expected (μ = 1.5 d^{-1}, Fig. 1A). Doubling the amount of Mn in the medium to 32.9 nmol L^{-1} Mn′ had no effect on the growth rate (1.1 and 1.2 d^{-1}, duplicate measurements) compared with the control. The efficiency of PSII was largely unaffected by any of the treatments; however, as the cultures began to deplete macronutrients in the media between days 3 and 4, F_v/F_m began to decline.

Single resource limitation experiments that examined Mn deficiency revealed slightly different responses to the addition of Fe or Mn. These low-Mn cultures were acclimated in medium that contained high concentrations of Fe′ (5.66 nmol L^{-1}). Control cultures grew at rates identical to the inoculum culture (0.92 and 0.93 vs. 0.93 d^{-1}). Curiously, the addition of more Fe to a level double that added in Fe-replete cultures reduced growth rate (Fig. 1B). The addition of Fe also slightly reduced F_v/F_m (Fig. 1B), which suggests that high Fe had a direct physiological effect. Any low concentration of Mn that may have been inadvertently added as a contaminant with the Fe stock was minor and insufficient to stimulate growth of these Mn-limited cultures. Microscopic analysis of the cultures sampled on day 2 confirmed that the

Table 1. Growth rates, cell volumes, and elemental quotas of *Thalassiosira pseudonana* grown under steady-state conditions in Fe- and Mn-replete and -deplete seawater (±1 SD, $n = 4$ or $n = 3$*) NA: not measured.

[Fe′] (nmol L^{-1})	[Mn′] (nmol L^{-1})	Growth rate (d^{-1})	Cell volume (fl)	Cellular [C] (mol L^{-1})	Cellular [Mn] (μmol L^{-1})	Cellular [Fe] (μmol L^{-1})
5.66	14.24	1.91 ± 0.04	93.4 ± 7.3	8.91 ± 0.10	191 ± 52	1460 ± 430
0.0815	14.24	1.30 ± 0.07	69.0 ± 7.1*	13.2 ± 0.67	263 ± 18*	70 ± 10*
5.66	1.42	1.55 ± 0.06	70.8 ± 8.0	NA	21.5 ± 1.4	NA
0.0815	1.42	1.00 ± 0.02	40.3 ± 5.6	NA	78.6 ± 8.4	NA
5.66	0.59	1.18 ± 0.11	30.4 ± 10.9	6.11 ± 0.35	14.7 ± 4.0	1640 ± 360
0.0815	0.59	0.84 ± 0.09	115 ± 9.6	9.45 ± 0.40	6.9 ± 1.2	15 ± 2
5.66	0.35	0.76 ± 0.14	NA	NA	NA	NA
0.0815	0.35	0	NA	NA	NA	NA

increases in fluorescence we observed represented increases in cell density and not just changes in chlorophyll fluorescence (control = 6,514 cells ml^{-1}, +Fe = 4,475 cells ml^{-1}, +Mn = 27,418 cells ml^{-1}, +Fe and Mn = 25,154 cells ml^{-1}, average of two replicates). Fluorescence per cell decreased with the addition of Mn, so the growth rates calculated for these treatments should be considered as conservative estimates (see below). Of interest, the fully acclimated Mn-deficient culture had a low F_v/F_m of ~0.4 (Fig. 1B). Increasing the concentration of Mn, with or without Fe, increased F_v/F_m and the growth rate. Differences in the response of F_v/F_m of *T. pseudonana* to low Mn and low Fe during steady-state growth and after the additions of metal allowed us to differentiate between Mn and Fe limitation and to document changes in cellular physiology.

