Increased metabolic requirements for manganese and copper in iron-limited marine diatoms

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Preface

Contributions of Authors

All data were collected by Graham Peers with the exception of data contained in Figures 1 and 2 and Table 2 of Chapter 3. These were generated by Sarah-Ann Quesnel as part of an undergraduate research project.

All ideas, experiments and written manuscripts were carried out in collaboration with my supervisor, Prof. Neil Price.

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Jenny-K, your buoyancy keeps me together. I love you and I can't wait for what happens next.

I thank you all and I apologize…it just took this long.

Abstract

Productivity in large areas of the world's oceans is limited by low concentrations of dissolved iron in surface waters. Phytoplankton have adapted to persist in these environments by reducing their requirements for iron (Fe) in key metabolic pathways, in some cases by replacing Fe-containing catalysts with their iron-free functional equivalents. This thesis examines the requirements and biochemical roles for copper (Cu) and manganese (Mn) in Fe-limited centric marine diatoms. A major finding of my research is that diatoms have elevated requirements for Mn and Cu when grown in Fe-deficient seawater. Iron deficiency induces oxidative stress and increases the cellular concentrations of toxic oxygen radicals and damage products in *Thalassiosira pseudonana*. The increased Mn-requirement is used, in part, to activate Mn-containing isoforms of the antioxidant enzyme superoxide dismutase. Cultures co-limited by Fe and Mn exhibit high levels of oxidative stress and an inefficient detoxification pathway that further reduces cell growth. Diatoms isolated from the metal poor open ocean require more Cu to divide than related species from metal-rich coastal waters. This pattern is in stark contrast to all other known nutritive trace metals. One part of the diatom Cu requirement that is independent of provenance is for efficient Fe transport. The additional Cu requirement of oceanic species appears to be due to the constitutive expression of a Cu-containing electron transport protein, possibly plastocyanin. Coastal species, which have higher Fe-requirements for growth, retain the Fe-containing functional homologue cytochrome c_6. By employing metals other than Fe within photosynthesis and antioxidant pathways, marine diatoms are able to increase their fitness in Fe-deficient environments. However, Mn and Cu also occur in low concentrations in the open ocean and thus may co-limit growth of natural populations of phytoplankton. Metal enrichment experiments in the Bering Sea reveal that additions of both Cu and Fe play a role in the ecology of the plankton community.

Résumé

La productivité biologique de grandes étendues océaniques du monde est limitée par les faibles concentrations de fer dissous dans les eaux de surface. Le phytoplancton s'est adapté pour survivre dans ces environnements en réduisant son besoin en fer (Fe) dans les voies métaboliques essentielles, dans certains cas, en remplaçant le Fe dans le métabolisme par des équivalents fonctionnels sans fer. Cette thèse étudie les besoins et les rôles biochimiques du cuivre (Cu) et du manganèse (Mn) dans les milieux pauvres en fer. Une découverte importante dans ma recherche est que les diatomées ont des besoins élevés en Cu et en Mn lorsqu'elles croissent dans un milieu marin déficient en fer. Une déficience en fer induit un stress oxydatif. Elle augmente la concentration cellulaire de radicaux libres de l'oxygène et de sous-produits toxiques pour *Thalassiosira pseudonana*. L'augmentation des besoins en Mn est utilisée en partie pour activer des isoformes contenant du Mn de l'enzyme antioxydante *dismutase superoxydique*. Des cultures limitées en fer et en manganèse démontrent des niveaux élevés de stress oxydatif et des voies métaboliques de détoxification déficientes qui réduisent encore plus la croissance cellulaire. Les diatomées isolées de zones océaniques pauvres en métaux dissous nécessitent plus de Cu pour se multiplier que des espèces similaires issues d'eaux côtières riches en métaux. Ce comportement contraste avec celui des autres oligo-éléments alimentaires. Une partie du Cu, essentiel aux diatomées, est nécessaire au transfert efficient du fer quel qu'en soit la provenance, soit côtière ou océanique, des diatomées. Les besoins supplémentaires en Cu des espèces océaniques sont pour l'expression d'une protéine de transfert contenant du Cu, possiblement la plastocyanine. Les espèces côtières qui ont un besoin en fer plus important pour leur croissance, expriment un homologue fonctionnel contenant du fer, le cytochrome c_6. En utilisant des métaux autres que le Fe pour la photosynthèse et dans les réactions antioxydantes, les diatomées marines sont capables d'augmenter les adéquations dans un environnement pauvre en Fe. Toutefois, les concentrations de Mn et de Cu peuvent être également faibles en haute mer dans

les océans et peuvent ainsi co-limiter la croissance des populations de phytoplanctons. Des expériences d'enrichissement en oligo-éléments (métaux) dans la mer de Béring ont indiqué que l'ajout de Cu et de Fe joue un rôle dans l'écologie des communautés de planctons.

Table of Contents

Index of Figures

Index of Tables

General Introduction

Primary production in several regions of the world's oceans is limited by the extremely low concentrations of iron (Fe) in the upper water column (the photic zone). In these regions the supply of Fe to the photic zone is much smaller than the Fe demand of the indigenous biota. Growth limitation by Fe has promoted some remarkable adaptations. Phytoplankton, single-celled photosynthesizing eukaryotes and prokaryotes, have developed a range of strategies to acquire scarce Fe or to reduce their metabolic requirements for it. In particular, diatoms from low Fe waters need considerably less Fe for photosynthesis than species from comparatively high metal seas. Other nutritive transition metals, namely manganese (Mn) and copper (Cu), may be important for survival in Fe-limited environments. This thesis investigates the phenomenon of Fe-regulated metal requirements in diatoms, employing a combination of physiological and biochemical techniques in the laboratory coupled with an investigation of natural phytoplankton communities in the field.

Biogeochemical cycling of Fe in the ocean.

The supply of Fe to the surface ocean occurs by aeolian deposits (with dust, Duce and Tindale 1991), through import of Fe from below the photic zone via upwelling or eddy diffusion (Coale et al. 1996a), and, in coastal areas, through riverine inputs of weathered terrestrial material and from the suspension of continental shelf sediments. These latter sources, plus their close proximity to dust sources, mean that coastal areas are rarely limited by Fe (although exceptions exist: Hutchins and Bruland 1998). Conversely, large areas of the open ocean receive very little input of Fe, and dissolved Fe concentrations can reach as low as 70 pM in surface waters, which severely limits the growth and reproduction of the native phytoplankton (Martin et al. 1989, Coale et al. 1996b, Price and Morel 1998).

The biogeochemistry of Fe in the ocean is controlled by its insolubility in oxygenated seawater and by biological uptake. Dissolved Fe is lower in surface than in deep waters because of biological uptake in the photic zone and the subsequent remineralization of the settling organic matter in deeper water (Price and Morel 1998). Iron's vertical profile is similar to that of other phytoplankton nutrients such as P, N or Si (Libes 1995). These nutrinets are largely remineralized below the photic zone and returned by mixing to the upper ocean layers. However, Fe(III), the dominant oxidation state of Fe in seawater, is highly insoluble and particle reactive. Dissolved Fe is quickly scavenged onto particles that eventually sink to the sediments. Consequently, macronutrients build up in the photic zone, as Fe-limitation prevents biological activity from removing these nutrients from the upper water column. Fe-limited areas such as those found in the Southern Ocean (Boyd et al. 2000), the equatorial Pacific (Coale et al. 1996) or the North-East (NE) Pacific gyre (Martin and Fitzwater 1988, Tsuda et al. 2003) are consequently termed High Nutrient, Low Chlorophyll (HNLC) waters.

With so little input of new Fe into the system, production in HNLC waters is highly dependent on recycled Fe. The largest pool of Fe in surface waters resides in the particulate phase (Price and Morel 1998), which is made up of biota and detritus. The regeneration of Fe from these particulate pools by grazing (Hutchins et al. 1993, Maranger et al. 1998) or viral lysis (Poorvin et al. 2004) supplies the majority of Fe to primary producers. Of the small quantities of Fe in the dissolved phase, over 99.5% is bound to organic ligands (Fe-L, Rue and Bruland 1995). These organic-Fe complexes prevent dissolved Fe from being scavenged out of the photic zone and may also reduce Fe-availability to the phytoplankton community. The relative bio-availabilities of Fe, Mn and Cu determines their influence on primary producers in the open ocean.

The biogeochemistry of Mn & Cu

Manganese and Cu are supplied to the surface oceans by the same mechanisms as Fe (rivers, aerosol deposition and upwelling), keeping their absolute concentrations low in the open ocean. However, relative to Fe, their concentrations are substantially higher. The key reason for this difference appears to be the relative solubilities of the ionic species in seawater, although several differences in their biogeochemical cycles are important to highlight here.

Unlike any other nutritive metal, Mn has a pronounced surface maximum (Bender et al. 1977, Landing and Bruland 1980). Surface concentrations normally range from 0.5 to several nM in most areas of the open ocean (Morgan 2000) and similar concentrations are low enough to limit the growth rates of some diatom species (Sunda and Huntsman 1986). Manganese is kept in surface waters by light catalyzed reduction of Mn-oxides and, unlike Fe, the re-oxidation rate of this Mn(II) is slow, preventing the insoluble Mn(IV) species from sinking out of the mixed layer. Below the photic zone, the biogeochemistry of Mn changes dramatically. Manganese-oxidizing bacteria remove Mn from the dissolved phase (Tebo et al. 2004). The microbialy produced Mn-oxides represent the majority of particulate Mn in waters such as the Sargasso Sea (Sunda and Huntsman 1988). These sinking particles transport Mn to the deep sea floor. Phytoplankton require Mn for photochemical processes and one would expect that they exert some influence on the biogeochemical cycling of Mn. Indeed, there is some evidence that the cycling of Mn is not due to redox behaviour alone (Moffett 1997). Judging from the surface enrichments of Mn in most areas of the world's oceans, it appears as though Mn is present in adequate amounts to supply phytoplankton demand.

The biogeochemistry of Cu in the surface ocean shares some similarities with Fe. Copper is scavenged from surface and intermediate waters and its distribution can reveal nutrient-type behaviour (Bruland 1980) when surface concentrations fall to ~0.5 nM. Compared to Fe(III), however, Cu(II) is relatively soluble, does not readily precipitate and its scavenging from the surface ocean is relatively inefficient compared to Fe. Additionally, dissolved Cu is almost entirely bound to organic ligands (Coale and Bruland 1988). Concentrations of Cu similar to those found in the open ocean are toxic to cyanobacteria and they exude ligands to bind the metal possibly to prevent its accidental uptake (Moffett and Brand 1996). Indeed, the majority of

Cu/plankton investigations revolve around copper's toxicity, not its function as a nutrient. If, in response to low Fe, more Cu is required for biochemical functions, the Cu-ligand complexes may reduce its availability to eukaryotic phytoplankton.

Ecology of Fe-deficient waters

The biogeochemical patterns described above result in a pelagic ecosystem that is constrained by the availability of Fe. Native phytoplankton, mostly small picoplankton (< 2 μm), are efficiently grazed by zooplankton (Cullen 1991). Fast growing ciliates, heterotrophic dinoflagellates or other small zooplankton can easily adjust their grazing rates to crop down the indigenous phytoplankton, keeping total biomass low (Price et al. 1994, Landry et al. 1997). Input of new Fe into HNLC waters accompanying events such as dust deposition (Bishop et al. 2002), upwelling of waters overlying Fe-rich continental shelf margins (Hutchins and Bruland 1998) or experimental Fe fertilization (i.e. Coale et al. 1996) results in marked changes to the community of primary producers. Invariably the community shifts from picoplankton to large, fast growing eukaryotes, normally diatoms. The diatoms grow, unchecked by grazing, until the N, P or Si accumulated in the photic zone is exhausted. The termination of the diatom bloom may result in a massive sinking event (Bishop et al. 2002).

The "biological pump" of CO_2 from the air-sea interface to deep waters could be strengthened considerably if these diatom blooms sink out of the water column (Kumar et al. 1995). John Martin and his colleagues proposed that the addition of Fe to the ocean could strongly influence atmospheric CO_2. The "Fe hypothesis" posits that lower atmospheric CO_2 levels seen during glacial events are due to Fe-enhancement of the biological pump in areas such as the Southern Ocean or the NE Pacific (Martin 1990). However, *in situ* fertilization experiments (i.e. Boyd et al. 2003) have shown that only a very moderate proportion of the bloom (if any at all) sinks to depth.

Diatoms exert strong influence on the cycling of elements in the ocean and form the base of the most productive marine fisheries (Falkowski et al 1998, Smetecek 1999, Walsh 1981), so the factors that lead to their survival or successes in the ocean are of great interest to oceanographers and ecologists. Compared to the dominant picoplankton in HNLC waters, diatoms are comparatively large and at a competitive disadvantage to acquire adequate Fe to maintain fast growth (Chisholm 1992). Therefore these phytoplankton are under severe pressure to reduce their metal requirements in the open ocean (Morel and Price 2003)

Adaptation to low Fe environments

Evolution in low metal environments has selected for flexibility in the metal requirements for photoautotrophic growth. For example, diatoms appear to reduce their Zn requirements with Cd or Co when Zn limits growth. This work began with the observation of a Zn and Cd/Co growth interaction (Price and Morel 1990), followed by the discovery that addition of Cd and Co restored the activity of carbonic anhydrase (CA, an enzyme that participates in the concentration of carbon dioxide) in Zn-limited diatom cultures (Morel et al. 1994). The work culminated by showing that Cd-specific CA replaces Zn-CA (Lane and Morel 2000) and that this interaction occurs in natural populations of phytoplankton (Cullen et al. 1999). Zinc has not yet been shown to be important in phytoplankton ecology and we have only just begun to ascertain how phytoplankton adapt to low Fe.

The majority of Fe required for photoautotrophic growth is used as irreplaceable cofactors in the protein complexes engaged in photosynthesis (Raven 1990, Raven et al. 1999, Strzepek and Harrison 2004). It is the inhibition of the light reactions of photosynthesis that results in reduced ecosystem production in HNLC areas (Greene et al. 1994, Behrenfeld et al. 1996). The photosynthetic apparatus of phytoplankton in the open ocean is under strong selection pressures to reduce its requirements for Fe. One short-term physiological adaptation to low Fe is to replace ferredoxin (containing a 2Fe-2S cluster) with flavodoxin, a functional homologue containing flavin as a cofactor instead of Fe (Hutber et al. 1977, LaRoche et al. 1995). However this replacement is not adequate to explain why diatoms from low-metal waters (termed "oceanic" strains throughout this thesis) have Fe-requirements nearly an order of magnitude lower than diatoms from relatively metal replete waters ("coastal" strains; Ryther and Kramer 1961, Sunda et al. 1991). Instead, oceanic diatoms have constitutively reduced the cellular concentrations of the Fe-rich components of photosynthesis, namely cytochrome b_6/f (6 Fe per complex) and photosystem I (12 Fe per complex), compared to coastal strains (Strzepek 2003, Strzepek and Harrison 2004). In diatoms, transport of electrons between cytochrome b_6/f and photosystem I is believed to be catalyzed by cytochrome c_6 (containing 1 Fe per complex, Raven et al. 1999). Plastocyanin replaces this protein in green algae, cyanobacteria and higher plants but is believed to be absent from diatoms (Sandmann et al. 1983, Raven et al. 1999). It may represent an important mechanism to reduce Fe-requirements. Iron is also required to activate a series of redox enzymes involved in respiration, nitrate assimilation, N_2 fixation, and for the detoxification of toxic oxygen radicals (Raven et al. 1999, Geider and LaRoche 1994).

In addition to reducing Fe-requirements diatoms also change their Fe-acquisition strategies. To gain access to the Fe-L ligand complexes that dominate the dissolved Fe pool in the ocean, diatoms extracellularly reduce Fe(III) to Fe(II) (Maldonado and Price 2001). In yeast (Stearman et al. 1996) and green algae (Herbik et al. 2004) the Fe(II) is re-oxidized to Fe(III) preceding transport by a Cu-containing oxidase. Employing alternate metals to either replace or acquire Fe may increase the fitness of natural diatom communities, if adequate resource is available.

Thesis overview

This thesis sets out to address several novel questions: Do marine diatoms change their requirements for Mn and Cu when Fe-limits growth? If so, what enzymes are using the additional Mn and Cu and does their absence impair the acclimation to low Fe? Are these same strategies employed by diatoms in HNLC regions of the sea?

Chapters 1 and 2 examine the use of Mn in the antioxidant pathways of the marine diatom *Thalassiosira pseudonana*, specifically within the enzyme superoxide dismutase. I show that this species increases its Mn-requirement in response to the increased oxidative stress associated with Fe-limited growth. Copper is involved in Fe-acquisition by diatoms isolated from high- and low-Fe environments and stimulates the growth of natural populations of phytoplankton in the Fe-limited Bering Sea (Chapter 3). Chapter 4 investigates the possibility that oceanic diatoms possess the Cu-containing redox protein plastocyanin. Each of these chapters establishes that the accumulation of Mn and Cu is essential for maintaining fitness in an Fe-limited environment.

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Chapter 1. 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 co-limit *Thalassiosira pseudonana* and *T. oceanica* so that maximum rates of cell division are achieved only when both resources are added simultaneously to cultures. Co-limited diatoms amended with either Fe or Mn alone show unique physiological responses, implying that the observed interaction between Fe and Mn is not due to substitution of one metal for the other. Iron deficiency increases the Mn quota of *T. pseudonana* by 3-times compared to 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 anti-oxidant enzyme, superoxide dismutase (SOD). The Mn content of the SODs increases by 1.8- to 2.8-times when Fe is limiting, suggesting that the SODs are Mn-containing 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.

Introduction

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 Fe dependent (Raven 1990). When Fe supply is insufficient to meet cellular demand, 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 growth rate slows.

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 rely on other metals and enzymes for their survival (Archibald 1983, Posey and Gherardini 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 to Fe in surface waters of the sea (Morgan 2000, Price and Morel 1998), 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 involved in C acquisition by phytoplankton. The concentrations of ρCO_2 and Zn influence the levels of the different carbonic anhydrases and their metal co-factors, 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. They show that Fe-limited diatoms produce greater amounts of reactive oxygen species and need more Mn to activate the anti-oxidant enzyme, superoxide dismutase. 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 reactive oxygen species. Field data obtained from published reports show that particulate matter is enriched in Mn in Fe-deficient compared to Fe-sufficient waters, suggesting that the Mn/Fe interaction observed here may occur in some parts of the sea.

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. Culture volume varied from 23 ml to 2 l according to the experiment. $MnCl_2$ and $FeCl_3$ were of the highest purity (ANALAR grade, BDH) as reagent grade $FeCl_3$ (Aldrich, BDH) contained trace amounts of Mn that masked the interaction reported here. The $FeCl_3$ used in this 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 pM. Iron was added to seawater bound to EDTA (1:1.05) and Mn was added directly to media containing 100 μM EDTA and other trace metals as reported (Price et al. 1988/89). The concentrations of inorganic Mn (Mn': Mn^{2+}, $Mn^{2+}Cl$, Mn_2Cl_2) and Fe (Fe': $Fe(OH)_2^+$, $Fe(OH)_3$, $Fe(OH)_4^-$) ions were calculated using MINEQL and as described (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 pM Fe' / 14.24 nM Mn'), Mn-deficient (5.66 nM Fe'/ 0.59 nM Mn'), or Fe- and Mn-deficient (81.5 pM Fe'/ 0.59 nM Mn') conditions. Following acclimation, Fe-deficient cultures were transferred into media enriched with Mn (32.9 nM Mn'), or Fe (5.66 nM Fe'), or Fe plus Mn (5.66 nM Fe' / 32.9 nM Mn'). Control cultures were transferred into the original Fe-deficient medium. Mn-deficient cultures were transferred into media enriched with Mn (14.24 nM Mn'), or Fe (16.8 nM Fe'), or Fe plus Mn (16.8 nM Fe' /

14.24 nM Mn'). Control cultures were transferred into the original Mn-deficient medium. Iron- and Mn-deficient cultures were transferred into media enriched with Mn (14.24 nM Mn'), or Fe (5.66 nM Fe'), or Fe plus Mn (5.66 nM Fe' / 14.21 nM 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) as 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, semi-continuous batch cultures (Maldonado and Price 1996). Cell densities and volumes were determined by microscopy using a Palmer-Maloney chamber, assuming a cylindrical shape (Hillebrand et al. 1999). Phytoplankton were harvested by gentle filtration (<10 mm Hg) onto pre-combusted GF/F filters for carbon analysis. Carbon content was measured on a Carlo Erba EA 1108. Metal quotas (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

Mid-exponential phase cultures of *T. pseudonana* were gently filtered onto acid-cleaned 2 μm, 25 mm diameter polycarbonate filters. The cells were resuspended in AQUIL containing no trace metals or EDTA. The suspension was then aliquoted into media identical to that used for growth but containing

1.42 nM ^{54}Mn' (carrier free) with either 0 nM Fe', 0.0815 nM Fe', or 5.66 nM 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 non-specific binding of Mn. Cell densities and dimensions were measured as described above and uptakes were normalized to cell surface area.

Superoxide dismutase (SOD)

Mid-exponential 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 no longer than 3 weeks before analysis. Cells were resuspended in 0.05 M phosphate buffer, pH 7.8 with 0.1 mM 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 16000 *g*. Protein concentrations of crude homogenates ranged from 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 μM cytochrome *c* by a xanthine/xanthine oxidase (XOD) couple (50 μM xanthine, 0.2 units XOD in 3 ml).

To quantify the relative change in Mn-content of SOD as a function of Fe nutritional state, 2 l cultures were grown under Fe-deficient (81.5 pM Fe') and Fe-sufficient (5.66 nM Fe') conditions with 14.24 nM 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 Fridovich (1971). The gel was soaked in a freshly-made solution of 0.028 mM riboflavin, 25 mM nitroblue tetrazolium and 0.028 M TEMED in 36 mM 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 coloured 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 prior to drying. The relative amount of ^{54}Mn-content associated with SODs was estimated from densitometry of digital images of either x-ray film or phosphorimager output.

Reactive oxygen species

Intracellular reactive oxygen species were detected by the method of Maxwell et al. (1999) using dichlororfluorescein diacetate (H_2DCF-DA; Sigma). H_2DCF-DA was added to a final concentration of 5 μM 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 photo-generated reactive oxygen species. Cells were collected at 15 000 *g* for 3 min and the supernatant diluted 4 –fold and measured in a spectrofluorometer with excitation and emission wavelengths of 488 nm and 520 nm, respectively. Values are reported as relative fluorescent units (RFU) 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 pM Fe'), high Mn (14.24 nM Mn') medium. The fully acclimated culture that was used as the inoculum for these experiments was Fe-deficient (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). Addition of Fe with or without Mn significantly stimulated growth as expected (μ = 1.5 d^{-1}, Figure 1.1A). Doubling the amount of Mn in the medium to 32.9 nM Mn' had no effect on growth rate (1.1 and 1.2 d^{-1}, duplicate measurements) like 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 Fe or Mn addition. These low Mn cultures were acclimated in medium that contained high concentrations of Fe' (5.66 nM). 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 (Figure 1.1B). The addition of Fe also slightly reduced F_v/F_m (Figure 1.1B) suggesting that the 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 increases in fluorescence we observed represented increases in cell density and not just changes in chlorophyll fluorescence (control = 6514 cells ml^{-1}, +Fe = 4475 cells ml^{-1}, +Mn = 27418 cells ml^{-1}, +Fe and Mn = 25154 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 conservative estimates (see below). Interestingly, the fully acclimated Mn-deficient culture had a low F_v/F_m of approximately 0.4 (Figure 1.1B). Increasing the concentration of Mn, with or without Fe, increased F_v/F_m and 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 following metal additions allowed us to differentiate between Mn and Fe limitation and to document changes in cellular physiology.

