6.05

Marine Bioinorganic Chemistry: The Role of Trace Metals in the Oceanic Cycles of Major Nutrients

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6.05.1 INTRODUCTION: THE SCOPE OF MARINE BIOINORGANIC CHEMISTRY

The bulk of living biomass is chiefly made up of only a dozen "major" elements—carbon, hydrogen, oxygen, nitrogen, phosphorus, sodium, potassium, chlorine, calcium, magnesium, sulfur (and silicon in diatoms)—whose proportions vary within a relatively narrow range in most organisms. A number of trace elements, particularly first

row transition metals—manganese, iron, nickel, cobalt, copper, and zinc—are also "essential" for the growth of organisms. At the molecular level, the chemical mechanisms by which such elements function as active centers or structural factors in enzymes and by which they are accumulated and stored by organisms is the central topic of bioinorganic chemistry. At the scale of ocean basins, the interplay of physical, chemical, and biological processes that govern the cycling of biologically essential elements in seawater is the subject of marine biogeochemistry. For those interested in the growth of marine organisms, particularly in the one-half of the Earth's primary production contributed by marine phytoplankton, bioinorganic chemistry and marine biogeochemistry are critically linked by the extraordinary paucity of essential trace elements in surface seawater, which results from their biological utilization and incorporation in sinking organic matter. How marine organisms acquire elements that are present at nano- or picomolar concentrations in surface seawater; how they perform critical enzymatic functions when necessary metal cofactors are almost unavailable are the central topics of "marine bioinorganic chemistry." The central aim of this field is to elucidate at the molecular level the metal-dependent biological processes involved in the major biogeochemical cycles.

By examining the solutions that emerged from the problems posed by the scarcity of essential trace elements, marine bioinorganic chemists bring to light hitherto unknown ways to take up or utilize trace elements, new molecules, and newer "essential" elements. Focusing on molecular mechanisms involved in such processes as inorganic carbon fixation, organic carbon respiration, or nitrogen transformation, they explain how the cycles of trace elements are critically linked to those of major nutrients such as carbon or nitrogen. But we have relatively little understanding of the binding molecules and the enzymes that mediate the biochemical role of trace metals in the marine environment. In this sense, this chapter is more a "preview" than a review of the field of marine bioinorganic chemistry. To exemplify the concepts and methods of this field, we have chosen to focus on one of its most important topics: the potentially limiting role of trace elements in primary marine production. As a result we center our discussion on particular subsets of organisms, biogeochemical cycles, and trace elements. Our chief actors are marine phytoplankton, particularly eukaryotes, while heterotrophic bacteria make only cameo appearances. The biogeochemical cycles that will serve as our plot are those of the elements involved in phytoplankton growth, the major algal nutrients—carbon, nitrogen, phosphorus, and silicon—leaving aside, e.g., the interesting topic

of the marine sulfur cycle. Seven trace metals provide the intrigue: manganese, iron, nickel, cobalt, copper, zinc, and cadmium. But several other trace elements such as selenium, vanadium, molybdenum, and tungsten (and, probably, others not yet identified) will assuredly add further twists in future episodes.

We begin this chapter by discussing what we know of the concentrations of trace elements in marine microorganisms and of the relevant mechanisms and kinetics of trace-metal uptake. We then review the biochemical role of trace elements in the marine cycles of carbon, nitrogen, phosphorus, and silicon. Using this information, we examine the evidence, emanating from both laboratory cultures and field measurements, relevant to the mechanisms and the extent of control by trace metals of marine biogeochemical cycles. Before concluding with a wistful glimpse of the future of marine bioinorganic chemistry we discuss briefly some paleoceanographic aspects of this new field: how the chemistry of the planet "Earth"—particularly the concentrations of trace elements in the oceans—has evolved since its origin, chiefly as a result of biological processes and how the evolution of life has, in turn, been affected by the availability of essential trace elements.

6.05.2   TRACE METALS IN MARINE MICROORGANISMS

6.05.2.1   Concentrations

Bioinorganic chemists are now interested in the overall concentration and chemical speciation of trace elements in cells (O'Halloran and Culotta, 2000). In parallel with the "genome" and the "proteome" in organisms, some have begun to talk of the "metallome" (Outten and O'Halloran, 2001) to designate the suite of trace-metal concentrations, and perhaps the topic of this section could be described as "marine metallomics." What emerges from studies of trace-element concentrations in various types of cells, chiefly in unicellular organisms, is that these cellular concentrations are maintained at reasonably similar proportions among organisms from widely different taxa. This is exemplified in Figure 1, which shows the trace-metal composition of a few species of eukaryotic marine phytoplankton in cultures (Ho *et al.*, in press). Averaging the data given in Figure 1 provides an extension to Redfield formula ($C_{106}N_{16}P_1$):