When *T. pseudonana* was grown in seawater that contained low [Fe] and low [Mn], the addition of either nutrient alone increased the growth rate significantly, but only the addition of both nutrients together allowed for a full recovery of growth (Fig. 1C). The Fe- plus Mn-amended cultures grew fastest at 1.37 and 1.46 d^{-1}, followed by the cultures that received an addition of Mn (1.12 and 1.16 d^{-1}), an addition of Fe (0.84 and 0.91 d^{-1}), and no additions (0.54 and 0.6 d^{-1}, calculated from days 2–4, analysis of variance [ANOVA], Tukey test; $P < 0.05$). The photosynthetic efficiency of PSII (F_v/F_m) was only increased by Mn, even though the addition of Fe alone increased growth. As was observed in the single resource limitation experiments, only Mn deficiency compromised PSII function, and the subsequent addition of Mn promoted its recovery. This result further demonstrated that the addition of Fe did not introduce significant levels of contaminating Mn (PSII did not recover in the +Fe treatment). Thus, growth stimulation by Mn (or Fe) was not caused indirectly by intracellular or extracellular changes in the availability of Fe (or Mn).

Table 2. Steady-state growth rate (d^{-1}) of *Thalassiosira oceanica* clone (1005) conditioned with different ambient Fe′ and Mn′ concentrations (±1 SD, $n = 3$).

[Fe′] (nmol L^{-1})	[Mn′] (nmol L^{-1})	μ (d^{-1})
5.66	0.33	0.75 ± 0.06
0.0156	0.33	0.59 ± 0.12
5.66	0.087	0.64 ± 0.08
0.0156	0.087	0.10 ± 0.02

Steady-state growth and metal content—The Fe-Mn interaction reported in the transient experiments was also observed in fully acclimated, semicontinuous batch cultures of *T. pseudonana* where Fe and Mn were added at low, limiting concentrations (Table 1). Steady-state growth rates were significantly slower in Fe- or Mn-deficient medium than in controls and were further reduced when both nutrients were simultaneously at low concentrations (one-way ANOVA, Tukey test; $P < 0.05$). The interdependence of the Mn and Fe requirement was most apparent when the resources were most growth limiting. At a [Mn′] = 0.35 nmol L^{-1}, for example, Fe-sufficient cultures grew well (Table 1), whereas Fe-deficient cultures were unable to survive ($n = 5$). Increasing the [Mn′] slightly to 0.59 nmol L^{-1} in the low-Fe medium increased the steady-state growth rate from 0 to 0.84 d^{-1}. Thus, the growth of *T. pseudonana* required more Mn in low- than in high-Fe medium and more Fe in low- than in high-Mn medium. A similar pattern of Mn : Fe interaction was also observed for steady-state growth of the oceanic diatom *T. oceanica* (Table 2).

Under single-resource limitation, the quotas of the limiting metals were greatly reduced (Table 1). Intracellular Fe quotas of *T. pseudonana* decreased by 20-fold as Fe became limiting and Mn quotas were reduced by one order of magnitude when Mn′ was reduced from 14.24 to 0.59 nmol L^{-1} (both results, one-way ANOVA, Tukey test; $P < 0.05$). At 14.24 nmol L^{-1} Mn′, Fe-deficient cells contained 1.3 times the concentration of Mn compared with Fe-sufficient cells, whereas, at 1.42 nmol L^{-1}Mn′, Q_{Mn} tripled from 21.5 to 78.6 μmol L^{-1}. Growth at low concentrations of Mn and Fe together caused a large increase in cell volume. This increase, compared with Fe-deficient cells (0.0815 nmol L^{-1} Fe′ and 14.24 nmol L^{-1} Mn′), greatly reduced the Q_{Fe} of Mn- and Fe-deficient cultures. The amount of Fe per cell remained statistically unchanged in the two treatments.

Short-term ^{54}Mn uptake—The uptake of ^{54}Mn at concentrations that limit the growth *T. pseudonana* were not inhibited by the presence of Fe in the medium (Table 3). The short-term rate of transport of cells incubated with 5.66 nmol L^{-1} Fe and 1.42 nmol L^{-1} Mn was identical to the steady-state rate (27 vs. 30 nmol Mn m^{-2} d^{-1}). Iron-deficient cells

Table 3. Short-term (ρ^{ST}) uptake of ^{54}Mn by *Thalassiosira pseudonana* grown under a variety of Mn and Fe concentrations. Short-term uptakes were performed on cells resuspended in media that contained 1.42 nmol L^{-1} Mn′ in the presence of 0, 0.0815 or 5.66 nmol L^{-1} Fe (±1 SD, $n = 3$).