When *T. pseudonana* was grown in seawater containing low [Fe] and low [Mn], addition of either nutrient alone increased growth rate significantly, but only addition of both nutrients together allowed for full recovery of growth (Figure 1.1C). The Fe- plus Mn-amended cultures grew fastest at 1.37 and 1.46 d^{-1}, followed by the cultures that received Mn addition (1.12 and 1.16 d^{-1}), Fe addition (0.84 and 0.91 d^{-1}) and no addition (0.54 and 0.6 d^{-1}, calculated from days 2-4, 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 observed in the single resource limitation experiments, only Mn-deficiency compromised PSII function and subsequent addition of Mn promoted its recovery. This result further demonstrated that addition of Fe did not introduce significant levels of contaminating Mn (as 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 Fe (or Mn) availability.

Steady state growth and metal content

The Fe-Mn interaction reported in the transient experiments was also observed in fully acclimated, semi-continuous batch cultures of *T. pseudonana* where Fe and Mn were added at low, limiting concentrations (Table 1.1). Steady state growth rates were significantly slower in Fe- or Mn-deficient media 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 nM, for example, Fe-sufficient cultures grew well (Table 1.1), while Fe-deficient cultures were unable to survive (n=5). Increasing the [Mn'] slightly to 0.59 nM in the low Fe medium, increased the steady state growth rate from 0 to 0.84 d^{-1}. Thus, 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 1.2).

Under single resource limitation the quotas of the limiting metals were greatly reduced (Table 1.1). 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 nM (both results, one way ANOVA, Tukey test, $p < 0.05$). At 14.24 nM Mn', Fe-deficient cells contained 1.3 times the concentration of Mn compared to Fe-sufficient cells, while at 1.42 nM Mn' Q_{Mn} tripled from 21.5 to 78.6 μM. Growth at low concentrations of Mn and Fe together caused a large increase in cell volume. This increase, compared to Fe-deficient cells (0.0815 nM Fe', 14.24 nM Mn'), greatly reduced the Q_{Fe} of Mn- and Fe-deficient cultures, as Fe per cell remained statistically unchanged in the two treatments.

Short-term ^{54}Mn uptake

Uptake of ^{54}Mn at concentrations growth limiting to *T. pseudonana* were not inhibited by the presence of Fe in the medium (Table 1.3). The short term rate of transport of cells incubated with 5.66 nM Fe and 1.42 nM Mn was identical to the steady state rate (27 compared to 30 nmol Mn m^{-2} d^{-1}). Iron-deficient cells 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 co-limit, and *T. weissflogii* because its Q_{Mn} was reported to increase when Fe-deficient (Harrison and Morel 1986). Iron deficiency increased SOD activity in both species by 1.4 and 1.6 times, respectively (Table 1.4; *t*-test, $p <$

0.05). In *T. weissflogii*, the increased SOD activity coincided with an increase in reactive oxygen species (ROS) generation, suggesting that the cells experienced greater oxidative stress under low compared to 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 Fe-containing by their characteristic inhibition by H_2O_2, but not by KCN. When non-denaturing PAGE gels were soaked in 4.15 mM H_2O_2 prior to staining, SOD activity was reduced by 70% (data not shown). Exposure of the gel to 3 mM 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 to Fe-replete cells. These results agreed well with the relative changes in SOD activity measured independently in whole cell extracts (Table 1.4).

Autoradiograms showed that virtually all of the protein-bound ^{54}Mn solubilized by our methods co-migrated with the SODs (Figure 1.2). 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 *Thalassiosira weissflogii* and *T. pseudonana* increased in Fe-limited cells by 2.8 and 1.8 times respectively, compared to 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 up-regulated.

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 *Thalassiosira 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 co-limitation 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 (see Silva and Williams 2001 for discussion of Mn-requiring enzymes) we limit our discussion to those biochemical pathways 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

Thalassiosira pseudonana exhibited a distinctive reduction in steady state growth rate, Q_{Fe}, and cell size in low Fe medium as expected (Table 1.1) (Sunda and Huntsman 1995, Maldonado and Price 1996). Examination of the growth data reported in Table 1.1 and 1.2 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 nM Mn') 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

nM Mn') (Table 1.1). At higher concentrations of Mn (14.2 nM Mn'), 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} (M cell-volume)) for diatoms growing at 1.42 nM Mn' was more than 2-fold faster when Fe was low than high. However, short term Mn transport rates were slightly slower for Fe-deficient than for Fe-sufficient cells (Table 1.3). Harrison and Morel (1986) made similar observations for the centric diatom *T. weissflogii.* They postulated that Fe interfered with Mn transport which resulted in the 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 Mn-uptake during short-term experiments (Table 1.3) and instead suggest that steady state Q_{Mn} increases during Fe-deficient growth because growth rate declines.

Steady-state Fe-deficiency had no effect on the maximum quantum yield of PSII in *T. pseudonana* (Figure 1.1A). F_v/F_m measured by the DCMU method was near the maximum theoretical value of 0.65 and not substantially different from what we measured in nutrient-replete cultures. This result was surprising, because low Fe is well known to affect PSII efficiency (Greene et al. 1992, *c.f.* McKay et al. 1997). The slow growth rate of the culture 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 (unpublished) 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, as few studies have been conducted under the latter conditions.

Reactive oxygen species are a byproduct of normal cellular metabolism and 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. Since 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 were apparently accepted by O_2. We cannot without a doubt assume that the electrons originated from water splitting, as 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 we used for ROS detection was a non-specific 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 Fe-containing 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 (Figure 1.2). 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-deficiency

Lowering Mn' in the medium reduced the steady state growth rate, Q_{Mn}, and cell volume of *T. pseudonana* (Table 1.1), 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 to Mn-sufficient cells (Table 1.1, 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). Manganese-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 1.1 and 1.2).

When the ratios of dissolved [Mn]:[Cu] or [Mn]:[Zn] are low in cultures of phytoplankton, Cu and Zn can out compete 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 2 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) (Figure 1.1B). 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 MnSODs are present in *T. pseudonana* (F. Wolfe, personal communication). Thus, the observed reduction in steady state growth rate under Mn deficiency can be explained by a decrease in water-splitting activity and supply of electrons to the reaction center of PSII. MnSOD 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, 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 to controls. It is unlikely that the added Fe inhibited Mn uptake judging from the results of the short-term experiments (Table 1.3). We cannot unequivocally explain the decrease on F_v/F_m, but 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

Assuming 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 co-limitation 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. Manganese-containing SOD cannot be synthesized in sufficient quantities to detoxify superoxide, one of the ROS produced, resulting in oxidative stress. Addition of Mn-alone increases the amount of electrons entering photosynthesis (judging from the recovery in F_v/F_m, Figure 1.1C) and decreases the levels of ROS (by activating Mn-SOD) caused by an inefficient, Fe-limited electron transport chain. Addition of Fe alone improves electron transfer efficiency, reducing the proportion of electrons lost to ROS production and providing more energy for growth. PSII function remains compromised (low F_v/F_m, Figure 1.1C), so the phytoplankton are unable to sufficiently liberate electrons from water for use in the electron transport chain and their MnSODs are inhibited. Only addition of both metals together allows for full recovery of photosynthesis and detoxification of superoxide, allowing maximal growth.

An alternative 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. 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 inter-replacement of the photosynthetic electron carrier proteins plastocyanin (Cu-containing) with cytochrome c_6 (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 co-limited transient experiment, however, appear to be inconsistent with this hypothesis because they show unique physiological responses of *T. pseudonana* to Fe and Mn additions. The observation suggests that Fe and Mn are not directly substitutable resources. 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 maxima 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, phytoplankton uptake of Mn(II) is thought to be the primary mechanism at play (Moffett 1997). 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 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 to total particulate Mn. Surface waters of the equatorial Pacific Ocean contain roughly 0.4 μg Chl *a* l^{-1} (Lindley and Barber 1998; Moffett 1997) corresponding 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 pM Mn compared to 20 pM Mn in all particulate matter (Gordon et al. 1998). The same exercise conducted using data from the Sargasso Sea shows that phytoplankton contain roughly 1% of the total particulate Mn pool (130 pM Mn, 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 (Figure 1.3). 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 80 pM (Martin et al. 1990). Steady state cultures of the oceanic diatom *T. oceanica* are growth limited at this [Mn'] and become severely growth limited when Fe is lowered concomitantly (Table 1.2). 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 co-limited cells. On a cellular basis, however, the Mn quota actually doubles in the co-limited compared to the Mn-limited cultures and the steady state transport rate is 30% faster. Thus, the co-limited 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 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 Fe-limited phytoplankton may indeed be relatively Mn-enriched.

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 HNLC regions obtained such a response.

Coale (1991), for example, observed an increase in diatom biomass after the addition of nM 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 Mn-addition on biomass or Fe accumulation 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 co-limitation of growth of coastal and oceanic diatoms. Only addition of both nutrients together allows for a full physiological recovery from nutrient limitation. The observed interaction and the biochemical manifestations documented in this paper 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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Table 1.1 Growth rates, cell volumes and elemental quotas of *Thalassiosira pseudonana* grown under steady state conditions in Fe and Mn-replete and -deplete seawater (± 1 standard deviation, n=4 or n=3*) na: not measured

[Fe'] (nM)	[Mn'] (nM)	Growth rate (d^{-1})	Cell volume (fl)	Cellular [C] (M)	Cellular [Mn] (µM)	Cellular [Fe] (µM)
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

Table 1.2. Steady state growth rates (d^{-1}) of *Thalassiosira oceanica* (clone 1005) conditioned with different ambient Fe' and Mn' concentrations (± 1 standard deviation, n=3).

[Fe'] (nM)	[Mn'] (nM)	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

Table 1.3. 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 containing 1.42 nM Mn' in the presence of either 0, 0.0815 or 5.66 nM Fe (± 1 standard deviation, n=3).

[Fe'] (nM)	[Mn'] (nM)	ρ^{ST} (nmol Mn m^{-2} d^{-1})		
		0 nM Fe'	0.0815 nM Fe'	5.66 nM 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 1.4. 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 standard deviation, n=4.

Species	[Fe'] (nM)	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	

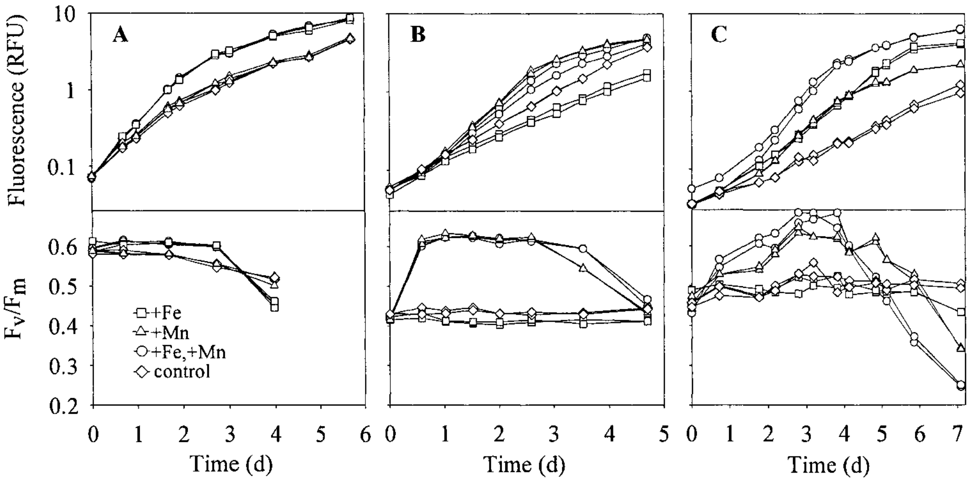

Figure 1.1. Growth and photosynthetic efficiency of *Thalassiosira pseudonana* during transient metal enrichment. *T. pseudonana* was acclimated to different metal conditions and inoculated into media containing additional Mn, additional Fe, additional Fe and Mn and medium identical to that used in acclimation (see 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 pM Fe'/ 14.24 nM Mn'). (B) *T. pseudonana* acclimated to Mn-deficient conditions (5.66 nM Fe'/ 0.59 nM Mn'). (C) *T. pseudonana* acclimated to Fe- and Mn-deficient conditions (81.5 pM Fe'/ 0.59 nM Mn').

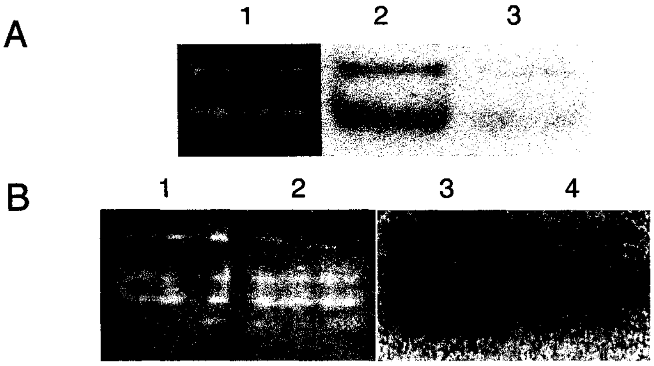

Figure 1.2. Enzymatic activity and ^{54}Mn autoradiograms of superoxide dismutases (SODs) as resolved on native protein gels. A) *T. weissflogii* Lane1: SOD activity (Fe-sufficient). Lane 2: ^{54}Mn (Fe-deficient). Lane 3: ^{54}Mn (Fe-sufficient) . B) *T. pseudonana* Lane 1: SOD activity (Fe-deficient). 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.

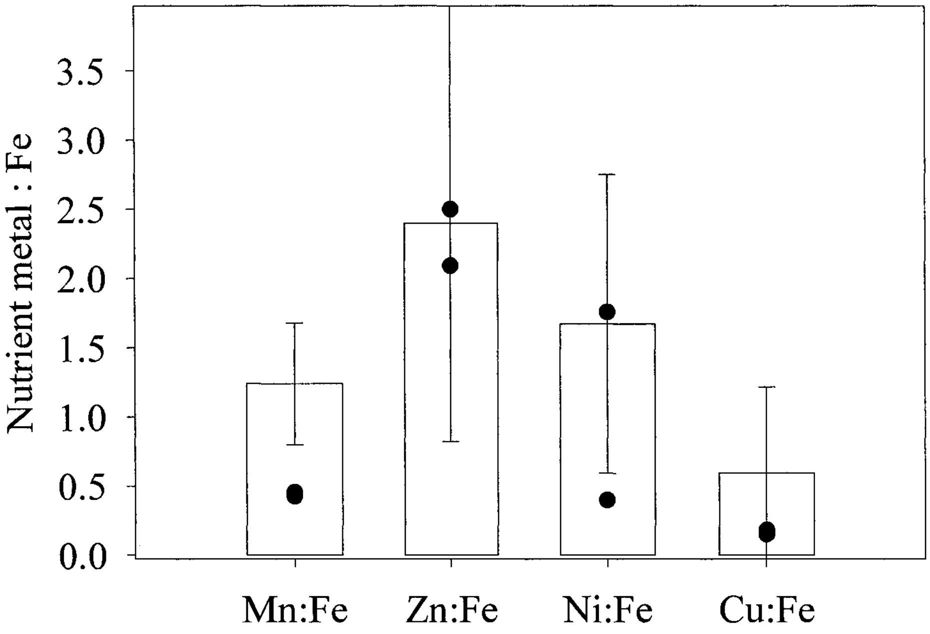

Figure 1.3. Ratios of nutrient metals to Fe in acetic acid leachable particulate material from waters around the Galapagos Islands (PLUMEX). Metal concentrations measured in samples collected at 20 m were taken from Gordon et al. (1998). Stations were categorized as Fe-deficient (white bars, error = 1 standard deviation, n=4) or Fe-sufficient (circles, n=2) according to phytoplankton photosynthetic performance (Lindley and Barber 1998).

Chapter 2. Iron and manganese limitation of the marine diatom *Thalassiosira pseudonana* induces chronic oxidative stress.

This chapter tests the hypothesis that Fe-deficiency induces oxidative stress in diatoms. It also explores the physiological effects of Mn-deficiency on photosynthesis and antioxidant defences and unifies the two effects in a single model of co-limitation.

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Abstract

In diatoms, the metabolic reactions that generate reactive oxygen species (ROS) and the enzymes that detoxify them are Fe and Mn dependent. We examined how Fe- and Mn-deficiency affected several oxidative stress parameters in *Thalassiosira pseudonana*. Superoxide dismutase activity (SOD) increased and the activity of Fe-containing ascorbate peroxidase (APX) decreased when Fe limited growth of this species. Under these conditions the rate of light-induced ROS production normalized to oxygen evolution was elevated, suggesting that a greater proportion of electrons liberated from water were donated to molecular oxygen forming ROS. The intracellular concentrations of oxidative damage products were also two times higher than those of metal replete cells. During Mn-deficient growth, SOD activities of *T. pseudonana* declined by 60% compared to those of metal-replete cells. Despite this reduction, ROS generation remained similar to that observed in the controls, but oxidative damage was higher. Photosystem II (PSII) fluorescence transients showed donor side inhibition of PSII, most likely because of insufficient Mn for water splitting and damage to PSII. In cells co-limited by Fe and Mn, SOD activities were equal to those of Mn-deficient cultures, ROS generation was the highest and damage products were elevated. PSII fluorescence transients were similar to those measured in cells limited solely by Mn. We conclude that co-limitation of diatom growth by Fe and Mn causes inhibition of the light reactions of photosynthesis and increases oxidative stress.

Introduction

Aerobic photosynthetic and respiratory pathways continually generate unstable reactive oxygen species (ROS) either by the direct transfer of energy or by the successive reduction of O_2 (Møller 2001, Foyer and Noctor 2000). The ROS thus formed include singlet oxygen, superoxide, hydrogen peroxide and the hydroxyl radical and are among the most damaging of cellular metabolic by-products. A suite of metal and metalloid cofactors are required to activate enzymes involved in the metabolism of these oxygen species (Raven et al. 1999). Some of the same elements also participate in the enzymes and pathways that generate ROS, such as the electron transport chain of photosynthesis. Physiological conditions that upset the equilibrium between production and detoxification of oxygen radicals result in accumulation of ROS and increase the probability of damage to cellular macromolecules (Foyer 1997). These conditions, collectively referred to as oxidative stress, occur during exposure to any number of acute or chronic environmental insults, including resource limitation.

One of the most severe constraints on photoautotrophic growth in the open ocean is a lack of elemental resources (Morel and Price 2004). Low concentrations of Fe, for example, are well documented to inhibit the photophysiology and growth of natural phytoplankton communities. The photosynthetic apparatus of these plankton recuperates following the enrichment of the water with nanomolar concentrations of Fe (Behrenfeld et al. 1996). This addition stimulates the growth of phytoplankton normally resulting in a bloom of large diatoms (Boyd et al. 2004). The role of other metals in structuring the phytoplankton community is less clear, although concentrations of Zn, Cu and Mn in the open ocean are low enough to limit diatom growth in culture (Sunda and Huntsman 1992, Peers et al. 2005 and Peers and Price 2004).

Iron- and Mn-containing proteins are employed by the light reactions of photosynthesis to assist in the light catalyzed transfer of electrons from water to $NADP^+$ or ferredoxin. Indeed, the majority of Mn and Fe required for autotrophic growth is contained in these proteins (Raven et al. 1999, Strzepek and Harrison 2004). Inhibition of the light reactions by either metal may lead to increased transfer of excitation energy to molecular oxygen or other unintended targets. Iron-starvation, for example, has been shown to disrupt the flow of electrons through photosynthesis and to result in damage to the reaction center proteins of photosystem II (PSII, Greene et al. 1992, Ivanov et al. 2000). Inactivation of any component of the photosynthetic electron transport chain would cause an accumulation of reducing equivalents that could lead to the formation of ROS. Much less is known about the effects of Mn-deficiency on the photophysiology of diatoms. Manganese is engaged by the oxygen evolving complex of PSII to remove electrons from water molecules (Raven and Falkowski 1997, Vrettos and Brudvig 2002). An anticipated outcome of Mn-deficiency is a decrease in electron flow through PSII, possibly increasing the probability of energy transfer to molecular oxygen or donor side inhibition of PSII (Aro et al. 1993).

In algal chloroplasts, ROS are either reduced by metallo-enzymes or scavenged by small organic molecules (Niyogi 1999). The breakdown of superoxide (O_2^-) is mediated by superoxide dismutase (SOD) containing either Fe or Mn in diatoms (Asada et al 1977, Wolfe-Simon et al. 2005) and its product, hydrogen peroxide (H_2O_2), is detoxified by ascorbate peroxidase (APX) in the chloroplast. APX is Fe-containing and employs ascorbate as a reactant (Asada 2000). Antioxidant enzymes also serve to detoxify ROS produced throughout the cell, although Fe-containing catalase replaces APX in other organelles and in the cytoplasm. The inhibition or genetic deletion of these enzymes leads to oxidative damage to photosynthetic organisms (i.e. Herbert et al. 1992, Rizhsky et al. 2002).

Because Fe and Mn are a source and sink for ROS, their low concentrations in the environment could have consequences for oxidative stress. Previously we observed that Fe-deficient cultures of *Thalassiosira pseudonana* accumulated Mn and that part of it was used to activate SOD (Peers and Price 2004). The increased Mn-requirement was thus hypothesized to result from elevated oxidative stress as ROS per cell was also increased under Fe-deficiency, despite upregulation of SOD. Here we investigate the oxidative stress response of *T. pseudonana* to low Fe and Mn by measuring changes in antioxidant enzyme activities, ROS generation and lipid peroxide concentration, an indicator of oxidative damage. Limitation by either metal appears to induce oxidative stress, but it remains unclear how this oxidative stress contributes to the overall growth reduction incurred by metal-deficiency.

Methods

Cultures and harvesting

Cultures of *Thalassiosira pseudonana* (clone 3H, CCMP strain 1335) were raised in edetic acid (EDTA) buffered synthetic seawater media (Price et al 1988/89). Cells cultured in the presence of either 1 μM Fe and 121 nM Mn (all values are for total metal concentrations), 1 μM Fe and 5 nM Mn, 40.8 nM Fe and 121 nM Mn or 40.8 nM Fe and 5 nM Mn resulted in metal replete, Mn-deficient, Fe-deficient or co-limited conditions, respectively. Details of metal concentrations and media composition may also be found in Peers and Price (2004). Cultures were harvested during mid-exponential growth phase onto 45 mm diameter, 3 μm pore size polycarbonate filters. Cells were immediately scraped off the filters and frozen in liquid nitrogen until further analyses.

SOD activity

Diatom cells were prepared for SOD activity measurements as described by Peers and Price (2004). Enzyme activities were measured in crude homogenates according to the methods of McCord and Fridovich (1969), as described by Flohe and Otting (1984). In this assay, one unit of SOD activity represents a 50% reduction in the oxidation rate of 10 μM cytochrome *c* by a xanthine/xanthine oxidase (XOD) couple (50 μM xanthine, 0.2 units XOD in 3 ml). Changes in A_{550} over time, corresponding to the progressive reduction of cytochrome *c* by superoxide, were observed in temperature controlled Cary 1E spectrophotometer (20°C). Total soluble protein was measured using the Bradford Assay (Sigma) with bovine serum albumin as a standard.

APX activity

Frozen cells were resuspended in APX buffer containing 50 mM KPO_4 buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM Na ascorbate and 0.05% Triton X-100 (v/v, all chemicals supplied by Sigma unless specified otherwise). Cells were disrupted by sonication and centrifuged as described in the SOD assay.