$$(C_{106}N_{16}P_1)_{\times 1000}Fe_8Mn_4Zn_{0.8}Cu_{0.4}Co_{0.2}Cd_{0.2}$$

The stoichiometric coefficients of this average formula are within a factor of 3 of the elemental proportions measured for almost all individual
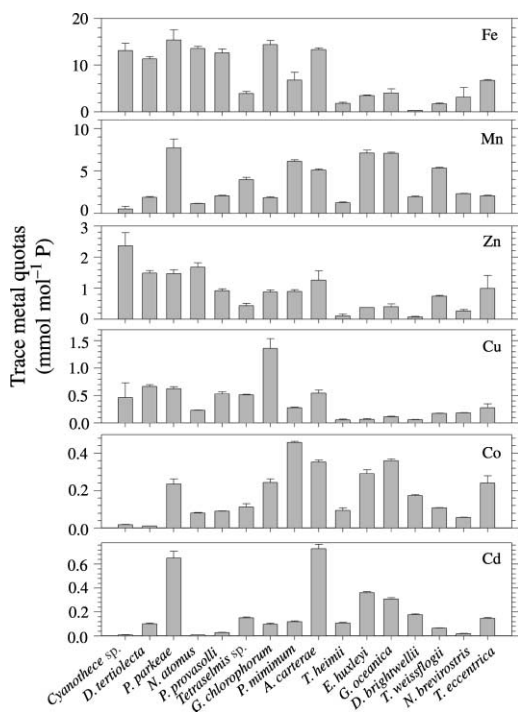
Figure 1 Cellular trace metals amounts normalized to phosphorus in a variety of phytoplankton. $Cd' = 20$ pM; $Co' = 20$ pM; $Cu' = 0.2$ pM; $Fe' = 200$ pM; $Mn' = 10$ nM; $Zn' = 20$ pM (source Ho *et al.*, in press).

Figure 2 Cellular zinc normalized to carbon (a) and specific growth rate (b) as functions of $\log(Zn')$ (unchelated zinc concentration) in a marine diatom *Thalassiosira weissflogii* (squares) and two clones of a marine coccolithophorid *Emiliana huxleyi* (triangles) (after Sunda and Huntsman, 1992).

species. As expected, iron and manganese are quantitatively the most important trace elements in marine phytoplankton, being on average about 10 times more abundant than zinc, copper, cobalt, or cadmium. But there are obvious differences among major taxa; e.g., green algae (which are rarely dominant in the oceans) have higher iron, zinc, and copper than diatoms or coccolithophores, which contain relatively higher proportions of manganese, cobalt, and cadmium.

To what extent do the data presented in Figure 1 reflect the physiology of the organisms or the composition of their growth medium? This is obviously a key question for marine metallomics and the extant literature on marine microorganisms indeed indicates particular attention given to the relation between the composition of organisms and their growth medium (e.g., Anderson and Morel, 1982; Hudson and Morel, 1990; Saito *et al.*, 2002; Sunda and Guillard, 1976; Sunda and Huntsman, 1992). Some of the differences between the data of Figure 1 and similar data published for *S. cerevisae* and *E. coli* may owe as much to differences in growth media as to biochemical differences among organisms.

The design of culture media, in which the concentration and speciation of trace elements are tightly controlled, has been critical to physiological studies of organisms whose natural medium
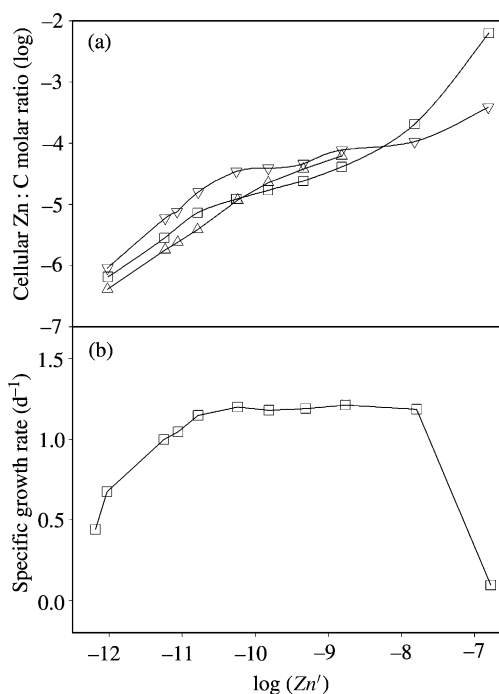
is trace-metal-poor seawater (Morel *et al.*, 1979; Price *et al.*, 1988/1989). In most instances, it has been found practical to control the bioavailability of trace metals by using strong chelating agents such as EDTA (designated Y). Because the rate of uptake of a metal, M, is usually proportional to its unchelated concentration (often designated as M' and sometimes referred to as the "inorganic" concentration of M), the chelated metal provides a convenient buffer that maintains M' at constant low values in the growth medium over the course of a batch culture. The value of M' can be precisely adjusted by choosing an appropriate ratio of the total concentrations of M and Y.