[Fe′] (nmol L^{-1})	[Mn′] (nmol L^{-1})	ρ^{ST} (nmol Mn m^{-2} d^{-1}) 0 nmol L^{-1} Fe′	0.0815 nmol L^{-1} Fe′	5.66 nmol L^{-1} Fe′
5.66	1.42	26.1 ± 5.1	27.5 ± 3.0	30.0 ± 1.9
0.0815	1.42	16.5 ± 1.7	22.4 ± 3.6	19.7 ± 3.1
5.66	0.59	23.5 ± 1.3	30.1 ± 5.8	25.2 ± 1.3
0.0815	0.59	16.5 ± 1.2	19.2 ± 1.6	18.7 ± 3.1

Table 4. Oxidative stress response of *Thalassiosira weissflogii* and *T. pseudonana* acclimated to steady state Fe-deficient and Fe-sufficient conditions. Values reported for superoxide dismutase (SOD) activity and reactive oxygen species generation are means ± 1 SD, $n = 4$.

Species	[Fe′] (nmol L^{-1})	SOD activity (units (mg protein)$^{-1}$)	Reactive oxygen species (RFU 10,000 cells^{-1})
T. weissflogii	5.66	2.69 ± 0.23	1.83 ± 0.13
	0.082	3.77 ± 0.23	3.17 ± 0.52
T. pseudonana	5.66	11.8 ± 1.25	
	0.082	18.5 ± 0.3	

appeared to transport Mn at slower rates than the Fe-sufficient cells, and their short-term rate of transport was roughly half their steady-state rate.

SOD activity—To examine the biochemical basis for the increased Mn requirement of low-Fe cells, we chose to focus on the coastal species: *T. pseudonana* because it was easy to colimit and *T. weissflogii* because its Q_{Mn} was reported to increase under Fe deficiency (Harrison and Morel 1986). Iron deficiency increased SOD activity in both species by 1.4 and 1.6 times, respectively (Table 4; *t*-test; $P < 0.05$). In *T. weissflogii*, the increased SOD activity coincided with an increase in the generation of ROS, which suggests that the cells experienced greater oxidative stress under low- than high-Fe conditions.

Three isoforms of SOD were detected in *T. weissflogii*, and four were found in *T. pseudonana*. Under Fe-replete conditions, these SODs were identified as containing Fe by their characteristic inhibition by H_2O_2 but not by KCN. When nondenaturing polyacrylamide gel electrophoresis gels were soaked in 4.15 mmol L^{-1} H_2O_2 before staining, SOD activity was reduced by 70% (data not shown). Exposure of the gel to 3 mmol L^{-1} KCN, a known inhibitor of Cu/Zn SODs (Butow et al. 1997), had no effect on the activities of SODs from *T. weissflogii*.

According to densitometry analyses, SOD enzyme activity increased by 1.3 times in Fe-deficient *T. weissflogii* and by 1.5 times in Fe-deficient *T. pseudonana* compared with Fe-replete cells. These results agreed well with the relative changes in SOD activity measured independently in whole-cell extracts (Table 4).

Autoradiograms showed that virtually all of the protein-bound ^{54}Mn solubilized by our methods comigrated with the SODs (Fig. 2). A few faint ^{54}Mn signals were observed in smaller proteins but only in Fe-deficient cells (data not shown). The ^{54}Mn content of the SODs of *T. weissflogii* and *T. pseudonana* increased in Fe-limited cells by 2.8 and 1.8 times respectively, compared with Fe-sufficient cells. Standard curves of the radioactive signal detected by densitometry were linear over the range of ^{54}Mn concentrations encountered in our experiments. All of the isoforms increased in activity relative to total protein, but the slowest migrating form in both species appeared to be preferentially upregulated.