Homogenates were stored on ice for no longer than one h before analyses: no loss of enzyme activity was observed during this time (data not shown). APX activity was measured according to the methods of Rijestenbil (2002). Protein homogenate (100 μl) was added to 800 μl of APX buffer in a quartz cuvette. The reaction was started by the addition of 100 μl of 2 mM H_2O_2. Cuvettes were inverted 3 times and the consumption of ascorbate was monitored for 1 min at 290 nm in a thermostated spectrophotometer at 20°C. Ascorbate consumed per unit time was calculated using ε_{290}=2.88 $mM^{-1}cm^{-1}$. Total protein was measured as described for SOD. One unit of APX is equal to 1 μmol of ascorbate consumed min^{-1}.

Lipid peroxidation

Cells were harvested for analysis as described above. Cells were resuspended in 2.5 ml 80:20 (v/v) ethanol:water mixture containing 0.01% (w/v) butylated hydroxytoluene to prevent further lipid oxidation. Cells were disrupted by sonication using a Branson Sonifier equipped with a microtip for 2 x 15 s bursts at full power and homogenates were kept on dry-ice before and between cycles. One ml of the resulting solution was added to 1 ml of 20% trichloroacetic acid (w/v) either alone or containing 0.65% (w/v) thiobarbituric acid (TBA). These solutions were then placed in a 95°C water bath for 25 min, removed and cooled to room temperature before centrifugation at 140000 *g* for 10 min. The absorbance of the clarified sample was measured in a Cary 1E spectrophotometer at 440, 532 and 600nm. Malionaldehyde equivalents were determined as per the equations derived by Hodges et al. (1999) and reported as thiobarbituric reactive substances (TBARS). These values were normalized to the total cell volume of the sample (determined as described in Peers and Price 2004).

ROS generation

Reactive oxygen species generation was estimated using H_2DCF-DA (Maxwell et al. 1999) by following the oxidative cleavage of H_2DCF to its fluorescent product, DCF. Two ml of *T. pseudonana* cultures growing in mid-exponential phase were incubated with 5 μM H_2DCF-DA in the dark, at 20°C for 20 min. Cells were then centrifuged at 15000 x *g* for 3 min and then resuspended in fresh media. Labelled cells were then either incubated in the light (equal to culturing conditions, 180 μmol quanta m^{-2} s^{-1}) or dark at 20°C for 15 min. Finally, cells were centrifuged for 3 min at 15000 x *g* to end the experiment. The supernatant was diluted 8-fold and DCF formation was measured using 488 nm excitation and 520 nm emission wavelengths in a Shimadzu (RF-5301 PC) spectrofluorometer. Fresh media yielded small amounts of fluorescent product, so all assays were accompanied by a blank treatment containing no cells. Fluorescence from the dark treatment was subtracted from the light treatment to yield a measurement of light generated ROS. Generation of fluorescent product over time was normalized to the rate of oxygen evolution in the light. Net oxygen evolution rates were measured with a calibrated Clark-type electrode (Hansatec) supplied with light equal to that used for growth for 15 min. Cells used for oxygen measurements were loaded with H_2DCF-DA as described above to control for any physiological changes due to handling. Oxygen evolution rates were corrected for respiratory oxygen consumption as measured during a 10 min exposure to darkness.

Fluorescence transient analysis

The fluorescent transient of PSII was measured using a Hansatec Plant Efficiency Analyzer (PEA). Mid-exponential growth phase cultures were dark acclimated for at least 15 min prior to filtering 30-60 ml of culture on Millipore AP glass fibre filters (10 mm diameter). This filter was immediately placed into a leaf-disc adaptor supplied by the manufacturer and cells were exposed to a saturating pulse of red light (650 nm peak) at an intensity of 3000 μmol quanta

m^{-2} s^{-1} light for 1or 10 s. Fluorescence readings are reported as the raw fluorescence (F) normalized to the maximal signal (F_m).

Pulse-amplitude modulated (PAM) fluorometry

Thirty ml of *T. pseudonana* cultures were harvested onto 25 mm diameter, 3 μm pore size polycarbonate filters and immediately resuspended into 3 ml of fresh media. Cells were dark acclimated at 20°C for at least 10 min prior to measurement. The maximum quantum efficiency of PSII (F_v/F_m) was measured in dark acclimated cells using a saturating pulse of 3000 μmol quanta m^{-2} s^{-1} supplied for 600 ms. The realized efficiency of PSII (Φ_{PSII}) was measured by supplying the same pulse of light to cells acclimated to an actinic light intensity of 150 μmol quanta m^{-2} s^{-1}. Both measurements were made using a Walz Water PAM (Walz instruments, Germany) and calculations were performed according the methods of Juneau et al. (2002).

Results

Fe-deficiency

Thalassiosira pseudonana grown in media containing 40.8 nM Fe was mildly Fe-deficient judging from its reduced growth rate compared to metal replete conditions (Table 2.1). The two antioxidant enzymes measured in this study responded in opposite ways to Fe-deficiency compared to the controls (Table 2.1). Superoxide dismutase activities, normalized to cellular protein, increased under Fe-limitation while APX activities were reduced by nearly 2.7 times. ROS generation rates normalized to photosynthetic oxygen evolution also increased during steady state Fe-deficiency compared to controls (Figure 2.1). Duplicate cultures yielded similar results. Cellular oxidative damage was estimated by measuring the total cellular concentration of thiobarbituric acid reactive substances (TBARS). Thiobarbituric acid reacts with malionaldehyde, a product of lipid peroxidation, to form a coloured complex and its assay is a common indicator of oxidative damage (Esterbauer and Cheeseman 1990). Cellular concentrations of TBARS increased in Fe-deficient cultures (Figure 2.2). Measurement of PSII fluorescence transients showed that the time to reach maximal fluorescence doubled compared to metal replete conditions (Figure 2.3).

Mn-deficiency

Manganese-deficiency reduced growth rates and SOD activities, and inhibited PSII in *T. pseudonana*. Superoxide dismutase activities were reduced by a factor of 2 compared to metal replete growth (Table 2.1). Ascorbate peroxidase activities were not measured in Mn-deficient diatoms. ROS generation per ng O_2 produced was virtually identical to that measured in metal replete cultures (Figure 2.1), but oxidative damage products increased significantly (Figure 2.2). PSII fluorescence kinetics showed that Mn-deficiency increased the time to reach maximal fluorescence by an order of magnitude (80 ms for metal replete conditions compared to 900 ms for Mn-deficient cells,

Figure 2.3). Fluorometry also revealed changes in PSII. The maximum potential quantum yield of PSII (F_v/F_m) and the realized efficiency of PSII (Φ_{PSII}) were significantly reduced by Mn-deficiency compared to metal replete conditions (Table 2.2).

Co-limitation

Cultures co-limited by both Mn and Fe had the slowest growth rates of any treatments. Total SOD activity was not significantly different from Mn-deficient cultures ($p>0.05$). However, ROS generation per ng O_2 evolved was greater than any of the other treatments in this study. Cellular concentrations of damage products were statistically identical to those observed during Fe-limitation (*t*-test, $p>0.05$ with Bonferroni correction). Finally, fluorescent transients for co-limited diatom cultures appeared to be similar to those for Mn-limitation (Figure 2.3).

Discussion

The formation of reactive oxygen species during metabolism is an unavoidable consequence of aerobic life that exacts a significant cost for growth of biota. The over-expression of ROS scavenging enzymes, for example, can increase the longevity of organisms (Orr and Sohal 1994) while the genetic deletion of these same enzymes can prove fatal (Carlioz and Touati 1986).

The light reactions of photosynthesis generate powerful reducing molecules and high concentrations of O_2. Thus, the potential for ROS formation and oxidative damage is high in algae and plants. Indeed, the Mehler reaction, a series of univalent reductions of molecular oxygen that occurs in close proximity to photosystem I (PSI) produces large quantities of ROS. It is believed to consume excess reductant generated by the light reactions (Asada 2000) and to adjust the ratio of ATP produced per unit NADPH (Kramer et al. 2004). Under steady state conditions, the Mehler reaction can consume between 10–30% of the reductant generated by the photochemical reactions in higher plants (Asada 2000) and up to 60% in the diatom *Cylindrotheca fusiformis* (Claquin et al. 2004). Oxygen radicals are constantly being formed by other components of the photosynthetic system as well (see discussion below) and photosynthetic organisms require a host of antioxidant enzymes to detoxify these radicals. The basal levels of oxidant defence (Table 2.1) and ROS generation (Figure 2.1) are evident in metal-replete cultures of *T. pseudonana.*

The formation of ROS leads inexorably to damage inside the cell, despite the antioxidant pathways. Reactive oxygen species deactivate enzymes and oxidize macromolecules producing damage products such as peroxidated lipids and proteins (Berlett and Stadtmann1997, Figure 2.2). Aerobic organisms adjust their metabolism to balance damage with repair mechanisms resulting in an oxidative equilibrium (Foyer 1997). However when the antioxidant pathways are overwhelmed and repair mechanisms are inadequate, oxidative stress occurs.

Oxidative stress in algae occurs during a wide range of disturbances including high light (Butow et al. 1997, Niyogi 1999), metal toxicity (Rijestilbil et al. 1994) and UV exposure (Rijestilbil 2002). Judging from the changes in antioxidant enzyme activities (Table 2.1), the increases in ROS generation (Figure 2.1) and in oxidative damage products (Figure 2.2), chronic Fe-limitation also appears to induce oxidative stress in diatoms. Algae also employ a suite of organic molecules that can scavenge ROS (Niyogi 1999). While these compounds are undoubtedly important in combating oxidative stress, we chose to focus our study on metallo-enzymes that would be directly affected by metal limitation.

Antioxidant enzymes under Fe-deficiency

Chronic Fe-deficiency increases the activity of SOD in *T. pseudonana* (Table 2.1). Diatoms contain either Fe- or Mn-SOD (Asada et al. 1977, Armbrust 2004), so the increase in activity could be due to either an upregulation of one or the other of the isoforms or both. We have shown that SOD co-migrates with ^{54}Mn in native electrophoresis gels and that its content of ^{54}Mn increases with activity during Fe-limitation (Peers and Price 2004) so the latter scenario seems unlikely. The genome of *T. pseudonana* contains several gene sequences for Fe- and Mn-SOD (Armbrust et al. 2004). Careful analyses of these sequences resolve several putative genes that possess conserved sets of amino acids for the active site of SOD (Hunter et al. 1997). In *Escherichia coli*, the metal specificity of SOD isoforms can be traced to changes in just two amino acid residues (a Gln residue differs in location). These characteristic residues are present in the putative FeSOD and MnSOD sequences (Peers and Price unpublished). However, expressed sequences tag (EST) databases assembled from metal replete cultures of both *T. pseudonana* and the model pennate diatom *Phaeodactylum tricornutum* do not resolve any transcribed FeSOD genes (www.avesthagen.sznbowler.com). We have yet to find evidence of an active FeSOD in *T. pseudonana*. Additionally, purified SODs from a related species, *T. weissflogii*, appear to be activated by Mn and not Fe (Appendix 2), despite the fact that they are inhibited by hydrogen peroxide, a characteristic of FeSOD. From these data we can conclude that MnSOD becomes increasingly important during Fe-deficiency, but cannot ascertain its relationship to Fe-containing isoforms in centric diatoms.

The product of SOD is hydrogen peroxide (H_2O_2) and its breakdown is catalyzed by ascorbate peroxidase (APX) in the chloroplast (Asada 2000). As APX requires Fe as a cofactor, it is not surprising that its activity is reduced under Fe-deficient growth (Table 2.1). The ROS probe used in this study is non-specific so we cannot discern between different reduced oxygen radicals. One other option for hydrogen peroxide detoxification that we did not investigate is the use of glutathione peroxidase (Se-containing) an enzyme that may replace APX (Price and Harrison 1988, Raven et al. 1999). The seleno-enzyme may be upregulated in response to low Fe, but clearly is inadequate to prevent ROS accumulation and oxidative damage. It is interesting to note that Fe-deficient pea leaves also have reduced APX activities accompanied by higher SOD levels (Iturbe-Ormaetxe et al. 1995) suggesting that the results observed here may be a common response of photosynthetic organisms to low Fe. A mutant of tobacco lacking APX accumulates higher concentrations of H_2O_2 than wild-type (Rizhsky et al. 2002) suggesting that the inhibition of APX during Fe-limitation may result in higher ROS generation as well.

ROS generation during Fe-deficiency

Reactive oxygen species generation during steady-state growth should be a function of metabolic rate, the proportion of electrons donated to O_2, the consumption of these ROS by detoxification, their reaction with macromolecules and their spontaneous dismutation (Foyer 1997). By normalizing to O_2 evolution rates we believe our measurements of ROS provide insight into the proportion of electrons "spilled" from photosynthesis that are available to damage macromolecules. Given the pattern in antioxidant enzymes described above, the higher levels of ROS generation may be due to the accumulation of H_2O_2 brought about by the inactivation of APX.

Iron-deficiency could result in several biochemical changes that would result in higher rates of electron donation to oxygen. Accumulation of a reduced plastoquinone (PQ) pool would result in ROS formation either directly from the PQ pool (Khorobrykh and Ivanov 2002) or at the donor side of PSII (Prasil et al. 2004). Electron transport between the photosystems (Greene et al. 1992, Ivanov et al. 2000) is inhibited by Fe-starvation, which could lead to an over-reduced PQ pool. However, we do not know if the redox state of the PQ pool changes during steady-state Fe-deficiency in *T. pseudonana.*

Additional ROS are known to be generated on the donor side of PSI via the Mehler reaction. Mehler activity involves the donation of one electron to oxygen from PSI followed by the rapid enzymatic detoxification of ROS (Asada 2000). Isoforms of SOD and APX are loosely associated with the thylakoid membrane in close proximity to the acceptor side of PSI (Ogawa et al. 1995 and Miyake et al. 1993). Loss of their activity under Fe- or Mn-limitation may lead to increased lifetime of the ROS generated by PSI and explain the observed increase in ROS generation. The measurements made here cannot identify the location of ROS generation.

While this study focuses on photooxidative stress and our ROS measurement controls for those ROS generated in the dark, we note that ROS occur throughout the cell. For instance, 1-5% of respiratory oxygen consumption in mitochondria results in partial oxygen reduction (Møller 2001). ROS accumulated at other sources may also respond to metal deficient conditions. Iron containing catalase, which is responsible for the consumption of hydrogen peroxide outside of the chloroplast, is inhibited by low Fe in pea plants (Iturbe-Ormaetxe, et al. 1995). This could cause accumulation of hydrogen peroxide produced from photorespiratory activity and from other sources besides photosynthesis.

Oxidative damage during Fe-deficiency

The net effect of oxidative stress is damage to cellular macromolecules and it is commonly evaluated using cellular lipid peroxide concentrations. Fe-deficient cells experience increased oxidative damage to their cellular components (Figure 2.2) despite increases in SOD activity, suggesting that an upregulation of SOD alone is insufficient to protect the cells from oxidative stress. Typically, organic macromolecules are not directly damaged by endogenous superoxide or hydrogen peroxide. The hydroxyl radical ($OH^{\cdot}$), an extremely powerful oxidant, is a reduced oxygen radical that is able to react with virtually all macromolecules. In biological systems, it is formed by the univalent reduction of H_2O_2 by a ferrous (or cuprous) ion (Imlay 2003).

Lipid peroxides could arise via oxidization by $OH^{\cdot}$ or perhaps through the action of singlet oxygen (this is discussed in greater detail when considering Mn-limitation below). Other macromolecules, such as DNA (Imlay et al. 1988) and proteins (Berlett and Stadtman 1997) are also damaged by the hydroxyl species. As hydroxyl radicals react with molecules at diffusion-limited rates, those molecules located close to the source of ROS and Fe^{2+} are likely substrates.

Hydroxyl radicals may be also be generated by PSI. Solvent exposed Fe-S clusters, such as those found in PSI, can be oxidized by superoxide causing the complex to become unstable and lose one Fe from the complex, rendering it inactive (Keyer and Imlay 1996, Imlay 2004). Indeed, oxidative damage to PSI results from the destruction of the Fe-S cluster (Sonoike et al. 1995, Sonoike 1996) and *Synechococcus* mutants devoid of a FeSOD are more susceptible to PSI damage (Herbert et al. 1992). As *de novo* synthesis of PSI is slow and the complex contains 12 Fe atoms (Chitnis 2001) the damage, degradation and synthesis of PSI complexes would increase the Fe demand to maintain consistent electron transport. Maintaining a functional, Fe-independent SOD in the chloroplast may reduce damage to the PSI complex during Fe-deficiency.

Mn-deficiency impacts SOD activities

Manganese has numerous catalytic functions in photoautotrophs but in diatoms the majority of the metabolically active Mn is in PSII and MnSOD (Raven et al. 1999, Appendix 3). SOD activities are reduced by Mn-deficiency suggesting that low Mn decreases oxidative stress or that the production of functional SOD is inhibited by the lack of Mn. The accumulation of oxidative damage products suggests that the cells are indeed experiencing greater oxidative stress (Figure 2.2). Interestingly, ROS production is similar to that observed under metal replete conditions (Figure 2.1) suggesting increased oxidative damage is not due to superoxide formation. Supporting this suggestion are results from populations of Mn-deficient Norway spruce that experience elevated oxidative stress compared to control populations despite elevated Cu/Zn SOD activities (Polle et al. 1992). We turn our attention to the inhibition of PSII by low Mn and how this could generate the observed pattern of oxidative stress.

Inhibition of PSII by Mn-deficiency

The maximum potential quantum efficiency of PSII (F_v/F_m) and the realized quantum efficiency of PSII (Φ_{PSII}) are both lower in Mn-deficient diatoms compared to metal replete cells (Table 2.2). Several lines of evidence suggest this damage is due to donor-side inhibition of PSII. A cluster of 4 Mn atoms is required to stabilize H_2O as its electrons are removed to replace those withdrawn from the photochemical reaction center of PSII (Vrettos and Brudvig 2002). The liberation of electrons from water may be inhibited by Mn-limitation, causing an accumulation of strong oxidizing radicals in the reaction center and subsequent damage to the PSII complex (Aro et al. 1993).

The kinetics of the fluorescence emission reflect the various redox transitions associated with primary photochemistry in PSII and the photosynthetic electron carriers. During Mn-limited photosynthesis, processes downstream of PSII seem to be effective at oxidizing PSII, resulting in the longer period of illumination required to reach F_m compared to metal replete cultures (Figure 2.3). The progressive closing of photochemical reaction centers leads to the increase in fluorescence seen in the first 0.2 ms of the fluorescence transient. If the reaction center of PSII has not been re-reduced, or its acceptor molecule has not been oxidized, then it remains closed to further photochemistry so that any additional absorbed light energy must be emitted as fluorescence or heat (Falkowski and Raven 1997). Further changes in fluorescence are due to the behaviour of fluorescence quenchers (such as electron transport, which opens the reaction center, or thermal dissipation of light energy) and the absorption of light energy. These results suggest that inhibition of PSII is not due to a restriction in the ability to oxidize PSII (which is termed acceptor-side inhibition), which would shorten the length of time it takes to reach F_m. The fluorescence transients indicate that the lesion is likely on the donor-side of PSII.

Damage to PSII increases the probability of excited chlorophyll molecules passing their energy directly to molecular oxygen, producing singlet oxygen (Hideg et al. 1998). Singlet oxygen will readily damage polyunsaturated lipid molecules initiating a chain reaction of lipid peroxidation (Niyogi 1999). It is important to note that the ROS probe used here does not react with singlet oxygen so lipid damage could result despite low SOD activities and similar ROS generation compared to that seen in metal replete cultures.

Low F_v/F_m is still observed in *T. pseudonana* grown in Mn-deficient conditions for months (data not shown). We believe this is the first time that a reduction in PSII efficiency over long time scales has been demonstrated. Perhaps this phenomenon will allow others to look at donor side inhibition of PSII *in vivo*, without the use of chemical inhibitors or thylakoid isolation.

Co-limitation

Following discussion of both Fe-deficiency and Mn-deficiency on their own it is now possible to interpret the results of their interaction at low concentrations. Peers and Price (2004) found that recovery of co-limited *T. pseudonana* after addition of either Fe or Mn alone was due to stimulation of different metabolic pathways. For instance, addition of Mn allowed for a recovery of PSII (and presumably MnSOD), but cells were still Fe-limited, most likely due to low concentrations of the photosystems (Strzepek and Harrison 2004). Results reported here build on these previous observations.

The passage of electrons through the electron transport chain of photosynthesis is inhibited by Fe-deficiency (Greene et al. 1992). When Mn-deficiency is imposed on top of this it appears that fewer electrons can enter the chain due to an inhibition of the oxygen-evolving complex of PSII (co-limited treatment, Figure 2.3). Iron-deficiency under Mn-replete conditions results in higher oxidative stress accompanied by a physiological upregulation of SOD. Active SOD is unable to accumulate during co-limitation (Table 2.1) and APX may also be low (but not measured), compromising the cell's ability to detoxify oxygen radicals. The increased ROS results in greater damage to cellular macromolecules (Figure 2.2) including the photosystems themselves. Iron and Mn co-limitation of diatom growth is therefore based on their apparently irreplaceable roles in cellular metabolism. This differs from the direct biochemical substitution of one metal for the other such as the interaction seen for Zn and Cd in diatoms (Lane and Morel 2000).

There is a relative increase in the costs associated with repair to damaged molecules during oxidative stress. Iron- and Mn-deficient growth rates may therefore not only be a result of reduced carbon fixation rates, but due to an increase in repair processes. Additionally, oxidative stress increases the rate of DNA damage and mutation rates (Imlay et al 1988, Evans and Cooke 2004), which may lead to cell death and reduced fitness of future generations.

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Table 2.1. Growth rates (μ), superoxide dismutase (SOD) and ascorbate peroxidase (APX) enzyme activities of *Thalassiosira pseudonana* cultures grown under metal-replete or metal-limiting conditions. Reported values equal means ± standard deviations (n=3). Enzyme activities with different letters are statistically different (SOD: ANOVA $F_{3,11}$=1563, p=0.00, Tukey; APX: t-test, t=12.1, p=0.001). APX was not measured in Mn-deficient or colimited cells.

Growth condition	μ (d^{-1})	SOD activity (Units mg^{-1} protein)	APX activity (Units mg^{-1} protein)
Metal replete	1.55 ± 0.06	12.1 ± 0.1^a	0.79 ± 0.03^a
Fe-deficient	1.00 ± 0.02	18.5 ± 0.3^b	0.30 ± 0.06^b
Mn-deficient	1.18 ± 0.11	5.0 ± 0.2^c	-
Co-limited	0.84 ± 0.09	5.56 ± 0.1^c	-

Table 2.2. Photosystem II fluorescence parameters of *Thalassiosira pseudonana* grown in steady-state Mn-deficient or metal-replete conditions. Parameters measured include the maximal potential efficiency of PSII (F_v/F_m) and the realized efficiency of PSII during constant illumination (Φ_{PSII}). All values represent means ± standard deviation from independent, fully acclimated cultures (*n*=3).