Discussion

The transient and steady-state growth results reported here are consistent in showing an interaction between Mn and Fe at low, limiting concentrations. The most complete data set we have is for *T. pseudonana*, but a similar pattern of interaction is also apparent in the oceanic species, *T. oceanica*. Because Fe limitation is a prevalent feature of large regions of the sea, we have focused our efforts on understanding the biochemical changes brought about by such limitation and the role of Mn in alleviating it. As described below, the model we propose to explain Fe/Mn colimitation of growth involves oxidative stress and the disruption of PSII function and electron flow through the photosynthetic electron transport chain. Although a number of Mn-containing enzymes may be regulated by Fe status (Silva and Williams 2001 for discussion of Mn-requiring enzymes), we limit our discussion to those biochemical pathways that are believed to dominate the Mn demand of diatoms: SOD and PSII (Raven 1990). We begin our discussion by outlining the primary effects of single resource limitation.

Fe deficiency—*T. pseudonana* exhibited a distinctive reduction in steady-state growth rate, $Q_{Fe,}$ and cell size in low

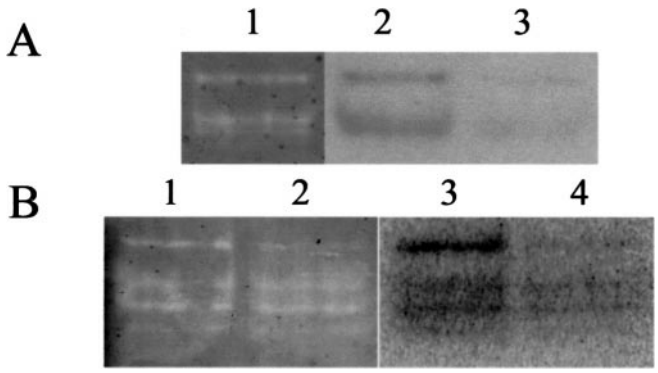

Fig. 2. Enzymatic activity and ^{54}Mn autoradiograms of SODs as resolved on native protein gels. (A) *T. weissflogii* Lane 1: SOD activity (Fe-sufficient). Lane 2: ^{54}Mn (Fe-deficient). Lane 3: ^{54}Mn (Fe-sufficient). (B) *T. pseudonana* Lane 1: SOD activity (Fe-deficient). Lane 2: SOD activity (Fe-sufficient). Lane 3: ^{54}Mn (Fe-deficient). Lane 4: ^{54}Mn (Fe-sufficient). Equal amounts of protein (75 μg) were added per lane.

Fe medium, as expected (Table 1) (Sunda and Huntsman 1995; Maldonado and Price 1996). Examination of the growth data reported in Tables 1 and 2 shows that both *T. pseudonana* and *T. oceanica* required higher concentrations of Mn to grow when Fe-deficient. Murphy et al. (1984) obtained the same result with *T. oceanica* but found no difference in the Mn requirements of *T. pseudonana* in high- and low-Fe media. The clearest example of Fe/Mn interaction in our data was seen at the lowest Mn level (0.35 nmol Mn′ L^{-1}), in which Fe-sufficient cells grew at 0.76 d^{-1} and Fe-deficient cells were unable to survive.

Manganese quota also increased substantially, but most noticeably and significantly ($P < 0.05$, ANOVA) at intermediate concentrations of Mn (1.42 nmol Mn′ L^{-1}) (Table 1). At higher concentrations of Mn (14.2 nmol Mn′ L^{-1}), the luxury uptake of Mn may have minimized the difference in the quotas of the high- and low-Fe cells. Excessive rates of consumption are typical of phytoplankton grown under conditions of nutrient oversupply (Droop 1974; Maldonado and Price 1996), so such an effect is not without precedent. An increase in Q_{Mn} could arise from an increase in the rate of Mn transport, a decrease in the growth rate, or a combination of the two. The steady-state Mn transport rate (viz. the product of steady-state growth rate (d^{-1}) and Q_{Mn} (mol L^{-1} cell volume)) for diatoms growing at 1.42 nmol Mn′ L^{-1} was more than twofold faster when Fe was low than when it was high. However, short-term Mn transport rates were slightly slower for Fe-deficient than for Fe-sufficient cells (Table 3). Harrison and Morel (1986) made similar observations for the centric diatom *T. weissflogii*. They postulated that Fe interfered with Mn transport which resulted in higher Q_{Mn} in Fe-deficient than in sufficient cells but did not test this hypothesis directly. Our results provide no evidence for Fe inhibition of the uptake of Mn during short-term experiments (Table 3) and instead suggest that steady-state Q_{Mn} increases during Fe-deficient growth because the growth rate declines.