Fluorescence parameter	Mn-replete	Mn-deficient
F_v/F_m	0.58 ± 0.01	0.40 ± 0.03
Φ_{PSII}	0.41 ± 0.02	0.29 ± 0.01

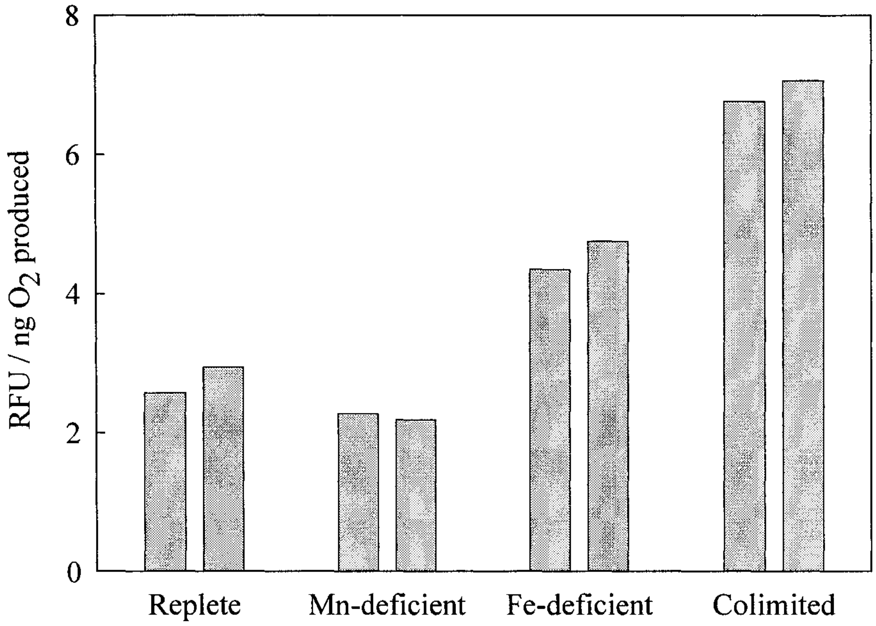

Figure 2.1. Steady-state reactive oxygen species (ROS) generation rates measured in metal-replete or metal-deficient cultures of *Thalassiosira pseudonana*. ROS generation values are normalized to photosynthetic oxygen evolution in each growth condition (*n*=2).

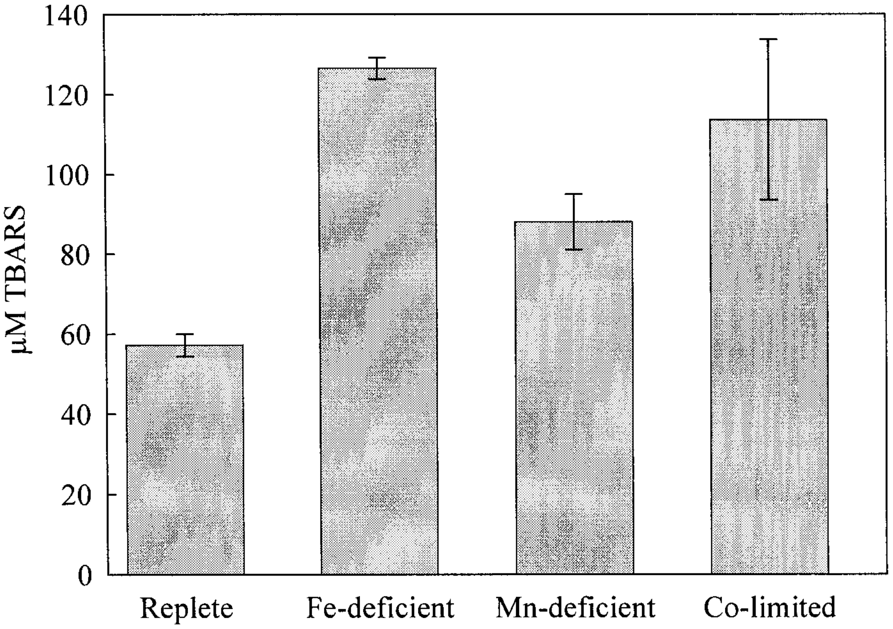

Figure 2.2. Cellular concentrations of peroxidated lipids measured in *Thalassiosira pseudonana* during steady-state growth under various metal conditions. Peroxidated lipids are estimated as thiobarbituric acid reactive substances (TBARS). Bars represent the means of 3 replicate cultures (error bars are ± standard deviation).

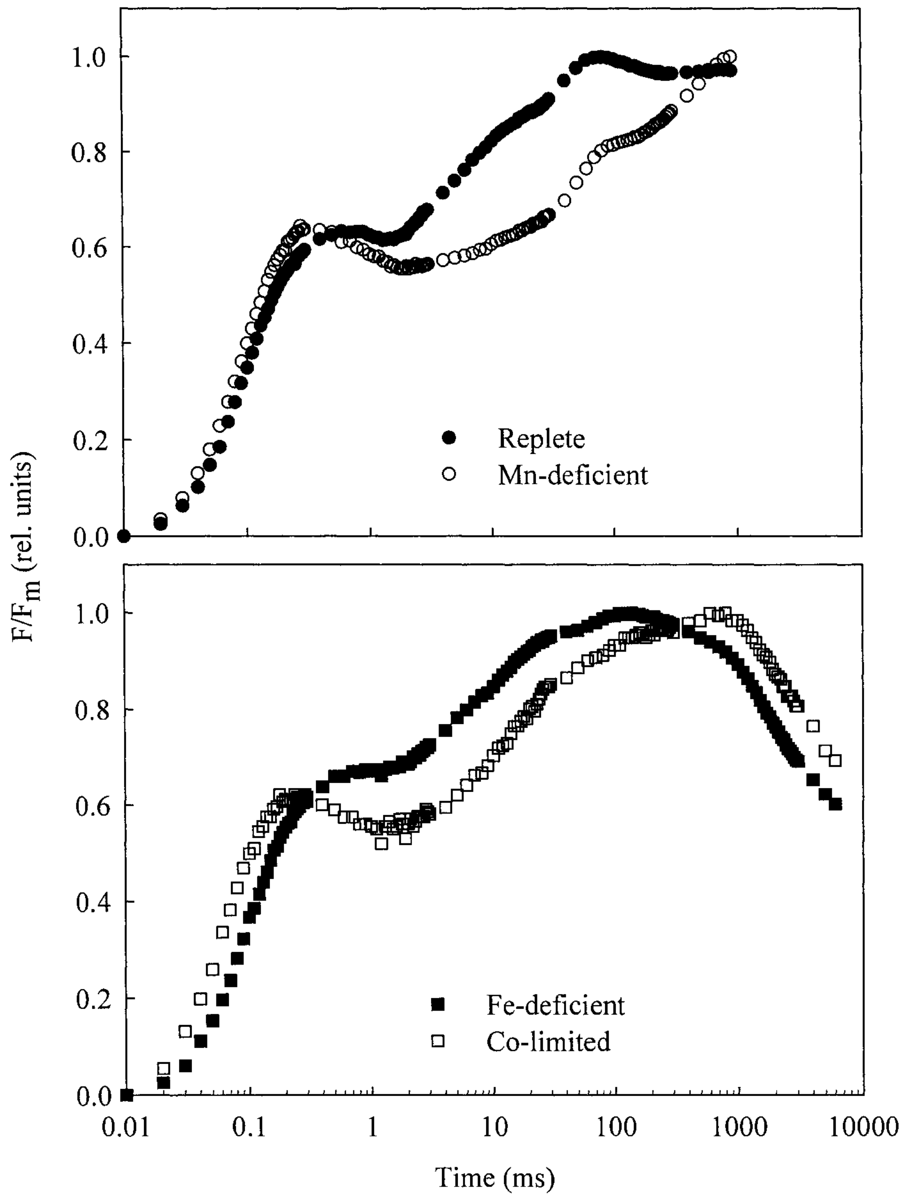

Figure 2.3. Photosystem II (PSII) fluorescent transients of *Thalassiosira. pseudonana* acclimated to various metal conditions. Metal replete and Mn-deficient cultures were exposed to saturating light for 1 s while Fe-deficient and co-limited cultures were exposed for 10 s. Displayed curves are the average of 3 replicates.

Chapter 3. Copper requirements for iron acquisition and growth of coastal and oceanic diatoms

While investigating the interaction between Fe and Mn in *Thalassiosira pseudonana,* I found that the addition of nanomolar concentrations of Cu increased the growth rate of Fe-deficient cultures. The next two chapters explore the interaction between limiting concentrations of Cu and Fe in marine diatoms.

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Abstract

Centric diatoms isolated from open ocean environments require higher concentrations of Cu for growth than their coastal counterparts. In artificial seawater medium containing <1 nM Cu, three coastal species maintained near maximum rates of growth, but the oceanic clones were unable to survive. Copper-limitation was more severe in the diatoms grown in low than in high-Fe seawater, suggesting that Cu and Fe were interacting essential resources. The interactive effect was in part the result of a Cu requirement for Fe transport. *Thalassiosira weissflogii* and *T. oceanica* had lower Fe-quotas and slower rates of Fe uptake when [Cu] was reduced in the medium. Brief exposure of Cu-limited cells to 10 nM Cu increased the instantaneous Fe uptake rate by 1.5 times in *T. oceanica.* Steady-state uptake rates of both species at high, growth-saturating concentrations of Fe were also Cu dependent. Oceanic species appeared to have an additional Cu requirement that was independent of Fe acquisition and likely responsible for their higher requirements compared to coastal species. Evidence for the importance of Cu in natural communities of phytoplankton was obtained from an incubation experiment performed in the Fe-limited basin of the Bering Sea. Addition of 2 nM Cu doubled the phytoplankton net growth rate compared to the untreated controls and, in the presence of extra Fe, increased the growth rate compared to the samples amended with Fe alone. The results suggest that Cu may be an important micronutrient for phytoplankton growth in low Fe regions of the sea because of its role in Fe-acquisition. Paradoxically, oceanic diatoms may be more susceptible to the effects of low Cu concentrations than coastal species.

Introduction

Low concentrations of Fe in the open sea are impeding autotrophic production (Tsuda et al. 2003) and must exert strong selection pressure on the Fe-deficient phytoplankton to economize the use of this limiting resource. Laboratory measurements have documented the results of this selection in showing a large difference in the Fe requirements of neritic and oceanic species. In offshore waters, where Fe concentrations are 10 to 100 times more dilute than in coastal seas, phytoplankton have reduced their need for Fe and are able to grow at levels well below those required for reproduction of coastal species (Brand et al. 1983; Maldonado and Price 1996; Ryther and Kramer 1961). Thus, oceanic phytoplankton have adapted to survive in Fe-impoverished waters. A similar habitat-related pattern of phytoplankton requirements is observed for other bioactive metals, including Zn and Mn (Brand et al. 1983; Tortell and Price 1996), which mirrors their availabilities in these environments like Fe (Bruland et al. 1991).

The majority of cellular Fe in algae is used in proteins required for light energy transduction (Raven 1990; Raven et al. 1999) so most attention has focused on these pathways to identify adaptive Fe-sparing strategies. Changes in the photosynthetic apparatus, ranging from the replacement of ferredoxin with flavodoxin (La Roche et al. 1996) to a general remodelling of the stoichiometry of photosynthetic components (Greene et al. 1991; Moseley et al. 2002) occurs in response to Fe-deficiency. Recent work by Strzepek and Harrison (2004) shows that an oceanic diatom has adjusted the architecture of its photosynthetic apparatus to reduce Fe-rich components compared to a coastal species. This work has provided the first biochemical explanation of how phytoplankton from the open ocean have reduced their dependence on Fe. In what other ways might oceanic and coastal phytoplankton differ in their adaptations to Fe availability in the environment?

One important physiological response of phytoplankton to low Fe involves their utilization of Fe organic complexes, which are the dominant chemical species in the sea. Under Fe-limiting conditions the marine diatom, *Thalassiosira oceanica*, develops the ability to reduce Fe bound to chelates and subsequently transports this Fe and utilizes it for growth (Maldonado and Price 2001). Field measurements confirm the importance of Fe(III) reduction by showing that natural communities also use Fe bound to strong organic complexes (Hutchins et al. 1999; Maldonado and Price 1999; Maldonado et al. 2001) and that the rates of Fe uptake are dependent on the Fe-nutritional state of the phytoplankton (Maldonado and Price 1999). In other eukaryotes that possess a high affinity Fe(III) reductive pathway, Cu is a necessary co-factor of an oxidase that is involved in the Fe transport reaction (Stearman et al. 1996). This Cu-containing oxidase (a multicopper oxidase, MCO) oxidizes the Fe(II) produced by the ferric chelate reductase and the Fe(III) thus produced is internalized by a permease. Sequence analysis of the genome of *T. pseudonana* has identified a gene with homology to the MCOs of other well characterized organisms (Armbrust et al. 2004), suggesting that a Cu requirement for Fe transport may also exist in diatoms.

Whether Fe nutritional state affects the Cu requirement of phytoplankton is presently unknown. We previously observed that diatoms adjust their requirements for Mn under Fe deficiency and hypothesized that the same may be true of Cu. In the case of Mn, Fe-deficient growth increased the requirements of diatoms for Mn because it induced oxidative stress and the need for Mn-containing superoxide dismutase (Peers and Price 2004). Iron limitation may likewise increase the need for Cu if high affinity Fe transport systems are up-regulated in Fe-limited phytoplankton.

While Cu-containing proteins such as the MCO (and others, such as plastocyanin) could be potentially important parts of the adaptive responses of phytoplankton to Fe-deficiency (Raven et al. 1999), their biosynthesis may present another problem because dissolved Cu concentrations in the ocean are very low. Indeed, total dissolved Cu in surface waters of the open ocean are in the nanomolar range and organic ligands maintain Cu^{2+} concentrations at pM (Coale and Bruland 1988; Moffett et al. 1990). Such concentrations may be inadequate for phytoplankton with an elevated Cu-demand induced by Fe-deficiency.

Here we report physiological data from a number of diatom species that suggest that natural selection in the metal impoverished open ocean has paradoxically resulted in diatoms that require higher ambient concentrations of Cu for growth than their coastal counterparts. Part of the diatom requirement for Cu is for efficient Fe-acquisition. Enrichment experiments in the Bering Sea confirm a stimulatory effect of Cu on net phytoplankton growth that can be interpreted as a direct result of Cu limitation of Fe acquisition or possibly primary Cu deficiency.

Methods

Culture

Six centric marine diatoms were obtained from the Provasoli-Guillard Culture Collection of Marine Phytoplankton (CCMP), West Boothbay Harbour, ME. Strains were assigned to coastal or oceanic provenance according to isolation information provided by the CCMP. The coastal strains were: CCMP 1335, *Thalassiosira pseudonana* (3H); CCMP 1336, *T. weissflogii* (ACTIN); and CCMP 1093, *T. minuscula* (WTSIO) and the oceanic strains were: CCMP 1003, *T. oceanica* (35-81); CCMP 1005, *T. oceanica* (13-1); and CCMP 1006, *T. oceanica* (B1SA). These diatoms were maintained at 20 °C under constant illumination of 180 μmol quanta m^{-2} s^{-1} provided by Phillips Cool White fluorescent bulbs except where noted. Cells were grown in acid-cleaned, 28 ml polycarbonate tubes and biomass was monitored by in vivo chlorophyll fluorescence using a Turner Designs 10-AU fluorometer. Specific growth rates (d^{-1}) were determined in fully acclimated, semi-continuous batch cultures (Maldonado and Price 1996).

Media

Diatoms were grown in artificial seawater medium (AQUIL, Price et al. 1988/89) containing the synthetic chelator ethylenediaminetetraacetic acid (EDTA) at a concentration of 100 μM. The Cu and Fe salts, $CuSO_4\ 5H_2O$ and $FeCl_3\ 6H_2O$, were of the highest chemical purity (ANALAR grade, BDH) and were added to the media bound to EDTA (1:1.05). All other trace metals were added as outlined in Price et al. (1988/89). Media were allowed to equilibrate for at least 24 h before use. The total concentration of inorganic Fe species (Fe': $Fe(OH)_2^+$, $Fe(OH)_3^0$, $Fe(OH)_4^-$) was calculated using MINEQL and as described (Sunda and Huntsman 1997). Both methods gave similar results. Addition of 1.29 μM, 40.8 nM, and 4 nM Fe to media that contained a background concentration of 5 nM resulted in a [Fe'] of 5.66 nM, 90.4 pM and 24.2 pM, respectively. The background concentration of Cu contaminants in our media (nutrient-replete AQUIL minus Cu) was undetectable by inductive-coupled plasma mass spectrometry (ICP-MS). Media containing no Cu additions are thus reported to contain 0 nM Cu. In Cu-enriched media, Cu was added at concentrations of 1, 3, 5, 8, and 21.4 nM, corresponding to concentrations of Cu' of 12, 37, 61, 98, and 260 fM as determined using the conversion of Sunda et al. (2005).

Fe quotas

Fully acclimated *T. oceanica* (CCMP 1005) and *T. weissflogii* (CCMP 1336) were inoculated into media containing $^{55}FeCl_3$ (10% of total Fe, all isotopes supplied by Perkin Elmer) and grown for at least 12 generations in the radioactive media. In these experiments, the growth irradiance was 200 µmol quanta m^{-2} s^{-1}. Cells were harvested in the mid-exponential phase of growth by gentle filtration onto 25 mm diameter, 2 µm pore size, polycarbonate filters (all membrane filters supplied by Poretics) and extracellular Fe was removed using the Ti-citrate protocol (Hudson and Morel 1989). Filters were placed in scintillation fluor (Ultima Gold, Beckman) for two days before counting on a Packard Tri-Carb scintillation counter. Cell density and volume were determined by microscopy as described (Peers and Price 2004).

Fe:C experiment

Thalassiosira oceanica (CCMP 1005) was acclimated to 4 nM Fe and 1 nM Cu, until the growth rates of successive transfers varied by less than 10%. A subsample of these acclimated cells was then inoculated into media containing $^{55}FeCl_3$ (78.2 mCi mg^{-1}) and $H^{14}CO_3$ (5.0 mCi $mmol^{-1}$) and cultivated for at least 12 generations. Cells were subsequently harvested and rinsed as described above. Inorganic ^{14}C was removed by exposing the filters to fuming HCl for 24 h after which scintillation fluor was added and the samples counted.

Short-term Fe uptakes

Thalassiosira oceanica (CCMP 1005) and *T. weissflogii* (CCMP 1336) were acclimated to Fe and Cu concentrations as described above. Only cultures in the mid-exponential phase of growth were used for uptakes. Cells were harvested by gentle filtration onto acid-cleaned (10% HCl, TraceMetal grade, Fisher Scientific) 25 mm diameter, 2 μm pore-size, polycarbonate filters and resuspended into media containing 50 nM $^{55}FeCl_3$ (10% radiolabel, 90% cold stock) complexed with 100 μM EDTA. This suspension was immediately subsampled for time-zero measurements and uptake was allowed to continue for 2-4 h. The experiments were terminated by filtration and the cells rinsed to remove surface-bound Fe as described above.

The role of Cu in Fe-transport was also assessed in short-term Cu addition experiments in which acclimated cells were initially resuspended in AQUIL with or without 10 nM Cu complexed with 100 μM EDTA. After one h of treatment, the cells were then refiltered, rinsed with metal-free AQUIL, and resuspended in radioactive uptake media. Iron uptake was measured as described above.

Metal enrichment of Bering Sea plankton communities

Seawater samples from 5 m depth were collected sequentially as the ship slowly steamed across a distance of ~20 km in the basin region of the Bering Sea (55.0°N, 178°E) in August 2003. A trace metal clean pumping system was used to dispense the water into 48, 1 l LDPE cubitainer bottles with Teflon-lined caps. The cubitainer bottles were cleaned before use with the following protocol: ethanol rinse, Micro-10 detergent soak (Fisher Scientific, overnight), and 10% HCl soak (TraceMetal grade, Fisher Scientific, 48 h at 50°C). Each bottle was rinsed extensively with 18.2 mΩ Milli-Q-H_2O (Millipore) between each cleaning step. The cleaned cubitainers were subsequently filled with 0.1% HCl (Baseline grade, Seastar Chemicals) for at least 2 weeks and finally rinsed and filled with clean seawater for 1 day before use. The 40 seawater samples were randomized and amended with either 2 nM Fe, 2 nM Cu, both metals together or no addition (control) in a Class-100 flow hood. There were 8-10 independent replicates of each treatment. Cubitainers were wrapped in PVC screens to supply approximately 30 % of incident irradiance and incubated on deck with flowing surface seawater. Duplicate cubitainers were sacrificed at each sampling time and an aliquot of each was filtered onto 25 mm GF/F filters (Whatman) for determination of chlorophyll *a* (Chl *a*, Coale 1991).

Statistics

All statistical calculations were performed using Systat v. 10.2 (Systat Software Inc.).

Results

The requirements for Cu of the oceanic and coastal diatoms were assessed during steady state growth in Aquil medium in which the total Cu concentration was varied from 0 to 21.4 nM Cu, corresponding to a maximum of 260 fM Cu'. An unexpected and surprising result was that all the oceanic strains required much higher Cu concentrations to achieve maximum rates of growth than the coastal species (Figure 3.1). This result was generally true regardless of the Fe concentration in the medium. At the lowest Cu concentration, which was below the detections limit of the ICP-MS analysis, none of the oceanic strains were able to divide whereas the coastal species grew at near maximum rates. Even with 5 nM Cu in the medium, growth of *Thalassiosira oceanica* was reduced compared to Cu-replete conditions. In the high Fe medium, when all resources were in excess, the reduction in diatom growth rate was most simply interpreted as a primary limitation by Cu. The growth reduction by Cu under limiting Fe, however, illustrated a multiplicative effect indicative of a Cu and Fe interaction that was inconsistent with single resource limitation. This interaction was clearly evident in *Thalassiosira oceanica* (CCMP 1003) at 1 nM Cu which grew at rates of roughly 0.5 d^{-1} in Fe-rich medium, but was unable to grow in the Fe-limiting medium. Chemical analysis showed no significant or detectable Cu contamination of the Fe-replete medium compared to the Fe-deplete and Fe-free media (data not shown). The other two oceanic strains showed the same type of response under low Fe with proportional reductions in growth rate as Cu declined.

Within the oceanic and coastal groups the diatoms responded similarly to Cu and Fe so we focused on one representative from each group for further study. *Thalassiosira oceanica* (CCMP 1005) and *T. weissflogii* (CCMP 1336) were chosen as the oceanic and coastal species, respectively. Iron quotas (Q_{Fe}) of these diatoms were measured under low and high Fe in the presence of varying amounts of Cu as a first step to elucidate the interaction (Table 3.1). Iron quotas of the coastal species under Fe-sufficient conditions were reduced when

Cu was omitted from the medium; a treatment that also significantly reduced growth (ANOVA, p=0.001, $F_{2,18}$=10.44, Tukey and ANOVA, p=0.012, $F_{2,18}$=5.67, Tukey, respectively). At 1 and 21.4 nM Cu, there were no significant differences in either growth rate or Q_{Fe}. *Thalassiosira weissflogii* appeared to be more sensitive to changes in Cu concentration in the medium under Fe-deficient than Fe-sufficient conditions, but Q_{Fe} did not decrease in proportion to growth rate. As shown in Figure 3.1, a reduction in ambient Cu at all Fe levels reduced growth rate in *T. oceanica* (Table 3.1). Volumetric Fe quotas, however, were only different between Cu treatments when cells were grown in mildly Fe-deficient conditions (t-test, p=0.006, t=5.41, df=4). Despite slower growth under Cu-deficiency, which might promote Fe accumulation if transport rates remained constant, Q_{Fe} did not increase in either species. Thus, Cu appeared to influence the accumulation of Fe by both the oceanic and coastal species.