Steady-state Fe-deficiency had no effect on the maximum quantum yield of PSII in *T. pseudonana* (Fig. 1A). F_v/F_m measured by the DCMU method was near the maximum theoretical value of 0.65 and was not substantially different from what we measured in nutrient-replete cultures. This result was surprising, because low Fe is well known to affect the efficiency of PSII (Greene et al. 1992; McKay et al. 1997). The slow growth rate of the culture that we examined here (0.6 μ_{max}), and its response to Fe enrichment, leaves no doubt that it was Fe limited. One possible explanation could be differences in the physiological status of the phytoplankton used in this and in other studies. Price (unpubl. data) found that F_v/F_m varied little across a range of steady-state, Fe-limited growth rates but that it declined greatly (~0.2) in cultures that had entered stationary phase in low-Fe medium. Analogous results were obtained by Parkhill et al. (2001) with N-limited and -starved cultures. Clearly, the lack of agreement between results obtained from Fe starvation and steady-state growth limitation warrants further study. For the remainder of the discussion, however, we assume that changes in photosynthetic biochemistry associated with Fe starvation are similar to those for steady-state Fe limitation, because few studies have been conducted under the latter conditions.

ROS are a byproduct of normal cellular metabolism and are commonly generated under conditions of metal overload by metal redox reactions (Chrichton 2001). They include such species as superoxide, hydroxyl radical, hydrogen peroxide, and organohydroperoxides, many of which are enzymatically detoxified. Under conditions of low, limiting Fe, ROS production rates were elevated above those of metal-replete cultures. Because the assay for ROS was conducted in the light and the controls were measured in the dark, the source of electrons for their production was presumably from the photosynthetic reactions. Thus, electron flow through photosynthesis was made less efficient by low Fe—a greater proportion and total quantity of the electrons was apparently accepted by O_2. We cannot assume without a doubt that the electrons originated from water splitting, because other light-mediated charge transfer reactions could also be involved, but the reduction in photosynthetic efficiency of Fe-deficient phytoplankton reported by Greene et al. (1992) is consistent with this proposal. The probe that we used for ROS detection was a nonspecific trapping agent that reacts with many ROS at different rates (Maxwell et al. 1999). We surmise that at least a portion of these species were superoxide, because SOD enzyme activity increased under the same conditions. The SOD and ROS measurements confirm that Fe limitation increases oxidative stress in diatoms.

All three of the SODs of *T. weissflogii* were putatively identified as containing Fe according to their characteristic inhibition by H_2O_2 but not by KCN (Butow et al. 1997). However, surprisingly, we found that Mn was associated with each of the isoforms and that it increased under Fe deficiency (Fig. 2). The increase in Mn content provides a biochemical mechanism to explain the observed increase in Q_{Mn}, although we cannot estimate from these data how much Mn was contained in SOD. We attempted to use ^{59}Fe as a tracer to examine whether the SODs also contained Fe, but we were unable to distinguish between ^{59}Fe in SOD and adjacent proteins on the gel.

Mn limitation—Lowering Mn′ in the medium reduced the steady-state growth rate, Q_{Mn}, and cell volume of *T. pseudonana* (Table 1), as reported in other studies on Mn limitation (Sunda and Huntsman 1983). We also observed a slight, but insignificant, increase in Q_{Fe} in the Mn-deficient compared with Mn-sufficient cells (Table 1, ANOVA; $P > 0.05$). Because diatoms grown at high Fe′ have been shown to accumulate Fe without any change in growth rate (Sunda and Huntsman 1995), it is possible that a low Mn-induced change in Q_{Fe} was obscured by luxury uptake (as described above for Mn). Mn-limited cells required higher concentrations of Fe in the media to grow at maximal rates, and growth rates of *T. pseudonana* and *T. oceanica* dropped precipitously when both nutrients were reduced in the medium (Tables 1, 2).