To evaluate independently the Cu dependence of Fe accumulation in *T. oceanica* and *T. weissflogii*, we measured short-term Fe uptake rates in cells acclimated to low and high Fe and Cu (Figure 3.2). The rates of Fe uptake were strongly dependent on the growth conditions of the cells. Rates of uptake by both species were significantly higher in the low than in the high Fe cells, as expected with Fe-limited growth. Additionally, when both species were grown under Fe-deficient conditions, the Cu-replete cells had the faster rates of Fe uptake. Iron-replete *T. oceanica* also had significantly higher rates of Fe-uptake when cultivated with high than with low Cu (ANOVA, p=0.001, $F_{3,32}$=11.2, Tukey and ANOVA, p=0.001, $F_{3,18}$=392, Tukey for *T. w.* and *T. o.*, respectively).

The Cu requirement for Fe transport was further documented in Cu enrichment experiments where Cu-deficient *T. oceanica* was briefly exposed to 10 nM Cu prior to measuring Fe uptake. In these experiments, cells were washed and resuspended in Cu-free medium after 1 h exposure to the Cu to avoid Cu carry-over in the uptake medium and any aqueous Fe-Cu interactions that could bias the results. As shown in Table 3.2, the instantaneous rate of Fe transport increased following brief Cu exposure. In both the high and low Fe acclimated cells, the relative increase in Fe transport rates was 1.5, although the results were only statistically significant ($p<0.05$) in the Fe-limited cells. Collectively, the results demonstrate that Cu was required for efficient Fe transport by both coastal and oceanic diatoms.

Because of the Cu requirement for Fe transport shown here, the low Cu growth data presented in Figure 3.1 could be interpreted as a direct effect of Cu on the Fe nutritional status of the phytoplankton. Low Cu could induce Fe limitation, because Cu is required for Fe transport. Indeed, when growth rate of *T. weissflogii* was plotted as a function of the steady state Fe transport rate all of the data points fell along a hyperbolic curve as expected if Fe transport were the determinant of growth rate (Figure 3.3). In this species, the effect of low Cu was to reduce the Fe transport rate and thereby reduce growth. Even in the presence of high concentrations of Fe, Cu influenced steady state Fe transport. The results for *T. oceanica* however, were dramatically different and suggest that Cu had a more important role in some other cellular process(es) that was independent of Fe uptake. Iron transport rate in the oceanic strain was Cu dependent (as illustrated by the slower rates of Fe transport in the presence of 1 compared to 21.4 nM Cu at each Fe concentration, Figure 3.3), but at low Cu the growth rate was disproportionately reduced. Under Fe-limiting conditions the transport rate of Fe appeared to control growth even at low Cu. These results suggest that the reduced growth rate under low Cu in the coastal diatom was primarily due to its reduced ability to acquire Fe. The oceanic species also required Cu for Fe transport, but had some other major requirement.

The Cu dependence of growth of the oceanic phytoplankter, *T. oceanica*, was examined over a range of Fe-deficient conditions at three different levels of Cu. Each Cu treatment resulted in a different linear correlation between Fe:C and growth rate (ANCOVA, $p=0.000$, $F_{2,16}=28.2$) (Figure 3.4). A reduction in the Cu concentration decreased growth rate at a constant Fe:C ratio. If Cu were involved in Fe-uptake alone, all treatments would be expected to be described by the same relationship between growth and cellular Fe.

The importance of Cu to a Fe-limited phytoplankton community in the Bering Sea was evaluated by a metal addition experiment. The experimental design consisted of 8-10 independent replicates per treatment. Iron enrichment increased the initial chlorophyll *a* concentration by 25-fold after 6 days of incubation (Figure 3.5). Control samples showed little change (a 1.2-fold increase) over the same period. Net phytoplankton community growth rates (d^{-1}) were estimated from linear regression of natural logarithm transformed concentrations of Chl *a* (shown in Figure 3.5). Values from day 2 to day 6 were used in the analyses to avoid the initial lag-phase as phytoplankton responded to the metal treatments. Addition of Cu alone significantly increased net community growth rate from 0.05 d^{-1} to 0.12 d^{-1} (ANCOVA, p=0.002, $F_{1,18}$=15.8) and additions of both Cu and Fe increased community growth rates to 0.77 d^{-1} compared to Fe alone (0.59 d^{-1}), although this result was only weakly significant (ANCOVA, p=0.11, $F_{1,13}$=2.8). Inexplicably, the Chl *a* concentrations measured on day 2.5 were abnormally high in the control and Cu-amended treatments. When these data were omitted from the analyses the difference between the treatments became even more significant.

Discussion

Copper requirements for growth

The habitat-related dependence of essential metal requirements of phytoplankton is well documented. Numerous studies show consistently that species of oceanic provenance are able to grow at much lower concentrations of metals than species from coastal regions (Brand et al. 1983; Maldonado and Price 1996; Tortell and Price 1996). Many of these metals, including Fe, Mn, and Zn, are more concentrated in coastal than in offshore waters (Bruland et al. 1991) so the differences in the biological requirements are thought to reflect phytoplankton adaptations to metal availabilities in the environment. In the case of Fe, these adaptations have reduced the metabolic requirements of the oceanic species to roughly one tenth of those of the coastal species (Maldonado and Price 1996).

The results reported here on the Cu requirements of six diatoms (Figure 3.1) are in stark contrast to all the other bioactive metals. They show that diatoms from oceanic environments require higher concentrations of Cu to grow than coastal isolates. Indeed, the oceanic diatoms we examined were unable to grow at concentrations of Cu that supported maximum growth rates of the coastal species. Total dissolved Cu varies from 10 nM in coastal waters to 1 nM in oceanic waters, similar to the pattern seen for other metals. As pointed out by Mann et al. (2002), however, the free Cu is kept reasonably constant across this gradient by complexation with organic ligands, so its availability may be similar in both environments. Thus, it seems somewhat paradoxical that oceanic species should have evolved higher requirements for Cu, unless they have some unique requirements that are necessary to inhabit these environments.

Copper limitation of phytoplankton growth has thus far only been reported in a few species of phytoplankton (Manahan and Smith 1973; Schenck 1984) so in this regard our results are noteworthy. More important is why have oceanic species evolved a requirement for relatively high concentrations of Cu for growth whereas coastal species need so little? Two obvious hypotheses come to mind. The first of these is that oceanic diatoms have inefficient Cu transport compared to coastal species and the second is that they require more metabolic Cu to grow. Although Cu uptake was not measured directly in our experiments, we can use published data (Sunda and Huntsman 1995*b*; Chang and Reinfelder 2000) to test this hypothesis. The relevant data are for *T. oceanica* and *T. weissflogii* from experiments conducted at low concentrations of Cu. In both diatoms, the calculated steady-state Cu uptake rates are ~25 µmol Cu l^{-1} cell volume d^{-1} at a pCu of 14.42 and 14.79, respectively. Thus, Cu transport per se does not appear to be fundamentally different in these two species. We surmise then that the results presented in Figure 3.1 are not a consequence of differences in Cu-acquisition between coastal and oceanic isolates, but that they may reflect differences in the amounts of metabolic Cu they require for growth.

Copper quotas of both *T. oceanica* and *T. weissflogii* are about 1 µmol Cu mol^{-1} C (Sunda and Huntsman 1995*b*; Chang and Reinfelder 2000) growing under the conditions described above. In neither case however were these quotas determined under Cu limitation and so are likely overestimates of metabolic Cu. Estimating the elemental requirement for growth of phytoplankton requires measurements of quotas under optimal or rate-limiting conditions and must consider the metabolic rate of the organism. So far these estimates do not exist for Cu. Luxury consumption increases the cellular content of many elements when they are abundant and can obscure the true metabolic requirements (Droop 1974). A good example of this phenomenon is cellular Fe in *T. oceanica* which increases concomitantly with increasing dissolved Fe even though growth rates remain constant and Fe-sufficient (Sunda and Huntsman 1995*a*).

We note that Sunda and Huntsman (1995*b*) measured a small (5%) decrease in growth rate of *T. oceanica* at the lowest Cu concentrations, suggesting that they may have been on the verge of achieving Cu-limited growth. The small decrease in growth rate observed in their experiments is surprising given the results we obtained. Indeed, Cu concentrations in both experiments were remarkably similar. At 1 nM Cu, for example, Sunda and Huntsman (1995*b*) measured near maximum growth rates of *T. oceanica* (CCMP 1005) whereas we observed a 50% decline in growth (Figure 3.1). Either Cu contamination was a confounding variable or some other differences in the composition of our media are the cause of this discrepancy. As discussed below, our results have established one important role for Cu in Fe transport in both coastal and oceanic diatoms. Measurement of Cu quotas under Cu-deficient conditions will be essential to establish the Cu requirements of these phytoplankton.

The role of Cu in Fe transport

High affinity iron transport in several model eukaryotic organisms requires Cu. In *Saccharomyces cerevisiae,* for example, Fe is first reduced by a Fe(III)-chelate reductase and then reoxidized during uptake (Stearman et al. 1996). The reoxidation step is Cu dependent, and is mediated by a multicopper oxidase (MCO) that has also recently been identified in the green alga, *Chlamydomonas reinhardtii* (Herbik et al. 2002; La Fontaine et al. 2002). Several of our physiological observations are consistent with a Cu-dependent step in Fe uptake by marine diatoms and suggest that an MCO may be involved.

Judging from changes in absolute growth rate, the copper requirements of the species we examined were enhanced by Fe-deficiency (Figure 3.1). Growth rates of the oceanic diatoms under Fe-limitation, for example, were even slower than under Fe-replete conditions when Cu was lowered in the medium. Thus, Cu and Fe were interacting essential resources, like Mn and Fe (Peers and Price 2004), and at low concentrations were co-limiting to growth. This interaction was less clear-cut in the coastal isolates, possibly because they were much more difficult to limit for Cu in the first place.

Direct evidence for the involvement of Cu in Fe transport of diatoms was provided by measurements of Fe quotas and short term uptake rates. Cellular Fe declined as Cu was lowered in the medium (Table 3.1), implying that transport rates of Fe were decreased. Indeed, the instantaneous Fe uptake rate was much slower in cells grown in low than in high Cu (Figure 3.2). This effect was most pronounced in the Fe-limited cells that had the fastest rates of Fe uptake. In Fe-replete cells, the effect of Cu was only statistically significant in the oceanic strain, although the mean rates of uptake by *T. weissflogii* were also greater in the high Cu grown cells. Exposure of Cu-depleted *T. oceanica* to 10 nM Cu_T (complexed with 100 μM EDTA) for 1 h increased Fe-transport rates by 1.5-fold (Table 3.2), regardless of the Fe-status of the cells. *Thalassiosira weissflogii* was not examined. Note, that the variability among the replicates of these experiments was quite high, likely because the additional steps of filtration and resuspension (see Methods) were detrimental to the cells. Despite this variation all individual experiments showed the same Cu stimulation. We note that treatment with Cu for one h was not sufficient to cause the Fe-transport rates to increase to the levels observed under Cu-replete conditions.

The results presented in Figure 3.3 show that Cu limitation of growth in the coastal diatom can be most simply interpreted as an indirect effect caused by a decrease in the steady state Fe transport rate. Although a number of other metabolic functions of Cu are undoubtedly important, they may be less sensitive to changes in Cu availability, perhaps because of their overriding importance or small Cu requirement. In Fe-replete cultures of *T. oceanica*, Cu-deficiency reduced Fe uptake but also caused a reduction in growth rate that was independent of Fe uptake, suggesting an additional, important role for Cu in this oceanic species (see below).

While our data are the first to show a link between Cu and Fe-acquisition in diatoms, previous studies have provided important evidence for the existence of such a pathway of Fe uptake. Extracellular Fe reduction, the first step of high affinity Fe-uptake, has been shown to occur in Fe-limited diatoms (Maldonado and Price 2000; Maldonado and Price 2001) and in natural plankton communities (Maldonado and Price 1999; Maldonado et al. 2001; Shaked et al. 2004). Many freshwater algae also possess this ability (Weger 1999). It should be noted that higher plants (Curie and Briat 2003) and yeast (Jones 2003) internalize Fe^{2+} directly, but in the case of yeast, this pathway has a much lower affinity for Fe compared to the MCO-dependent pathway. In the Fe-transport pathways of higher plants there has been no documented role for a MCO.

The genome of the coastal diatom *T. pseudonana* has recently been sequenced and shown to contain a gene homologous with the MCOs of other eukaryotes and to possess a ferric chelate reductase (Armbrust et al. 2004). Although we have no evidence for a Cu requirement in an MCO, we have detected MCO activity in partially purified membrane fractions from these diatoms (Quesnel and Price, unpubl.).

As the majority of dissolved Fe in surface waters of the open ocean is bound to ligands (Rue and Bruland 1995) a Fe(III)-chelate reductase system like that described for other eukaryotes may be an important way for phytoplankton to acquire Fe (Maldonado and Price 1999, 2000, 2001). Hutchins et al. (1999), Maldonado and Price (1999) and Maldonado et al. (2001) have all reported that natural populations of plankton are able to obtain Fe bound to a variety of ligands, possibly through a Fe(III)-chelate reductase. Such a pathway could be compromised by a lack of Cu so that the reduced Fe may be free to diffuse away from the cell before it is oxidized and transported.

Copper requirements in oceanic diatoms

Possible explanations for the higher Cu requirement of the oceanic species could include a greater need for Cu in Fe transport as discussed above. However, oceanic and coastal diatoms take up Fe at similar, diffusion-limited rates (Sunda and Huntsman 1995a) so one would have to postulate that the oceanic strains have a less Cu-efficient Fe-uptake system. If additional Cu were required for Fe-uptake it does not appear to confer any apparent advantage to oceanic diatoms and is therefore unlikely.

The results of Figure 3.4 illustrate that Cu limitation in the oceanic diatom was independent of Fe. Decreasing the Cu concentration in the medium from 21.4 to 8 nM decreased growth rate at a constant cellular Fe:C ratio. At the extreme, growth rate at 3 nM Cu was completely independent of Fe:C. If the only significant effect of Cu-limitation was on Fe-acquisition (through inhibition of a MCO), then we would expect a single relationship between Fe:C and growth.

A number of other metabolic uses for Cu are well documented in cellular metabolism. Notable Cu-containing enzymes include those such as Cu/Zn superoxide dismutase, and the Cu-containing electron transport protein plastocyanin (Raven et al. 1999). We have observed that superoxide dismutases in diatoms contain Mn, not Fe (Peers and Price 2004), so it is unlikely that any Fe-sparing effect would be accomplished by replacing this enzyme with a Cu-containing homologue. The interreplacement of the photosynthetic electron transport proteins plastocyanin and cytochrome c_6 (Fe-containing) may also lead to substantial metal savings (Raven et al. 1999). Photosynthetic organisms that possess both proteins produce one or the other depending on cellular metal status (Wood 1978). Diatoms (and other chromophyte algae) are thought not to possess plastocyanin (Inda et al. 1999; Raven et al. 1999), but so far only a few species from coastal regions have been examined. Strzepek and Harrison (2004) were unable to detect cytochrome c_6 in *T. oceanica*, suggesting that this species either contains very low cellular concentrations of the protein or that the protein is completely absent. Such an absence may be compensated by a Cu redox protein such as plastocyanin. Indeed, using rapid fluorescence induction techniques we have found that Cu-deficiency impairs photosynthetic electron transport in oceanic but not in coastal diatoms and that this effect is independent of Fe-nutrition (Peers and Price, unpubl.). We are currently examining the role of Cu in photosynthetic reactions of diatoms.

Finally, no discussion of important Cu-containing enzymes would be complete without mention of cytochrome *c* oxidase, the terminal enzyme of respiratory electron transport. We have not investigated the effects of Cu-deficiency on respiration rates but perhaps oceanic diatoms have a higher biomass-specific respiratory demand than coastal species which would increase their Cu-requirement.

Oceanographic relevance

Research into the importance of dissolved copper in the ocean has primarily revolved around the free cupric ion and its potential toxicity to natural populations of plankton. Algal species are known to differ greatly in their sensitivities to Cu and their abundances in natural waters are thought in part to be influenced by variations in environmental Cu concentrations (Brand et al. 1986: Mann et al. 2002). Of particular interest are the cyanobacteria, the most sensitive of the species, which are able to produce organic ligands to complex Cu and render it non-toxic. The structure of the ligands they produce is unknown, but their Cu binding strength is remarkably similar to the natural Cu ligands in the sea (Moffett and Brand 1996). One hypothesis is that the Cu-sensitive phytoplankton may regulate the bioavailability of Cu in the open ocean by secreting organic ligands, thereby modifying the chemistry of their environment to allow survival. While these ligands may be important in reducing the toxicity of Cu to cyanobacteria in the ocean (Mann et al. 2002) they also lower the biological availability of the Cu to all other organisms. Conceivably, reduced bioavailability of Cu could be deleterious to the growth of eukaryotic phytoplankton or to other species with high Cu requirements.

Very little is known about the forms of dissolved Cu that are available to eukaryotic phytoplankton for growth. Most studies show that biological activity is proportional to the free cupric ion concentration (Sunda and Guillard 1976), but some recent observations suggest that organic complexes may also be used. Hudson (1998) calculated that the diffusive supply of dissolved, inorganic Cu species was too slow to supply most diatom cultures with the Cu they require for maximal growth and concluded that some must be acquiring Cu from Cu-EDTA complexes (although the same type of calculations showed that this was not the case for *T. weissflogii*). Diatoms are able to reduce Cu(II) bound to organic ligands (Jones et al. 1987) and may be able to use these forms of Cu for growth just as they do for Fe. Given the importance of Cu in Fe acquisition, we hypothesized that Cu could be a co-limiting resource in low Fe regions of the sea.

To test this hypothesis we conducted an enrichment experiment in the basin region of the Bering Sea. Iron was the primary limiting resource, judging from the large increase in chlorophyll after addition of 2 nM Fe (Figure 3.5, LeBlanc et al. unpubl.). Copper stimulated a small, but significant increase in net chlorophyll production, an effect that was observed even in Fe-amended samples (albeit this latter effect was weakly significant). There are three possible ways of interpreting these data: addition of Cu stimulated Fe-acquisition, addition of Cu stimulated growth independently of Fe or addition of Cu inhibited grazers resulting in a net accumulation of Chl *a*. This latter explanation was proposed by Coale (1991) to account for the Cu-induced increase in plankton biomass in the subarctic Pacific Ocean. However, in a separate experiment, grazing rate was unaffected by addition of 2 nM Cu_T (R. Kudela, pers. comm.), so we suggest that, in this case, net Chl *a* accumulation was not due to grazer inhibition. Thus, in this region of the Bering Sea Cu and Fe were co-limiting resources. Our grow out results however cannot discern the mechanism by which Cu stimulated the phytoplankton. According to our lab results, at low ambient Cu concentrations, Cu addition would be expected to stimulate phytoplankton growth in the presence and absence of Fe. At present we do not know how widespread Cu-deficiency is in the Bering Sea nor whether it is a general feature of other Fe-limited waters. Collectively, our results show that Cu is required for the acquisition of Fe by diatoms and advocate a role for Cu-limitation in regulating phytoplankton growth in the sea.

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Figure 3.1. Steady state growth rates (d^{-1}) of (A-C) oceanic and (D-F) coastal strains of diatoms in Fe-sufficient and -deficient media containing different concentrations of Cu. The oceanic diatoms, *Thalassiosira oceanica* CCMP 1005 (panel A), CCMP 1003 (panel B) and CCMP 1006 (panel C) were grown with 1.29 μM Fe_T (Fe-sufficient) and 4 nM Fe_T (Fe-deficient). The coastal diatoms, *T. weissflogii* CCMP 1336 (panel D), *T. pseudonana* CCMP 1335 (panel E), and *T. minuscula* CCMP 1036 (panel F), were grown with 1.29 μM (Fe-sufficient) and 40.8 nM Fe_T (Fe-deficient). No Fe-deficient data were collected for CCMP 1036. Data points represent means and error bars the standard error (n = 6-14). Error bars are smaller than symbol when not visible.

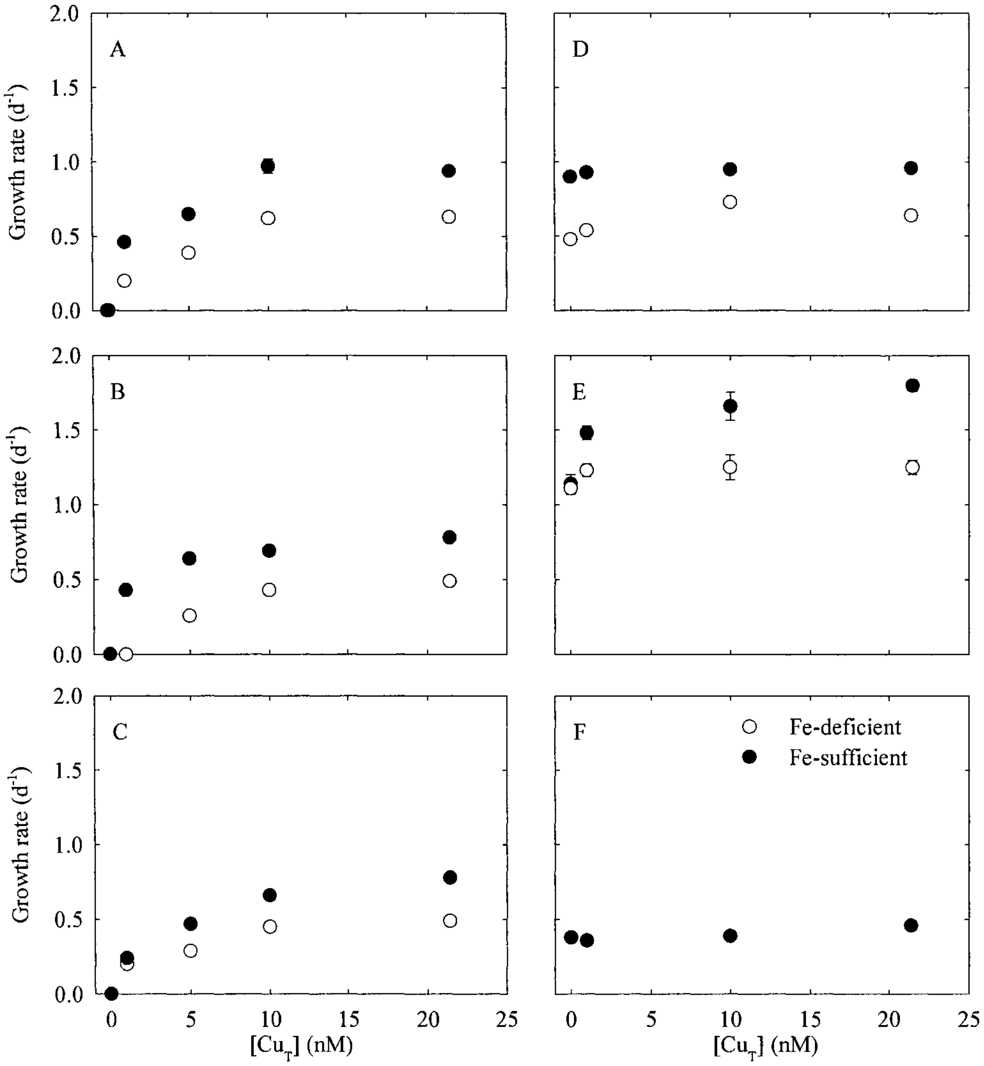
A
B
C
D
E
F
Growth rate (d^{-1})
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
[Cu$_T$] (nM)
○ Fe-deficient
● Fe-sufficient

Table 3.1. Volumetric Fe quotas (Q_{Fe}, mM Fe cell-volume) and specific growth rates (μ, d^{-1}) of a coastal (*T.w.*: *Thalassiosira weissflogii,* CCMP 1336) and an oceanic (*T.o.*: *Thalassiosira oceanica*, CCMP 1005) diatom grown at different concentrations of Fe and Cu complexed with 100 μM EDTA. Values reported are means ± standard errors.

Species	[Fe] (nM)	[Cu] (nM)	*n*	Q_{Fe} (mmol Fe l^1 cell-V)	μ (d^{-1})
T. w.	1250	21.4	9	0.78 ± 0.05	0.94 ± 0.04
	1250	1.0	6	0.88 ± 0.09	0.87 ± 0.03
	1250	0	6	0.48 ± 0.05	0.75 ± 0.03
	40.8	21.4	6	0.20 ± 0.01	0.62 ± 0.03
	40.8	1.0	6	0.13 ± 0.00	0.46 ± 0.03
	40.8	0	3	0.16 ± 0.01	0.27 ± 0.03
T. o.	1250	21.4	9	0.48 ± 0.06	0.89 ± 0.02
	1250	1.0	6	0.42 ± 0.07	0.58 ± 0.04
	40.8	21.4	3	0.15 ± 0.01	0.90 ± 0.02
	40.8	1.0	3	0.073 ± 0.004	0.59 ± 0.01
	4.0	21.4	6	0.023 ± 0.001	0.69 ± 0.03
	4.0	1.0	9	0.025 ± 0.002	0.39 ± 0.05

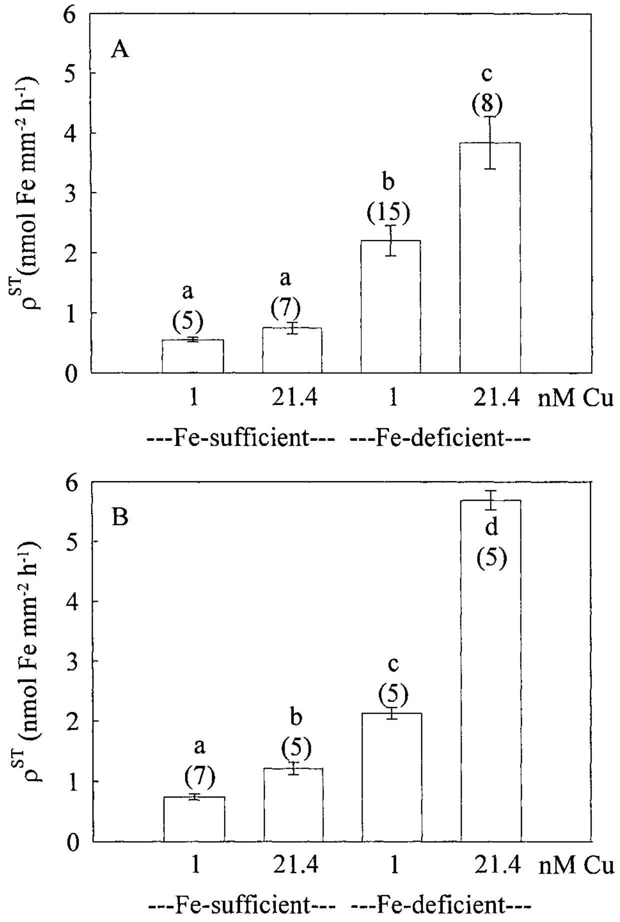

Figure 3.2. Short-term Fe uptakes (ρ^{ST}) of a coastal and oceanic diatom, *Thalassiosira weissflogii* (CCMP 1336) (A) and *T. oceanica* (CCMP 1005) (B), acclimated to different concentrations of Fe and Cu. Uptake rates were measured using 50 nM Fe equilibrated with 100 μM EDTA. Values are reported as means ± standard error. Treatments marked with different letters are significantly different (p<0.05, ANOVA, Tukey) and the numbers in parentheses represent the number of independent replicates.

Table 3.2. Effect of 1 h Cu exposure on the short-term Fe uptake rate (ρ^{ST}) of Cu-deficient cultures of *Thalassiosira oceanica* (CCMP 1005) grown in Fe-sufficient and -deficient seawater. Acclimated cultures were harvested and resuspended in growth medium with (+ Cu) and without (- Cu) Cu addition (10 nM). Iron uptake rates were measured after the cells were resuspended in seawater medium containing 50 nM ^{55}Fe equilibrated with 100 μM EDTA. Values are reported as single measurements of replicate cultures. Significance of the treatment was calculated using paired *t*-tests.

[Fe] (nM)	[Cu] (nM)	ρ^{ST} (nmol Fe m^{-2} h^{-1}) - Cu	+ Cu	% increase	significance
4.0	1.0	1.36	2.04	151	
		1.64	2.86	175	*p*=0.02
		0.80	1.31	163	
		1.90	3.24	170	
1250	1.0	0.39	0.53	135	
		0.80	1.02	126	*p*=0.11
		0.83	1.29	155	

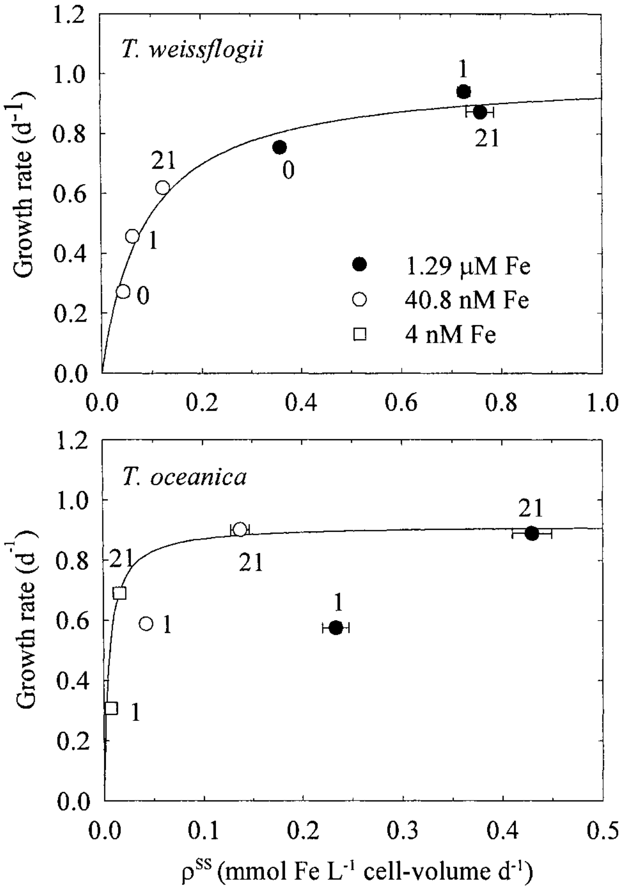

Figure 3.3. Growth rate as a function of steady-state Fe uptake rate (ρ^{SS}) of a coastal and oceanic diatom, *Thalassiosira weissflogii* (CCMP 1336) and *T. oceanica* (CCMP 1005). Copper concentrations (nM) are indicated by the numbers beside the individual data points. Plotted lines are hyperbola fit by a least squares fitting procedure (Sigmaplot 10) to all data (*T. weissflogii*) or to the high Cu (21.4 nM) data (*T. oceanica*). Data points are means $\pm$ standard errors (n=3-9).

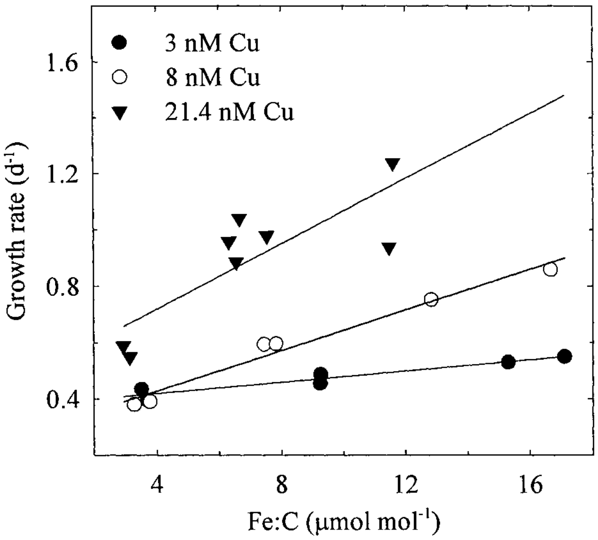

Figure 3.4. Growth rates of fully acclimated cultures of *Thalassiosira oceanica* (CCMP 1005) as a function of Fe:C ratio measured in Fe-deficient cells grown at three different concentrations of Cu. Iron concentrations ranged from 1.2 to 10 nM. Plotted lines represent best-fit regression lines with the following equations: 3 nM Cu, $y = 0.010x + 0.379$, $r^2 = 0.923$; 8 nM Cu, $y = 0.036x + 0.284$, $r^2 = 0.974$; 21.4 nM Cu, $y = 0.058x + 0.487$, $r^2 = 0.689$.

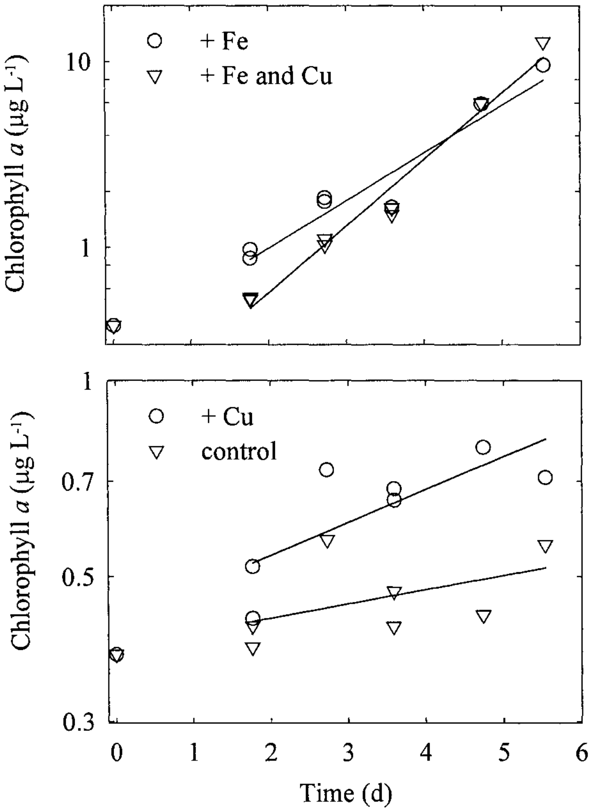

Figure 3.5. Net biomass accumulation (μg Chl *a* l^{-1}) of Bering Sea phytoplankton community following trace metal enrichment. Replicate seawater samples were amended with 2 nM Fe (+Fe), 2 nM Cu (+Cu), 2 nM Fe and 2 nM Cu (+Fe +Cu), or nothing (control) and monitored for 6 days. Each data point for each treatment was obtained from an independent replicate (single bottle). On most days two replicate bottles of each treatment were sampled for chlorophyll *a*. The linear regression lines through the data points (days 2-6) were fit using the least-squares fitting procedure of Systat 10.2. Note difference in scale between graphs.

Chapter 4. A role for copper in the photosynthetic reactions of an oceanic diatom

One of the most intriguing findings of Chapter 3 was that oceanic strains of *Thalassiosira* needed higher ambient concentrations of Cu to grow than coastal strains. Chapter 4 illustrates that this growth phenomenon is indeed due to a higher metabolic requirement. This chapter also explores the possibility that diatoms from a low Fe-environment have either acquired or retained biochemical functions that reduce their dependency on Fe, but that increase their Cu-requirements.

Prepared for submission to *Nature*

Abstract

Diatoms isolated from open ocean environments require higher concentrations of Cu to grow than their coastal counterparts. In one such species from the Sargasso Sea, *Thalassiosira oceanica*, the elevated requirement is due to a 10-fold greater metabolic need for Cu compared to a related coastal *Thalassiosira* species. Here we report that under Fe-replete growth, the oceanic diatom possesses a highly soluble, redox active Type I Cu-protein with no trace of cytochrome c_6 (cyt c_6). Protein extracts of the coastal species lack a Type-I Cu signal, but contain a Fe protein with a redox spectrum typical of cyt c_6. Photosystem II (PSII) fluorescence transients show that photosynthetic electron transport is inhibited by Cu-deficiency in the oceanic but not the coastal species. Thus Cu appears to have a constitutive metabolic role in the light reactions of photosynthesis in the oceanic diatom. These results suggest that intense selection pressure imposed by extremely low environmental concentrations of Fe has resulted in higher Cu requirements for photosynthesis in oceanic diatoms, possibly due to replacement of cyt c_6 with plastocyanin. This biochemical switch reduces the demand for Fe, but increases requirements for Cu which is also at near limiting concentrations in the open sea.

Results and Discussion

Concentrations of dissolved Cu in the surface waters of the open ocean average between 1-3 nM but can be as low as 0.4 nM (Bruland 1980, Coale and Bruland 1988, Moffett et al. 1990). Similar concentrations of this metal limit the growth of oceanic, but not of coastal diatoms (Figure 4.1, Peers et al 2005). This observation deviates from the pattern previously described for all other phytoplankton micronutrients. Typically, phytoplankton isolated from waters with very low concentrations of metals, like those found in the open ocean, have lower requirements for these micronutrients than species isolated from comparatively metal rich seas closer to the continental shelves (Brand et al. 1983, Sunda et al. 1991, Tortell and Price 1996). This pattern is clearly demonstrated for the Fe-requirement of different phytoplankton as shown in Figure 4.1A. The discrepancy we observe here appears to be due to a higher metabolic Cu requirement for growth in the oceanic strains (Figure 4.2). Indeed, when *T. oceanica* (isolated from the metal impoverished Sargasso Sea) is grown under Cu limiting conditions intracellular cellular quotas (Q_{Cu}) are ten times greater than those of a comparably Cu-deficient coastal strain, *T. weissflogii* (2.76 & 3.53 μM vs. 0.28 ± 0.16 μM , $n = 2$ and $n = 4$ ± stdev., respectively). These values represent the first estimates of metabolic Cu in diatoms.

Through a series of selective precipitations we identified a highly soluble, Type I Cu protein in protein extracts from *T. oceanica* that was not present in similar preparations from *T. weissflogii* (Figure 4.3). Instead, reduced minus oxidized spectra of protein extracts from *T. weissflogii* showed a peak at ~554 nm. This maximum corresponds to cyt c_6 (Quinn and Merchant 1998), which is believed to be the sole electron transport protein between the cytochrome b_6/f (cyt b_6/f) complex and PSI in diatoms and other chlorophyll *c* containing algae (Raven et al 1999). We found that Fe-replete *T. oceanica* did not contain measurable cyt c_6, as reported by Strzepek and Harrison (2004). Using an extinction coefficient of 4.5 mM^{-1} cm^{-1} for a Type I Cu protein (Quinn and Merchant 1998) and estimates of the total initial biomass used for partial purification we calculate that *T. oceanica* contains ~ 3 μM cellular Cu in these protein(s). So the difference in Q_{Cu} between the coastal and oceanic diatoms can be attributed to the presence of a highly soluble Type-I Cu protein(s). The Q_{Cu} measured in *T. weissflogii* represents basal metabolic requirements for Cu within enzymes such as cytochrome *c* oxidase (Da Silva and Wlliams 2001) and the multicopper oxidase required for Fe-transport (LaFontaine et al. 2002, Peers et al. 2005). Indeed, Cu limitation in the coastal strain induces secondary Fe-deficiency by inhibiting Cu-dependent Fe-uptake pathways (Peers et al. 2005).

According to changes in the fluorescence transient, Cu-deficiency in the oceanic, but not in the coastal strain causes a lesion in electron transport downstream of PSII (Figure 4.4). Transients in the Cu-deficient cells indicate that PSII reaction centers saturate faster than controls. More strikingly, Cu-limitation increases the duration at which fluorescence is at 98% of its maximum by nearly 10 times, from 300 ± 10 ms in metal replete diatoms to 2200 ± 100 s (*n* =3). Thus, the results suggest that components subsequent to PSII are being oxidized slowly under Cu-deficiency compared to metal replete conditions. This change happens regardless of Fe-status (data not shown). These transients suggest a lesion in electron transport downstream of PSII or a Cu electron carrier. A similar pattern has been found in mutants of the green alga *Chlamydomonas*, which have lowered levels of PSI (Moseley et al. 2000). Using a separate fluorescent technique (pulse-amplitude modulated fluorescence: PAM) we observed that the maximum potential efficiency of PSII (F_v/F_m) of Cu-limited diatoms is identical to that of control cultures (0.55/0.55 and 0.55/0.56, respectively, *n*=2). This indicates that there is little damage to PSII. However, the realized efficiency of PSII (assayed under conditions of constant illumination approximating growth irradiance) is reduced by low Cu (0.12/0.14 vs. 0.31/0.33 for metal replete conditions, *n*=2), further illustrating a bottleneck in photosynthesis downstream of PSII. Meanwhile, Cu-limitation had very little affect on the fluorescent transient in the coastal diatom, *T. weissflogii*, suggesting no direct role for Cu in photosynthesis (Figure 4.4). No PAM measurements were made on this species.

Work by Strzepek and Harrison (2004) convincingly demonstrated that selection pressures in low-Fe waters cause a reduction in the biochemical Fe-demands of the oceanic diatom investigated here. *T. oceanica* has constitutively reduced the Fe-rich components of photosynthesis, the photosystem I (PSI) and cyt b_6/f complexes, compared to *T. weissflogii*. These results explain the observed differences in the Fe-contents of coastal and oceanic strains of phytoplankton (Figure 4.1A, Ryther and Kramer 1961, Sunda et al. 1991, Maldonado and Price 1996) and support the observed physiological changes in constituents associated with Fe-limitation in other photosynthetic algae (Spiller and Terry 1980, Moseley et al. 2002 and Desquilbet et al. 2003). In this census of photosynthetic components, *T. oceanica* did not appear to contain cyt c_6 (Strzepek and Harrison 2004), which, in higher plants and green algae, is known to have a functional homologue in the Cu-containing protein, plastocyanin (PC) (Raven et al 1999). The absence of the redox peak corresponding to cyt c_6 in *T. oceanica* grown in Fe-replete conditions coupled with the presence of the Type-I Cu protein and the fluorescence behaviour described above strongly suggests the presence of PC in the oceanic diatom. Using measured ratios of cyt c_6 : PSI found in *T. weissflogii* (~1.5, Strzepek and Harrison 2004) and published cellular concentrations of PSI in *T. oceanica,* we estimate that PC concentration, if present in *T. oceanica*, would be 2.2-4.8 μM. These values are very similar to our estimates of the cellular concentration of the Type I Cu protein(s) measured above. The replacement of Fe-containing cyt c_6 with PC would result in the reduction of total cellular Fe demand by roughly 10% (using PC concentrations estimated above and total Q_{Fe} for *T. oceanica* from Maldonado and Price 1996).

Besides reducing total Fe-demand, an additional benefit of using PC instead of cyt c_6 maybe the Fe-independent regulation of photosynthesis. Under Fe-deficient conditions, cellular concentrations of the PSI and cyt b_6/f complexes in *T. weissflogii* are higher than those constitutively expressed by *T. oceanica*, but growth rates for the coastal species drop considerably while growth of oceanic strain remains unchanged. The presence of PC in *T. oceanica* would allow for rapid turnover of the PSI complex that is independent of Fe-status perhaps explaining how the oceanic species maintains high photosynthetic rates with very little PSI. Indeed, it has been proposed that linear electron transport in higher plants is controlled by the amount of PC relative to PSI (Schottler et al. 2004). In this model, a bottleneck to electron flow upstream of PSI prevents over-reduction and damage to this complex. Perhaps expression of PC is another way in which oceanic phytoplankton have evolved to low Fe.

Changes in ocean chemistry over geologic time are hypothesized to be responsible for the differences in the evolved biochemical metal requirements of phytoplankton groups (Saito et al. 2003, Quigg et al. 2003). For instance, concentrations of 0.1 μM Fe in the *Proterozoic* ocean allowed organisms to exploit the chemical versatility of Fe for a host of biochemical reactions and they evolved a large dependence on this element for growth (Da Silva and Williams 2001). These requirements remain imprinted in the light reactions of photosynthesis despite the fact that today Fe is a limiting resource in the ocean (Greene et al. 1991, Tsuda et al. 2003). As [Fe] fell, oxygenation of the surface ocean also increased the solubility of Cu (Saito et al. 2003) providing a suitable substrate for biochemical substitutions for Fe for those algae evolving in the new environment. Chlorophyll *b* containing organisms (green algae and prasinophytes, the "green line") are reported to have higher cellular Cu concentrations than chlorophyll *c* containing organisms (diatoms and coccolithophorids, the "red line", Quigg et al. 2003, Ho et al. 2003) leading to the suggestion that these groups first flourished in distinctly different chemical environments (Katz et al. 2004). The two lines have plastids derived from two separate endosymbiotic events and their Cu-requirements appear to segregate with the occurrence of PC (Raven et al. 1999). So the putatative presence of PC in the oceanic diatom and its apparent absence from the coastal strain is particularly surprising as the two species investigated here are closely related according to rRNA sequences (Ambrust and Galindo 2001). Our results raise the question of why does PC appear to be absent from the coastal species?

If the endosymbiont progenitor of the "red line" contained PC, then it may have been lost in algae that exist in high Fe environments (hence the apparent constitutive expression of cyt c_6 in *T. weissflogii*; Strzepek and Harrison 2004, Inda et al. 1999). It may be that low concentrations of bioavailable Cu, indeed nearly all Cu is bound to ligands in coastal seawater (Moffett et al. 1997), coupled with higher relative bioavailable Fe have allowed for the loss of PC in coastal diatoms. Conversely, oceanic diatoms selected in an environment with comparable concentrations of Cu but with extremely low concentrations of Fe may have acquired PC through lateral gene transfer, a possibility as viruses of *Prochlorococcus* have been shown to contain PC genes (Lindell et al 2004). We have purified a protein from *T. oceanica* using immunoprecipitation with an antibody raised against *Chlamydomonas reinhardtii* PC (provided courtesy of S. Merchant, UCLA). Its N-terminal sequence agrees with no PC sequence published thus far, but internal *de novo* sequence fragments suggest some similarity to green algal PC (data not shown). The purification of the putative PC is currently underway.

We propose that the extremely low concentrations of Fe in the open ocean have selected for the use of other metals to substitute for scarce Fe. In diatoms it appears as though some of these substitutions have become constitutive in the case of MnSOD (Peers and Price 2004) or the apparent use of PC in *T. oceanica* reported here. This increased demand for other trace metals will influence their biogeochemical cycling in Fe-poor waters and possibly lead to co-limiting conditions for phytoplankton growth.

Methods

Axenic cultures of *Thalassiosira oceanica* (strain CCMP 1005) and *Thalassiosira weissflogii* (strain CCMP 1336) were obtained from the CCMP West Boothbay Harbour ME. Cultures were grown in artificial seawater medium (AQUIL) in acid-cleaned polycarbonate flasks at 20°C and under 180 μmol quanta m^{-2} s^{-1} light supplied by Cool White fluorescent bulbs. Growth rates, cell volumes and cell densities were all calculated as previously described (Peers and Price 2004).

Cu-quotas

Diatom cultures were grown in semi-continuous batch cultures in 0.5 l of medium containing 21.4, 2.5, 1 or 0 nM Cu and a full complement of all other trace metals. Details of medium preparation and free Cu concentrations are given in Peers et al. (2005). Diatoms were fully acclimated to given growth conditions (<10% variation in growth between successive transfers) before analysis. Cells were harvested onto acid cleaned (10% TraceMetal grade HCl, Fisher Scientific) 25 mm diameter, 2 μm pore-size polycarbonate filters housed in an acid-cleaned polysulfone filtration apparatus using a gentle vacuum. Collected cells were rinsed with 25 ml AQUIL medium containing 100 μM EDTA with no added metals. Filtered diatoms were digested in acid cleaned (10 % HNO_3, TraceMetal grade, Fisher Scientific) quartz crucibles with the addition of 5 ml Baseline grade HNO_3 (Seastar Chemicals). Digests were covered and heated to 80°C. After 2 h, the filter was removed from the clarified solution and the sample was gently taken to dryness overnight. Residue was resuspended in a 10% solution of Baseline HNO_3 and stored in acid-cleaned Teflon vials until analysis by atomic absorption spectroscopy (Perkin Elmer Analyst 800 equipped with a Zeeman Graphite Furnace). Filter blanks, treated as above, were below detection limits. Copper concentrations in the digest were normalized to total filtered cell volume.