When the ratios of dissolved [Mn]:[Cu] or [Mn]:[Zn] are low in cultures of phytoplankton, Cu and Zn can outcompete Mn for uptake sites resulting in Mn deficiency (Sunda and Huntsman 1998). Such inhibition of Mn uptake was unlikely to have occurred in our cultures, because the concentrations

of Cu and Zn were well below those that elicit an antagonistic response. We tested for transport interference, however, by increasing and decreasing Cu and Zn concentrations by a factor of two and found no effect on the Fe/Mn interaction described here (data not shown). Sunda and Huntsman (1998) suggested that concentrations of Mn, Zn, and Cu found in the open ocean could lead to the suppression of Mn uptake in natural phytoplankton communities. Such an interaction would be expected to be even more problematic for Fe-limited phytoplankton that have an increased Mn demand, as shown here.

Steady-state, Mn-deficient growth reduced F_v/F_m to ~0.4, a level substantially lower than that observed in nutrient-replete cultures (~0.62) (Fig. 1B). This reduction in PSII efficiency was not surprising, because Mn is required in the water-splitting complex (Raven 1990), and its absence should impede entry of electrons into the photosynthetic electron transport chain. Theoretical calculations identify the water-splitting complex as the major Mn-containing constituent of phototrophic cells (Raven, 1990). As discussed above, Mn also appears to be used in SOD. We cannot rule out the possibility that the SODs also require Fe, given the results of the inhibitor assays; however, genome sequence analysis suggests that only Mn SODs are present in *T. pseudonana* (F. Wolfe, pers. comm.). Thus, the observed reduction in the steady-state growth rate under Mn deficiency can be explained by a decrease in water-splitting activity and the supply of electrons to the reaction center of PSII. Mn SOD is also likely to be compromised under low Mn, increasing oxidative stress and the requirements for energy and resources to repair damaged cellular constituents.

In the transient experiments, the addition of Fe to Mn-deficient cultures that already contained high [Fe] appeared to affect cell metabolism. The added Fe caused a slight reduction in F_v/F_m and in growth rate compared with controls. It is unlikely that the added Fe inhibited the uptake of Mn, judging from the results of the short-term experiments (Table 3). We cannot unequivocally explain the decrease on F_v/F_m, but we propose that excess Fe accumulated by the cells could participate in oxygen radical generation (Chrichton 2001). This would challenge an antioxidant system inhibited by low Mn (see above) and result in compromised physiology and growth.

Low Fe and Mn interaction—If we assume that the main effect of Mn deficiency is to reduce the number of electrons that enter the photosynthetic electron transport chain and that Fe deficiency reduces the efficiency of electron transport, we offer the following hypothesis to explain the Fe/Mn colimitation results. Under low Fe and low Mn, growth is slow because fewer electrons enter the photosynthetic electron transport chain and, of those that do, a large proportion are ''leaked'' to molecular oxygen, forming ROS. Mn-containing SOD cannot be synthesized in sufficient quantities to detoxify superoxide, one of the ROS produced, resulting in oxidative stress. The addition of Mn-alone increases the amount of electrons entering photosynthesis (judging from the recovery in F_v/F_m, Fig. 1C) and decreases the levels of ROS (by activating Mn SOD) caused by an inefficient, Fe-limited electron transport chain. The addition of Fe alone improves the efficiency of electron transfer, reducing the proportion of electrons lost to ROS production and providing more energy for growth. PSII function remains compromised (low F_v/F_m, Fig. 1C), so the phytoplankton are unable to sufficiently liberate electrons from water for use in the electron transport chain, and their Mn SODs are inhibited. Only the addition of both metals together allows for the full recovery of photosynthesis and the detoxification of superoxide, allowing maximal growth.

An alternate hypothesis to describe the interaction involves the direct substitution of Mn for Fe when Fe is limiting growth (or vice versa). Indeed, the up-regulation of Mn-containing SODs under Fe deficiency could represent a compensatory response to offset a decrease in Fe-containing isoforms (Campbell and Laudenbach 1995). As discussed above, we cannot rule out the possibility that the diatom SODs also contain Fe. The substitution of Mn for Fe in a single protein has been shown in cambialistic (exchangeable) isoforms of SOD, which are able to maintain activity with either metal in the active site (Meier et al. 1982).