Chlorophyll fluorescence

The fluorescent transient of PSII was measured using a Hansatec Plant Efficiency Analyzer (PEA Hansatec, Norfolk, England). Mid-exponential growth phase cultures were dark acclimated for at least 15 min prior to filtering 30-60 ml of culture onto Millipore AP20 glass fibre filters. The filter was immediately placed into a leaf-disc adaptor supplied by the manufacturer and exposed to a saturating pulse of red light (650 nm) at an intensity of 3000 μmol quanta m^{-2} s^{-1} supplied for 20 s. Fluorescence readings are reported as the fluorescence (F) normalized to the maximal signal (F_m). Other fluorescence induction parameters were measured using a pulse amplitude modulated fluorometer (Water PAM, Heinz Walz, Germany). Diatoms were concentrated from mid-exponential cultures by filtration onto 25 mm diameter, 2 μm pore size filters (Poretics). Filtered cells were immediately resuspended in approximately 5 ml of AQUIL medium and dark acclimated for at least 10 min prior to measurement. The maximum quantum efficiency of PSII (F_v/F_m) was measured in dark acclimated cells using a saturating pulse of 3000 μmol quanta m^{-2} s^{-1} supplied for 600 ms. Actinic illumination of samples with 150 μmol quanta m^{-2} s^{-1} of light allowed for the measurement of the realized efficiency of PSII (Φ_{PSII}, both parameters were calculated according to Juneau et al. 2002).

Visible spectroscopy of redox active metalloproteins

Diatoms were grown under metal replete conditions in 20 l carboys with vigorous bubbling. Late-exponential growth phase cultures were harvested by positive pressure onto 142 mm diameter, 3 μm pore size polycarbonate filters. Cells were quickly scraped off the filters and stored in liquid nitrogen until further handling. Spectra were generated from protein preparations as described by Navarro et al. (2004). Diatoms were resuspended in 50 mM KPO_4 buffer, pH 7.0 and this slurry was passed through an ice-cold Yeda press 3 times. NaCl (50 mM, final concentration) was added to the slurry which was then sonicated for 1 min on ice using a Branson sonicator equipped with a micro-tip. Protein concentrations in this homogenate were 214 mg ml^{-1} and 2.25 mg ml^{-1} for *T. oceanica* and *T. weissflogii*, respectively (BCA assay, Sigma). The homogenate was centrifuged for 20 min at 10 000 x *g* and the pellet discarded. The supernatant was put through a series of selective precipitations in the order of 0.01 M streptomycin sulphate, 50% saturated ammonium sulphate and finally 98% saturated ammonium sulphate. After addition of each salt the solution was allowed to stir, on ice, for 1 h before centrifugation for 20 min at 12 000 x *g* and the resulting supernatant was used in the subsequent precipitation. The final pellet was resuspended in 0.01 M Tris-HCl (pH 7.5) and allowed to dialyse at 4^{o}C overnight against the same buffer. The resulting protein preparation was then centrifuged for 10 min at 16 000 x *g* and the soluble protein was used for spectrophotometry (Cary 1E, 1cm pathlength, quartz cuvette).

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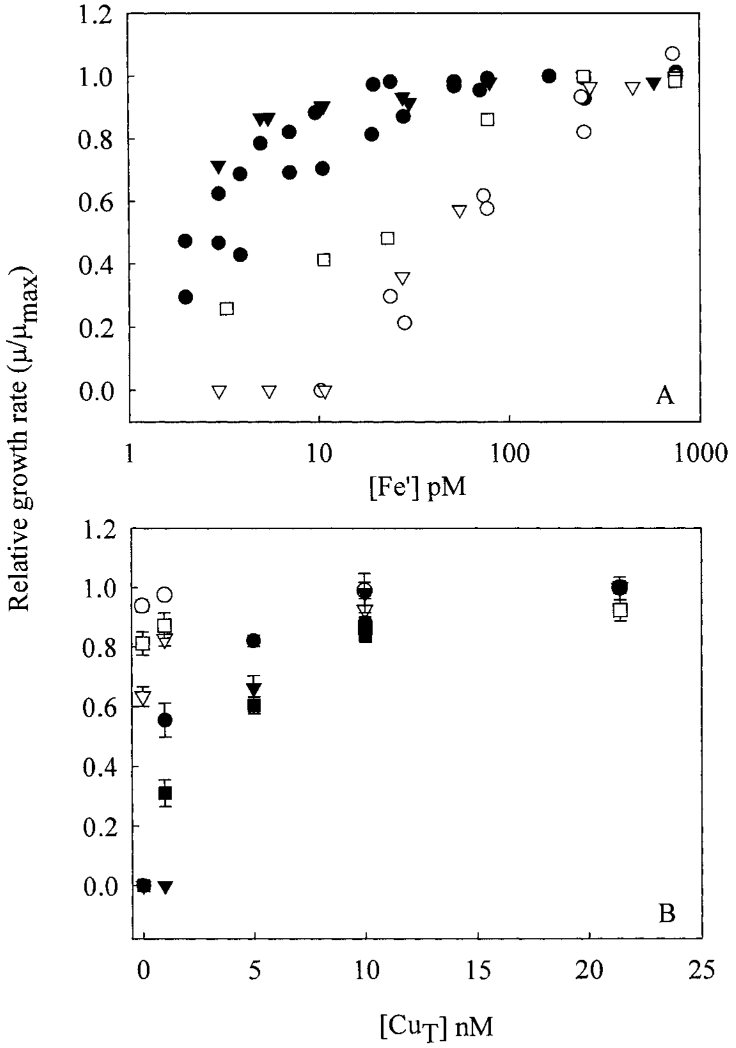

Figure 4.1. Relative growth rates of oceanic (closed symbols) and coastal (open symbols) phytoplankton cultured in varying concentrations of Fe (A) or Cu (B). Iron concentrations are reported in terms of total inorganic iron (Fe') and different algal species are represented as different symbols: data taken from Sunda and Huntsman (1996). Copper concentrations are reported as total added Cu (Cu_T) with each symbol representing the average ± standard error growth rate of a different species (data taken from Peers et al. 2005).

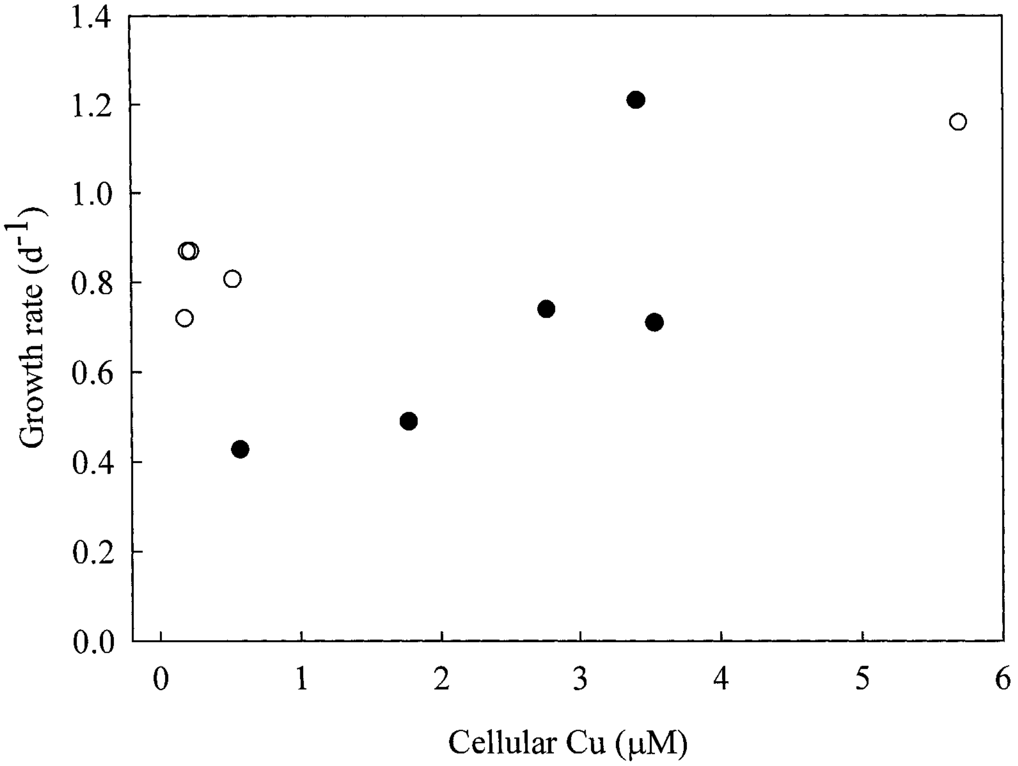

Figure 4.2. Cellular copper concentrations of the coastal *Thalassiosira weissflogii* (open circles) and oceanic strain *T. oceanica* (filled circles). Diatoms were cultured in either Cu-sufficient conditions (21.4 nM Cu_T) or under Cu deficient conditions (no added Cu for *T. weissflogii* and either 1 or 2.5 nM Cu_T for *T. oceanica*).

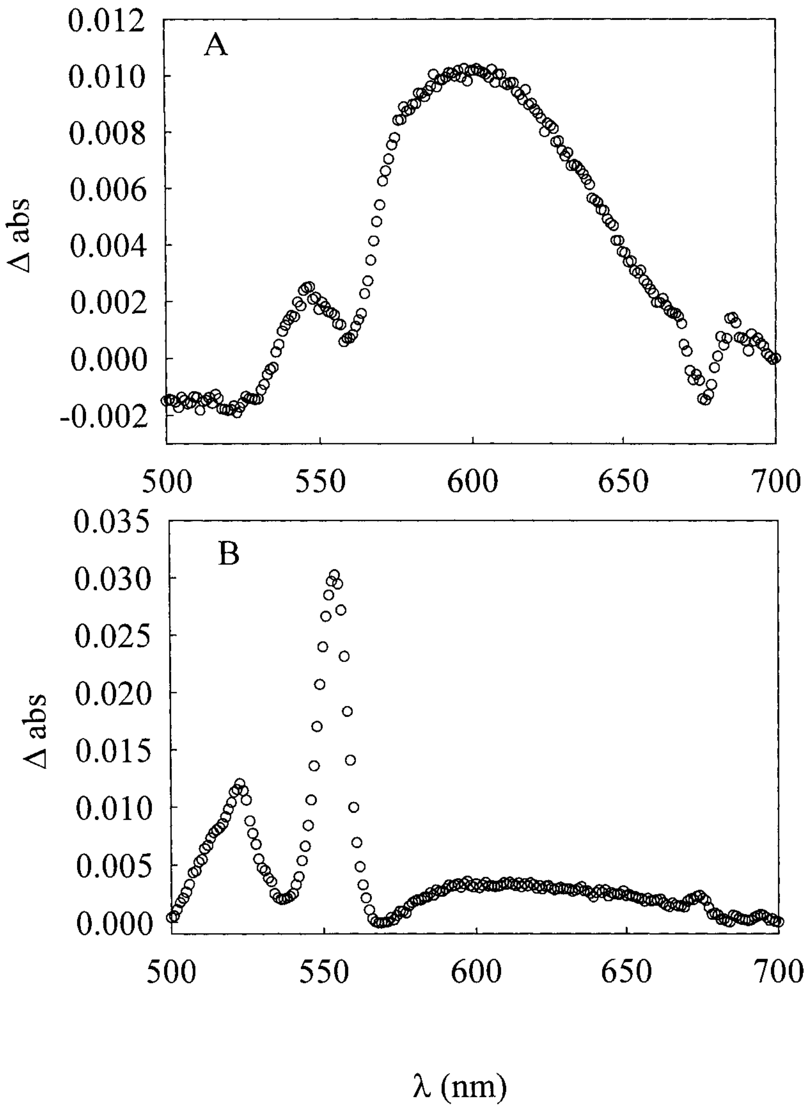

Figure 4.3. Identification of redox active metallo-proteins in extracts of *T. oceanica* (A) and *T. weissflogii* (B). Crude protein homogenates were subjected to a series of selective precipitations (see Methods) and then reduced with 1 mM sodium ascorbate or oxidized with 1 mM potassium ferricyanide(*T. oceanica*: oxidized minus reduced, *T. weissflogii*: reduced minus oxidized). Curves represent the average of 3 scans.

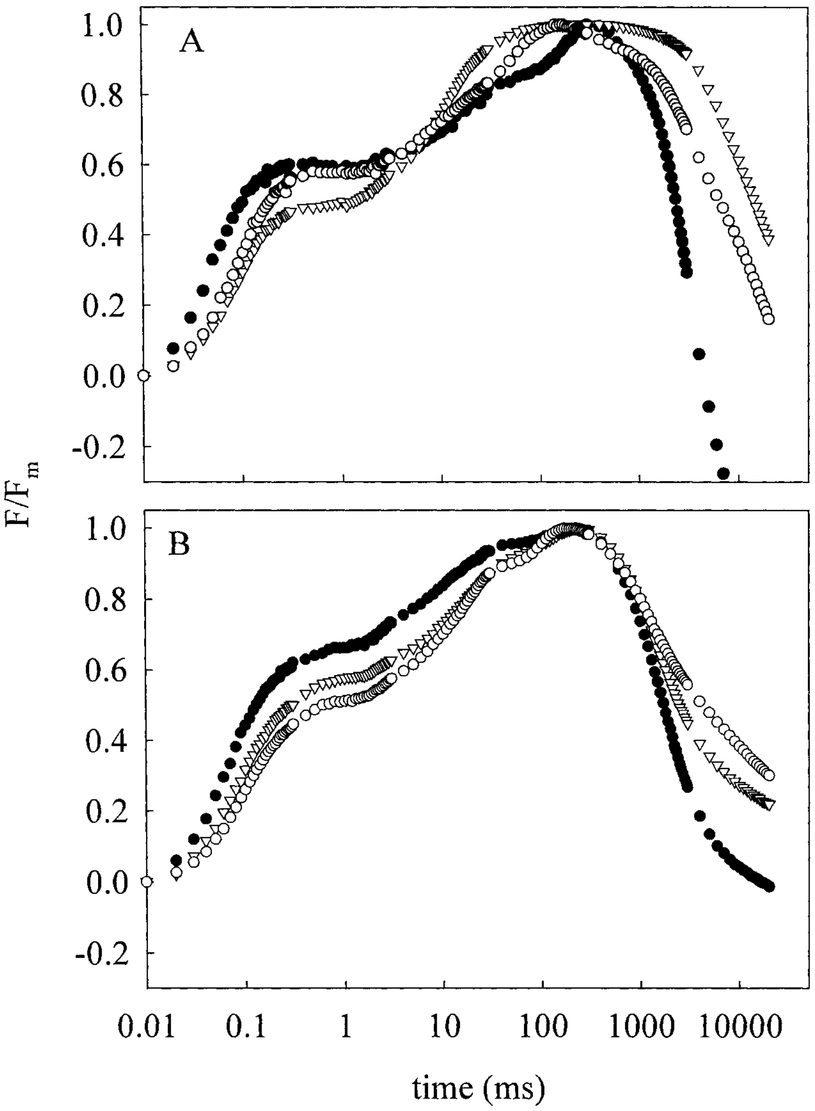

Figure 4.4. Photosystem II (PSII) fluorescence transients of *Thalassiosira oceanica* (A) and *Thalassiosira weissflogii* (B). Symbols are representative transients from Fe-deficient (solid circles), Cu-deficient (open triangles) or control (open circles) diatom cultures. Fluorescent transients of colimited (both Fe and Cu) cultures were identical to Cu-deficient traces from each species respectively (data not shown).

Synopsis

This thesis employs a multidisciplinary approach to characterize changes in the trace metal requirements of marine diatoms in response to low Fe. Using a combination of physiological, biochemical and ecological methods, I establish that diatoms require more Mn and Cu when grown in low Fe environments. The major findings of this thesis are summarized as follows: Increases in Mn content are necessary, in part, to activate the SOD isozymes required to combat oxidative stress invoked by Fe-deficiency. Manganese-deficiency also increases oxidative stress. How such oxidative damage reduces the fitness of diatoms remains unclear. Copper is required for the efficient acquisition of Fe in diatoms, presumably in a multi-copper oxidase. Finally, diatoms isolated from metal-poor habitats have higher metabolic requirements for Cu compared to related species that inhabit metal-rich environments. The additional Cu requirement appears to be for a highly soluble, Type-I Cu protein associated with photosynthesis, strongly suggesting plastocyanin. The lack of cytochrome c_6 in the oceanic species implies that the type-I Cu protein is its functional replacement in electron transport.

Chapter 3 illustrates that Fe and Cu enrichments in the Bering Sea stimulate growth of the indigenous phytoplankton, suggesting that metals other than Fe may be important in structuring the ecology of HNLC communities. To observe the influence of Mn and Cu in natural communities, we must target the physiological processes investigated here for measurement in the field.

Future directions

I propose that Mn and Cu play a key role in antioxidant defence and electron transport in Fe-limited diatoms. During unperturbed growth or following the addition of Fe, the community may not be able to acquire adequate Mn or Cu to activate these pathways. While phytoplankton production is clearly stimulated by Fe in HNLC regions, diatoms may not be completely released from metal stress. The inhibition of electron transport or the accumulation of oxygen radicals will make diatoms more vulnerable to photoinhibition.

Photoinhibition of PSII, measured as a reduction in the F_v/F_m parameter, is due to oxidative damage of the complex (Niyogi 1999). This metric is low in resource-limited seas and its recovery following Fe-addition to natural communities is commonly used as an indicator of phytoplankton revitalization. Interestingly, addition of Fe alone to some phytoplankton communities does not allow for full recovery of PSII efficiency (Greene et al. 1994, Behrenfeld et al. 1996, and Tsuda et al. 2003). While not discussed in his report, Cullen (1991) shows that additions of Cu in addition to Fe reduced the incidence of photoinhibition in Equatorial Pacific plankton. These examples suggest that a complete recovery of photosynthesis is not necessarily dependent on Fe alone. Observations and experiments in natural systems may reveal that the processes described in this thesis occur in the sea.

Selection in Fe-deficient environments has resulted in diatoms that require very little ambient Fe to grow but only recently have the biochemical mechanisms behind this adaptation been characterized. My work expands this knowledge to show that other transition metals are essential for acclimation to low Fe. The increased Mn- and Cu-requirements likely contribute to the survival of diatoms in the open ocean and, in turn, may profoundly influence how vast areas of the world's oceans function in the absence of Fe.

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Appendices

Appendix 1. Purification of superoxide dismutase from *Thalassiosira weissflogii*

Superoxide dismutase was purified from approximately 80 l of *T. weissflogii* cultures. Cells were grown in sterilized coastal seawater supplemented with NO_3^-, PO_4^{3-} and SiO_4^{2-} equal to the concentrations found in AQUIL. Cultures were bubbled vigorously with air filtered through sterile 0.2 μm pore-size Teflon filters (Whatman). Once the cultures reached stationary phase, cells were harvested with positive pressure onto several 142 mm, 5 μm pore size polycarbonate filters. The resulting cell paste was scraped off the filters and frozen in liquid nitrogen for several days before further manipulation. Twenty g of cell paste was resuspended in 60 ml ice cold Buffer A (50 mM potassium phosphate buffer (KPO_4), 0.5 mM EDTA, pH7.8) containing 0.1% Triton X-100. Cells were disrupted, on ice, by sonication using a Branson Sonifier on full power (50% duty cycle) for 3 x 1 min bursts with breaks to ensure the homogenate did not heat significantly. Some of this homogenate was reserved for later experiments involving total protein and immuno-quantification. The suspension was centrifuged at 144000 x g for 60 min. The supernatant was carefully removed for ammonium sulphate precipitation and the cell debris was discarded. Two ammonium sulphate precipitations were performed; 35% followed by 45% saturated ammonium sulphate (pellets were collected by spinning at 10000 x g for 10 min). Pellets were discarded after each cut. The remaining proteins were precipitated using 80% saturated ammonium sulphate, the supernatant was discarded and the proteins were resuspended in 20 ml Buffer B (10 mM KPO_4, 0.1 mM EDTA, pH 7.8). The solution was dialyzed overnight against Buffer B (2 l with one buffer change, using Spectropore membrane tubing (Fisher), 3500 molecular weight cut-off) and any remaining precipitated protein was removed by centrifugation for 25000 x g for 10 min. All subsequent chromatography work was performed at 4°C using a BioRad LPLC pump and fraction collector. The protein preparation was then loaded onto a DEAE cellulose (Sigma) column pre-equilibrated against Buffer B. Proteins were eluted from the column with a linear gradient of 0.1 M KCl to 0.4 M KCl in Buffer B (360 ml total volume) followed by 40 ml 0.1 M KCl, 80 ml 0.2 KCl and then 80 ml 0.4 KCl (all in Buffer B). Collected fractions were sub-sampled and assayed for SOD as described in Chapter 2. Fractions containing SOD activity were pooled and concentrated using an Amicon filter (Millipore, 5000 MWCO). Concentrated proteins were dialyzed overnight against 20 mM KPO_4, pH 7.2, as described above. This preparation was loaded onto a second column of hydroxyapatite (BioRad) equilibrated with the same buffer. Proteins were eluted from the column using a linear gradient of 10 – 200 mM KPO_4 (200 ml total volume, pH 7.2). Fractions containing SOD activity were concentrated using Centricon-10 ultrafilters (Millipore) and then dialyzed against buffer B. This crude purification contained three isoforms of SOD and a few contaminating proteins. To further separate SOD, the preparation was then loaded onto a native PAGE (12 % Acrylamide) PREP Gel (Biorad) and proteins were eluted at 160 V. Fractions with SOD activity were sub-sampled to establish the separation of isoforms using native PAGE (as described in Chapter 1). Fractions containing one isoform of SOD (assayed as described in Chapter 1) were concentrated and submitted for N-terminal sequencing (see below). The remaining SOD was pooled for polyclonal antibody production.

N-terminal sequencing

Three isoforms of *T. weissflogii* SOD, separated as described above were submitted for amino-terminal sequence analyses at the Sheldon Biotechnology Center of McGill University. Purified protein was deposited onto PVDF membrane and amino acid sequence was determined via an Edman degradation protocol using an Applied Biosystems Procise Sequencer. The N-terminal amino acid sequences of the three SOD isoforms were as follows:

Fast migrating isoform - FRELAXDDPE
Middle isoform - DIPASIQ(X)AR
Slow migrating isoform - DIPASI(G)A

Antibody generation

Polyclonal antibodies against SOD were raised in rabbits according to standard protocols (Harlow and Lane 1998).

Western Blots

Approximately 1 μg of partially purified *T. weissflogii* SOD, prepared as described above, was used for immunodetection on membranes. Protein isoforms were either isolated using native PAGE or denaturing PAGE techniques (Chapter 1 and Harlow and Lane 1998, respectively). Proteins were transferred onto PVDF membranes using standard protocols (25 mM Tris, 190 mM Glycine with 50% methanol for 14 h at 30 V). Native SOD was also transferred onto nitrocellulose using a slot-dot apparatus that filters a protein solution across the membrane using a light vacuum. Membranes were blocked with blocking buffer (5% w/v Carnation non-fat milk in Tris-buffered saline, TBS) over night at 4^{o}C or for 1 h at room temperature. All subsequent manipulations were performed at room temperature and all antibody dilutions and rinses employed blocking buffer. Membranes were then challenged with primary antibody contained in rabbit serum diluted 1:400. Membranes were rinsed for 3 x 5 min and then incubated for 1 h with secondary antibody (goat anti-rabbit IgG, conjugated to alkaline phosphatase, Southern Biotechnology,) diluted 1:3000 (1:4000 for slot blot). Membranes were rinsed for 4 x 5 min followed by 3 quick rinses with alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$ in 100 mM Tris, pH 9.5). Alkaline phosphatase was localized using standard procedures (Harlow and Lane) and membranes were rinsed in distilled water to end the reaction. Bands were dark purple on a lightly stained background.

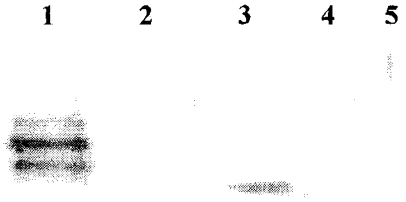

Figure A.1.1 – Antibody detection of *Thalassiosira weissflogii* SOD. Lane 1 shows SODs resolved with native PAGE techniques and detected with post-immune serum. Lanes 2 and 3 show SOD isoforms resolved using SDS PAGE and detected with pre- and post-immune serum, respectively. Lanes 4 and 5 are slot-blots of native SOD preparations using pre- and post-immune serum, respectively.

Appendix 2. In vitro metal replacement study of *Thalassiosira weissflogii* SOD.

The three isoforms of SOD from *T. weissflogii*, purified as described in Appendix 1, were tested for metal specific activity. SOD preparations were diluted 1:6 in buffer containing 10 mM Tris, 3M guanidine and 20 mM 8-hydroxyquinoline sulfonate. The pH was adjusted to 3.1 as specified, but the pH immediately began to rise. After 30 min the solution was placed in 10,000 MWCO dialysis tubing and dialyzed overnight against 10 mM Tris, 3M guanidine, 1 mM EDTA (pH 7.8). All buffer exchanges were kept at 4°C. Samples were then dialyzed against 50 mM Tris, 0.1 mM EDTA (pH 7.8) for 5 h. At this point, SOD was removed from the dialysis tubing and split into 3 different treatments. Apo(SOD) was immediately frozen (-30°C), Fe(SOD) was dialyzed against 50 mM Na acetate, pH 5.5 with 1 mM $Fe(SO_4)_2(NH_4)_2$, and Mn(SOD) was dialyzed against 50 mM Tris, pH 8.1, with 1 mM $MnCl_2$. After 24 h in their respective treatments, all samples were dialyzed for a further 24 h against 50 mM Tris, 1 mM EDTA (pH 7.8). Samples were removed from the dialysis tubing and frozen (-30°C) for SOD activity measurement on a later date (as described in Chapter 2).

Table A.2.1. *In vitro* SOD activities with different metal cofactors. SOD purified from *Thalassiosira weissflogii* was denatured and demetalated and then refolded in the presence of 1.0 mM $Fe(SO_4)_2(NH_4)_2$, 1.0 mM $MnCl_2$ or no additional metal (Apo). Activities are expressed as a percentage of activity of the native enzyme before metal stripping. MnSOD from *Escherichia coli* was used as a control.

Metal treatment	*T. weissflogii* SOD	*E. coli* MnSOD
Apo	20%	0%
Mn	90%	100%
Fe	13%	0.5%

Appendix 3. Enzyme-linked immunno-sorbent assay (ELISA) for *Thalassiosira weissflogii* superoxide dismutase

In an effort to directly quantify the contribution of SOD to total cellular metal quota, I developed an ELISA assay that is able to detect very low concentrations of SOD. The general method is a sandwich style technique that first immobilizes antibody on the well plate before being challenged by the antigen. This method is useful when the antigen source is in a rich protein matrix (such as crude cell homogenate) which can out-compete the antigen of interest for protein binding sites on the solid support. The method is based on the protocol of Suzuki et al. (2002).

All IgG used in this protocol was purified from immunized rabbit serum using the Pierce ImmunoPure protocol as described by the manufacturer. The secondary antibody in this assay is biotinylated polyclonal anti-SOD IgG, which was prepared as follows. IgG (1 mg ml^{-1}) was dialyzed overnight against 1 l of 0.1 M $NaBO_3$ (pH 8.8) at 4^{o}C. Biotin-N-succinylamide (100 μg, all chemicals from Sigma unless specified) was dissolved in DMSO and added to the IgG and kept on ice for 3.5 h. The reaction was stopped with the addition of 1 M NH_4Cl (final concentration). The biotinylated IgG was dialyzed against 500 ml of Tris-buffered saline solution overnight. The resulting solution was frozen in 100 μl aliquots at -30^{o}C. Protein samples were pre-cleared using pre-immune serum before analysis to remove any non-specific binding.

Wells of a 96-well plate (Immunolon 2HB, Thermo LabSystems) were coated overnight with 100 μl of primary antibody (2 μg ml^{-1} IgG in freshly made 0.05 M $NaCO_3$, pH 9.6) at 4^{o}C . Following two quick rinses with distilled water, residual protein binding sites were blocked with 300 μl undiluted SuperBlock in Tris-Buffered Saline (TBS, Pierce) for 3 x 5 min. Plates were rinsed with SuperBlock diluted 1:10 in TBS and all subsequent dilutions and rinses used this buffer. 100 μl of either *T. weissflogii* homogenate, SOD standards or buffer (blanks) were incubated at room temperature for two h with gentle agitation.

Proteins were rinsed from the plates and wells were rinsed for 3 x 5 min with agitation. IgG-Biotin (250 ng ml^{-1}) was then added to the sample and incubated for 2 h at room temperature. Wells were rinsed as previously described and the wells were incubated with a 1:200 000 dilution of avidin-horseradish peroxidase conjugate for 15 min. Plates were rinsed twice as described above and then 2 x 5 min with TBS alone. Finally, plates were rinsed twice with freshly made 0.1 M citrate (pH 5.0) and then 100 µl of enzymatic substrate reagent was added (60% w/v o-phenylenediamine and 0.01% H_2O_2 v/v in citrate buffer). The horseradish peroxidase reaction was stopped with the addition of 50 µl 1 N sulphuric acid (about 30 min total reaction time). The absorbance at 490 nm was measured in a microplate reader.

Using a dilution series, the linear portion of the standard curve was found to lie between 11.7 and 1.34 ng ml^{-1} SOD. By dilution of *T. weissflogii* protein to a concentration of 2.43 µg ml^{-1} we found that the quantity of SOD was within this linear range and corresponded to a concentration of 3.85 ng ml^{-1}.

Table A.3.1. Contribution of SOD to total metal quota in *Thalassiosira weissflogii*. Concentrations of SOD in crude cell homogenates from *T. weissflogii* were estimating using a Sandwich ELISA technique using polyclonal antibodies developed against the 3 isoforms of SOD described here. The cellular concentration of Cu/Zn SOD from *Saccharomyces cerevisiae* is reported for comparison.

SOD/total protein (mg g^{-1})	1.59
Protein $cell^{-1}$ (g l^{-1})[a]	60
Cell volume (fl)[a]	1000
$[Mn]_{SOD}$ (μM)	7.5-15
$[Mn]_{PSII}$ (μM)[b]	30-60
Q_{Mn} (μM)[c]	11-46.5
[Cu/Zn SOD] in *Saccharomyces* (μM)[d]	10

[a] Average values from measurements reported in this thesis and Price (2005).

[b] Calculated from Strzepek and Harrison (2004), assuming 4 Mn per PSII complex.

[c] From Harrison and Morel (1986).

[d] Rae et al. (1999)

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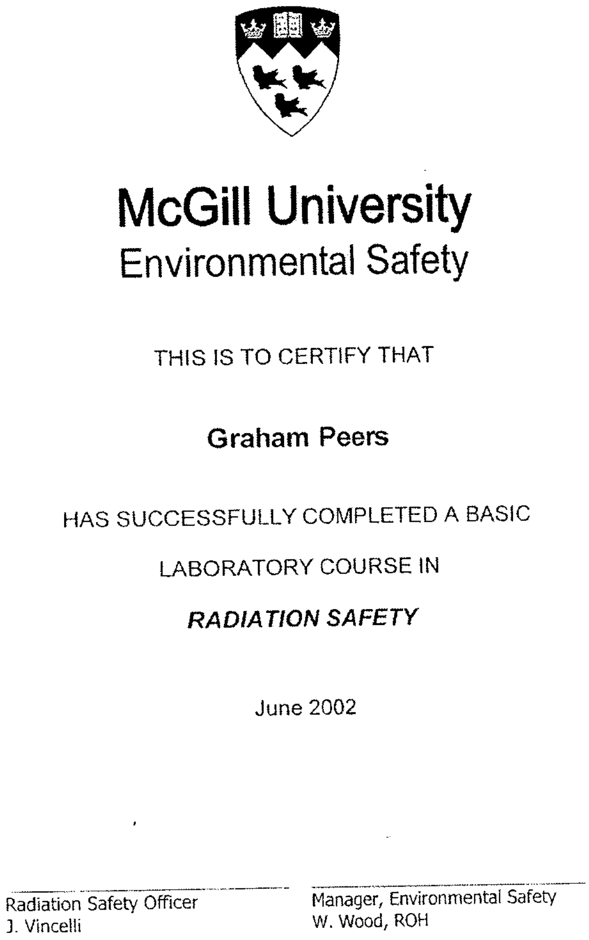
Appendix 4. Radiation certificates



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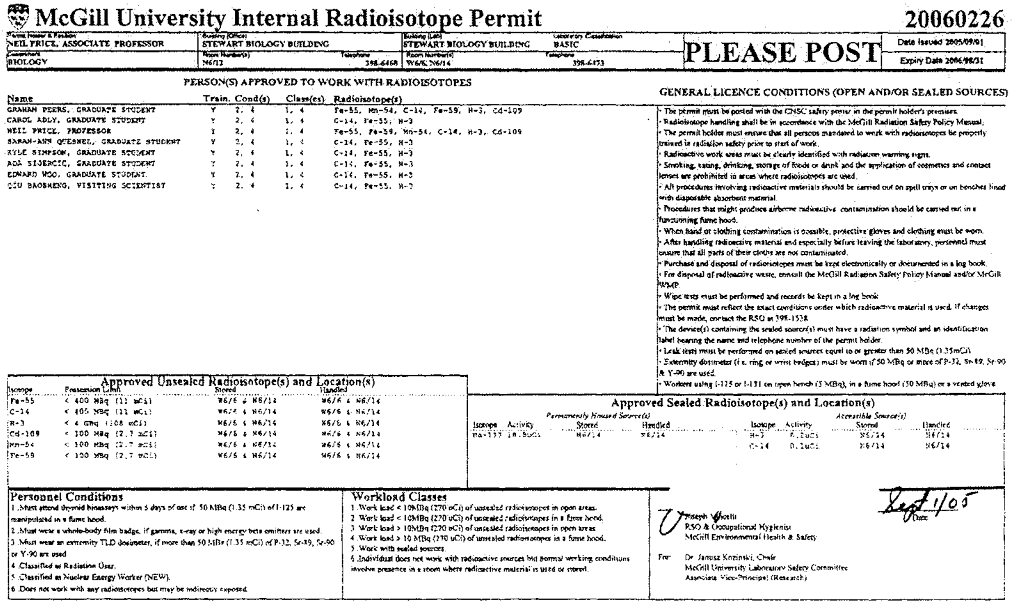
June 2002

Radiation Safety Officer
J. Vincelli

Manager, Environmental Safety
W. Wood, ROH



McGill University Internal Radioisotope Permit

20060226

Permit Holder & Position	Building (Office)	Building (Lab)	Laboratory Classification		
NEIL PRICE, ASSOCIATE PROFESSOR	STEWART BIOLOGY BUILDING	STEWART BIOLOGY BUILDING	BASIC	PLEASE POST	Date Issued 2005/09/01
Department BIOLOGY	Room Number(s) N6/13 Telephone 398-6468	Room Number(s) W6/6, N6/14	Telephone 398-6473		Expiry Date 2006/08/31

PERSON(S) APPROVED TO WORK WITH RADIOISOTOPES

Name	Train.	Cond(s)	Class(es)	Radioisotope(s)
GRAHAM PEERS, GRADUATE STUDENT	Y	2, 4	1, 4	Fe-55, Mn-54, C-14, Fe-59, H-3, Cd-109
CAROL ADLY, GRADUATE STUDENT	Y	2, 4	1, 4	C-14, Fe-55, H-3
NEIL PRICE, PROFESSOR	Y	2, 4	1, 4	Fe-55, Fe-59, Mn-54, C-14, H-3, Cd-109
SARAH-ANN QUESNEL, GRADUATE STUDENT	Y	2, 4	1, 4	C-14, Fe-55, H-3
KYLE SIMPSON, GRADUATE STUDENT	Y	2, 4	1, 4	C-14, Fe-55, H-3
ADA SIJERCIC, GRADUATE STUDENT	Y	2, 4	1, 4	C-14, Fe-55, H-3
EDWARD WOO, GRADUATE STUDENT	Y	2, 4	1, 4	C-14, Fe-55, H-3
QIU BAOSHENG, VISITING SCIENTIST	Y	2, 4	1, 4	C-14, Fe-55, H-3

GENERAL LICENCE CONDITIONS (OPEN AND/OR SEALED SOURCES)

- The permit must be posted with the CNSC safety poster in the permit holder's premises.
- Radioisotope handling shall be in accordance with the McGill Radiation Safety Policy Manual.
- The permit holder must ensure that all persons mandated to work with radioisotopes be properly trained in radiation safety prior to start of work.
- Radioactive work areas must be clearly identified with radiation warning signs.
- Smoking, eating, drinking, storage of foods or drink and the application of cosmetics and contact lenses are prohibited in areas where radioisotopes are used.
- All procedures involving radioactive materials should be carried out on spill trays or on benches lined with disposable absorbent material.
- Procedures that might produce airborne radioactive contamination should be carried out in a functioning fume hood.
- When hand or clothing contamination is possible, protective gloves and clothing must be worn.
- After handling radioactive material and especially before leaving the laboratory, personnel must ensure that all parts of their cloths are not contaminated.
- Purchase and disposal of radioisotopes must be kept electronically or documented in a log book.
- For disposal of radioactive waste, consult the McGill Radiation Safety Policy Manual and/or McGill WMP.
- Wipe tests must be performed and records be kept in a log book
- The permit must reflect the exact conditions under which radioactive material is used. If changes must be made, contact the RSO at 398-1538
- The device(s) containing the sealed source(s) must have a radiation symbol and an identification label bearing the name and telephone number of the permit holder.
- Leak tests must be performed on sealed sources equal to or greater than 50 MBq (1.35mCi).
- Extremity dosimeter (i.e. ring or wrist badges) must be worn if 50 MBq or more of P-32, Sr-89, Sr-90 & Y-90 are used.
- Workers using I-125 or I-131 on open bench (5 MBq), in a fume hood (50 MBq) or a vented glove

Approved Unsealed Radioisotope(s) and Location(s)

Isotope	Possession Limit	Stored	Handled
Fe-55	< 400 MBq (11 mCi)	N6/6 & N6/14	N6/6 & N6/14
C-14	< 400 MBq (11 mCi)	N6/6 & N6/14	N6/6 & N6/14
H-3	< 4 GBq (108 mCi)	N6/6 & N6/14	N6/6 & N6/14
Cd-109	< 100 MBq (2.7 mCi)	N6/6 & N6/14	N6/6 & N6/14
Mn-54	< 100 MBq (2.7 mCi)	N6/6 & N6/14	N6/6 & N6/14
Fe-59	< 100 MBq (2.7 mCi)	N6/6 & N6/14	N6/6 & N6/14

Approved Sealed Radioisotope(s) and Location(s)

Permanently Housed Source(s)				Accessible Source(s)			
Isotope	Activity	Stored	Handled	Isotope	Activity	Stored	Handled
Ba-133	10.5uCi	N6/14	N6/14	H-3	0.2uCi	N6/14	N6/14
				C-14	0.2uCi	N6/14	N6/14

Personnel Conditions

1. Must attend thyroid bioassays within 5 days of use if 50 MBq (1.35 mCi) of I-125 are manipulated in a fume hood.
2. Must wear a whole-body film badge, if gamma, x-ray or high energy beta emitters are used.
3. Must wear an extremity TLD dosimeter, if more than 50 MBq (1.35 mCi) of P-32, Sr-89, Sr-90 or Y-90 are used
4. Classified as Radiation User.
5. Classified as Nuclear Energy Worker (NEW).
6. Does not work with any radioisotopes but may be indirectly exposed.

Workload Classes

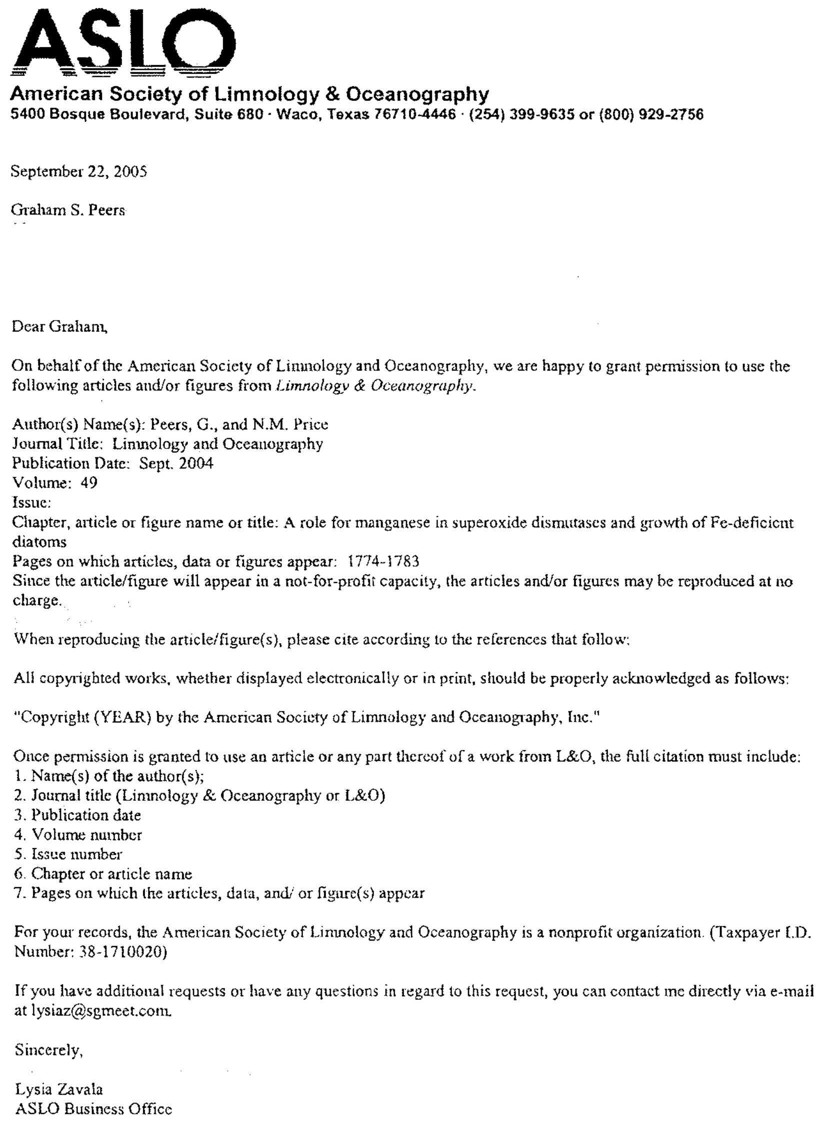
1. Work load < 10MBq (270 uCi) of unsealed radioisotopes in open areas.
2. Work load < 10MBq (270 uCi) of unsealed radioisotopes in a fume hood.
3. Work load > 10MBq (270 uCi) of unsealed radioisotopes in open areas.
4. Work load > 10 MBq (270 uCi) of unsealed radioisotopes in a fume hood.
5. Work with sealed sources.
6. Individual does not work with radioactive sources but normal working conditions involve presence in a room where radioactive material is used or stored.

Joseph Vincelli
RSO & Occupational Hygienist
McGill Environmental Health & Safety

For: Dr. Janusz Kozinski, Chair
McGill University Laboratory Safety Committee
Associate Vice-Principal (Research)

Sept 1/05
Date

Appendix 5 Copyright permits



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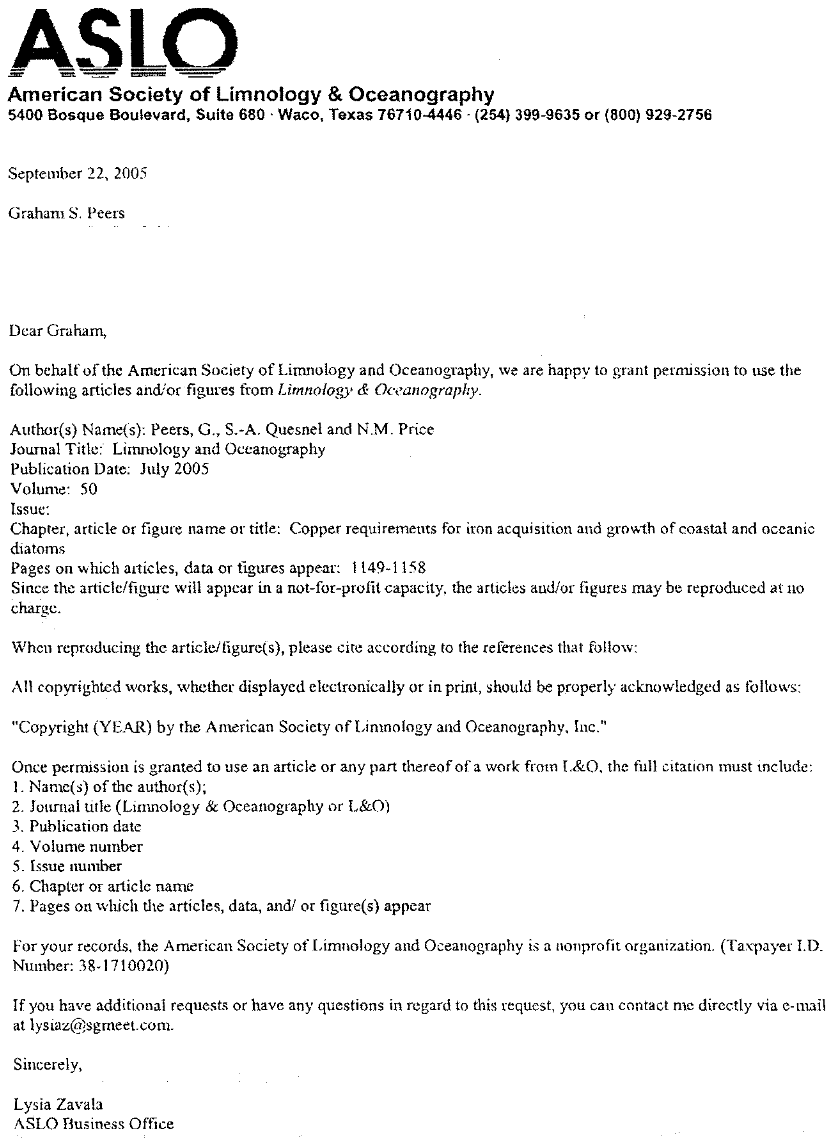

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