The above mechanism is similar to other metal replacement pathways described in phototrophs, such as the replacement of the photosynthetic electron carrier proteins plastocyanin (Cu containing) with cytochrome c6 (Fe containing) in *Chlamydomonas* (Merchant and Bogorad 1986), and the replacement of Zn-carbonic anhydrase in *T. weissflogii* with Cd- or Co-containing isoforms when Zn is low (Lane and Morel 2000). Data from the colimited transient experiment, however, appear to be inconsistent with this hypothesis because they show unique physiological responses of *T. pseudonana* to the addition of Fe and Mn. The observation suggests that Fe and Mn are not directly substitutable resources. The addition of either metal stimulates growth, but F_v/F_m remains low in the added Fe culture and increases in the added Mn culture.

Biogeochemical cycling of Mn—The biogeochemical cycle of Mn in surface waters of the sea involves an exchange between dissolved Mn^{2+} [Mn(II)] and a particulate pool of Mn oxides [Mn(IV)] and biogenic Mn of uncertain redox state (Morgan 2000). Aeolian supply continuously enriches surface seawater with Mn. Manganese-oxidizing bacteria dominate the formation of the particulate fraction in the Sargasso Sea (Sunda and Huntsman 1988) and are believed to be important below the photic zone in the Pacific Ocean as well (Cowan and Bruland 1985). They oxidize Mn(II) to Mn(IV) only in deep water, because sunlight inhibits their activity near the surface (Sunda and Huntsman 1988). Sunlight also promotes the reductive dissolution of MnO_x and contributes to the maintenance of a surface Mn(II) concentration maximum that is atypical of biologically active nutrients.

Bacteria do not play a dominant role in the formation of particulate Mn in all parts of the sea. In the upper water column of the equatorial Pacific Ocean, for example, the phytoplankton uptake of Mn(II) is thought to be the primary mechanism at play (Moffett 1997). The production of particulate Mn is stimulated by light and inhibited by azide, and the resultant particles are not dissolved by ascorbate. As with other biological metals (Price and Morel 1998), regeneration

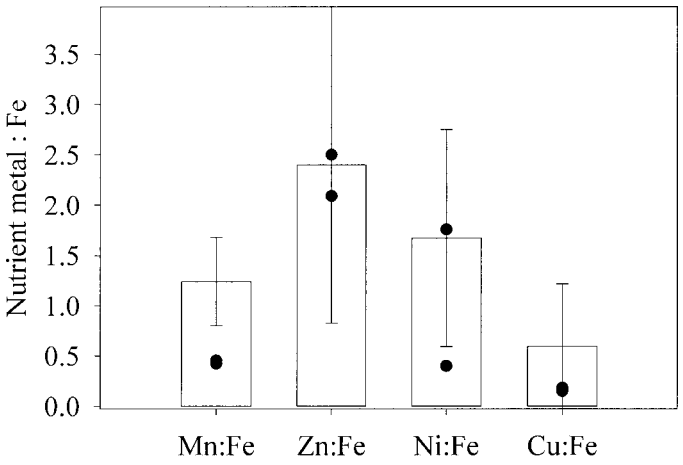

Fig. 3. Molar ratios of nutrient metals to Fe in acetic acid leachable particulate material from waters around the Galapagos Islands (PLUMEX). Metal concentrations measured in samples collected at 20 m were taken from Gordon et al. (1998). Stations were categorized as Fe-deficient (white bars, error = 1 SD, $n = 4$) or Fe-sufficient (circles, $n = 2$) according to the photosynthetic performance of phytoplankton (Lindley and Barber 1998).

by grazers likely releases the particulate Mn back into solution.

We can independently estimate the importance of phytoplankton to particulate Mn production by calculating their steady-state Mn content and comparing it with total particulate Mn. Surface waters of the equatorial Pacific Ocean contain ~0.4 μg Chl *a* L^{-1} (Lindley and Barber 1998; Moffett 1997), which corresponds to 1.7 μmol phytoplankton C L^{-1}. If these phytoplankton have similar Mn : C ratios as an oceanic diatom in culture (11.4 μmol mol^{-1}, Sunda and Huntsman 2000), then they would contain 13–38 pmol Mn L^{-1}, compared with 20 pmol Mn L^{-1} in all particulate matter (Gordon et al. 1998). The same exercise, conducted using data from the Sargasso Sea, shows that phytoplankton contain ~1% of the total particulate Mn pool (130 pmol Mn L^{-1}; Sunda and Huntsman 1988).

The equatorial Pacific Ocean is Fe limited (Coale et al. 1996), so phytoplankton may have high Mn requirements as shown here for *Thalassiosira* species. We predict that Fe-deficient phytoplankton in these waters should be enriched in Mn relative to Fe-sufficient phytoplankton. Data from the PLUMEX experiment near the Galapagos Islands provide a preliminary field test of this hypothesis. Stations occupied in this region can be differentiated on the basis of phytoplankton Fe nutritional status according to quantum yield of photosynthesis (Greene et al. 1991; Lindley and Barber 1998). To estimate the biogenic particulate metal concentration, we have focused on the acetic acid leachable fraction, which makes up most of the total particulate Mn in shallow depths. Results of the analysis show that plankton from Fe-deficient stations are clearly enriched in Mn relative to Fe-sufficient stations (Fig. 3). None of the other bioactive metals measured in the particulates show this pattern. Thus, these field data are consistent with our laboratory findings and suggest that Mn plays an increasingly important role in phytoplankton in the Fe-deficient sea.

In surface waters of the Southern Ocean, dissolved [Mn] can be as low as 80 pmol L^{-1} (Martin et al. 1990). Steady-state cultures of the oceanic diatom *T. oceanica* are growth limited at this [Mn′] level and become severely growth limited when Fe is lowered concomitantly (Table 2). Our results for the coastal diatom *T. pseudonana* show that at low, co-limiting concentrations of Fe and Mn, the Q_{Mn} does not increase as it does when ambient Mn is slightly higher. This result appears to arise because of the large increase in cell volume in the colimited cells. On a cellular basis, however, the Mn quota actually doubles in the colimited compared with the Mn-limited cultures and the steady-state transport rate is 30% faster. Thus, the colimited cells maintain or increase their cellular Mn demand although the volumetric quota declines. Quota measurements of *T. oceanica* or some other oceanic phytoplankter are required to evaluate whether or not the Mn content is increased under conditions similar to those of the Southern Ocean. Judging from the equatorial Pacific data of Gordon et al. (1998), we believe that Fe-limited phytoplankton may indeed be relatively enriched with Mn.

According to our results, Mn enrichments might stimulate phytoplankton growth in Fe-deficient waters if surface concentrations of Mn are low. Indeed, experiments conducted in two different high nutrient, low chlorophyll (HNLC) regions obtain such a response. Coale (1991), for example, observed an increase in diatom biomass after the addition of nanomolar concentrations of Mn. Diatom communities also responded to the addition of Fe, but there was no appreciable change in treatments with other metals. Buma et al. (1991) showed a similar stimulation of phytoplankton growth in the Southern Ocean, but the plankton collected at a second station did not exhibit the same response. Scharek et al. (1997) and Sedwick et al. (2000) observed no significant effects of the addition of Mn on biomass or the accumulation of Fe in samples collected from the Southern Ocean. These latter results suggest that ambient concentrations of Mn in HNLC areas may be sufficient for phytoplankton to meet their Mn demand.

In summary, we find an interaction between low concentrations of Mn and Fe that causes a colimitation of growth of coastal and oceanic diatoms. Only the addition of both nutrients together allows for a full physiological recovery from nutrient limitation. The observed interaction and the biochemical manifestations documented in the present article suggest that phytoplankton in HNLC regions may have increased Mn requirements for growth. These requirements may be most difficult to fulfill in the Southern Ocean, where [Mn] is lower than [Fe] (Martin et al. 1990) and where low light increases the demand for both metals (Raven 1990).

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