A general observation is that the cellular concentration of an element—its so-called cellular "quota"—varies in a sigmoidal fashion as a function of its concentration in the medium. This is illustrated in Figure 2(a) for zinc in two model species, a marine diatom and a coccolithophore (Sunda and Huntsman, 1992). Over a reasonably wide range of unchelated metal concentration, an organism regulates its metal quota by adjusting its metal uptake rate as well as, sometimes, its metal export rate. If other conditions are also optimal, this regulated metal quota normally allows the organism to grow at its maximum rate, μ_{max} (Figure 2(b)). Below the regulated range, however, the cellular quota of the trace

metal can become too low and growth rate decreases—a straightforward case of a limitation when the organism cannot take up the metal at a rate sufficient to maintain optimal growth.

Above the regulated range, the organism accumulates the metal uncontrollably by simple diffusion of some metal species (usually electrically uncharged complexes) through the membrane or by leakage through the transport system of another metal. For example, at high concentrations, zinc enters the diatom cell in part through the manganese transport system (Sunda and Huntsman, 1998b). At some point, the intracellular metal concentration becomes toxic and the growth rate decreases. Because a decreased growth rate results in an even faster rate of metal accumulation (the metal quota being no longer "diluted" by growth), growth often stops abruptly as M' is increased above some critical threshold in the growth medium.

The unchelated concentration of a given metal in the growth medium is not the only parameter that determines the corresponding metal quotas illustrated in Figure 1. The medium concentrations of other elements can also play an important role. This occurs principally under three types of conditions:

(i) Another trace element may effectively substitute for an essential trace element M and the relative environmental concentrations of both then affect the M quota. This phenomenon is described as "biochemical substitution" and its best-documented evidence is the alternative use of zinc, cobalt, or cadmium as a metal center in the carbonic anhydrases in diatoms (Lane and Morel, 2000a; Lee *et al.*, 1995).

(ii) Quite often, another trace element may compete with M for transport or somehow interfere with its uptake. Such "competitive inhibition" has been demonstrated, e.g., for the accumulation of manganese in coastal marine phytoplankton which can be hindered by high concentrations of metals such as copper or cadmium (Sunda and Huntsman, 1996).

(iii) In most cases, the regulated cellular quota of M is affected by the biochemical need for that element and thus depends on a variety of environmental parameters. This is illustrated in several of the examples discussed in the following sections. The most dramatic of these is the case of nickel. The nickel content of phytoplankton in culture is generally quite low and not even reported in Figure 1 (because it was not added to the medium and is usually below detection in the biomass). But the nickel quota increases dramatically when the organisms are grown on urea (rather than ammonium or nitrate) and they need nickel as a metal center in urease (Price and Morel, 1991).

6.05.2.2 Uptake

The concentrations of essential trace elements are extremely low in the waters of the open ocean, typically in the nanomolar to picomolar range as illustrated in Figure 3. Zinc, copper, cadmium, and nickel all show nutrient-like vertical distributions; they are depleted in surface waters as a result of uptake by the biota and increase in concentration at depth as a result of the remineralization of sinking organic matter. Iron and cobalt, which have low deep-water concentrations (~0.6 nM and ~0.05 nM, respectively), sometimes also display nutrient-like depletion in surface waters (Figure 3). Dissolved manganese concentrations usually exhibit a surface maximum as a result of the indirect photochemical reductive dissolution of manganese oxide (Sunda and Huntsman, 1988). More complex distributions are observed near shore as the result of fluvial inputs and in some cases over anoxic sediments on the continental margin (Sundby *et al.*, 1986; Thamdrup *et al.*, 1994).

In addition to being depleted at the surface by biological uptake, most of the essential trace metals (with the apparent exception of manganese and nickel) are chelated by strong organic ligands that maintain the unchelated metal concentrations, M', at extremely low values—between 10^{-15} for Co' and 10^{-11} for Zn' (Rue and Bruland, 1995; Saito and Moffett, 2001a; Moffett, 1995; Bruland, 1989; Achterberg and van den Berg, 1997; Martin and Gordon, 1988; Sunda, 1984; Sunda and Hanson, 1987). The presence of these chelating agents has been inferred as a result of electrochemical measurements showing that the metals are not labile (i.e., that they do not react with a strong ligand or cannot be reduced at the appropriate voltage at an electrode surface) but their chemical structures are still unknown. They are certainly of biogenic origin: some may be metalloproteins or cellular ligands in various stages of remineralization; some may be specific chelators released by the biota for the purpose of dissolving, transporting, or detoxifying metals. For example, the chelated iron in seawater is probably a mixture of iron–siderophore complexes (see below) and of various species of hemes and Fe–S clusters. We note that recent data on the speciation iron in the top 500 m of the open ocean indicate a large colloidal component and a depletion of chelated iron (Wu *et al.*, 2001). In some situations, such as photodegradation of surface water metal-ligand complexes or vertical mixing of unchelated metals to the surface, the excess of metals over chelators results in a dramatic increase in unchelated metal concentrations. This has been observed for copper in the Sargasso Sea and can lead to copper toxicity to the ambient flora (Mann *et al.*, 2002).
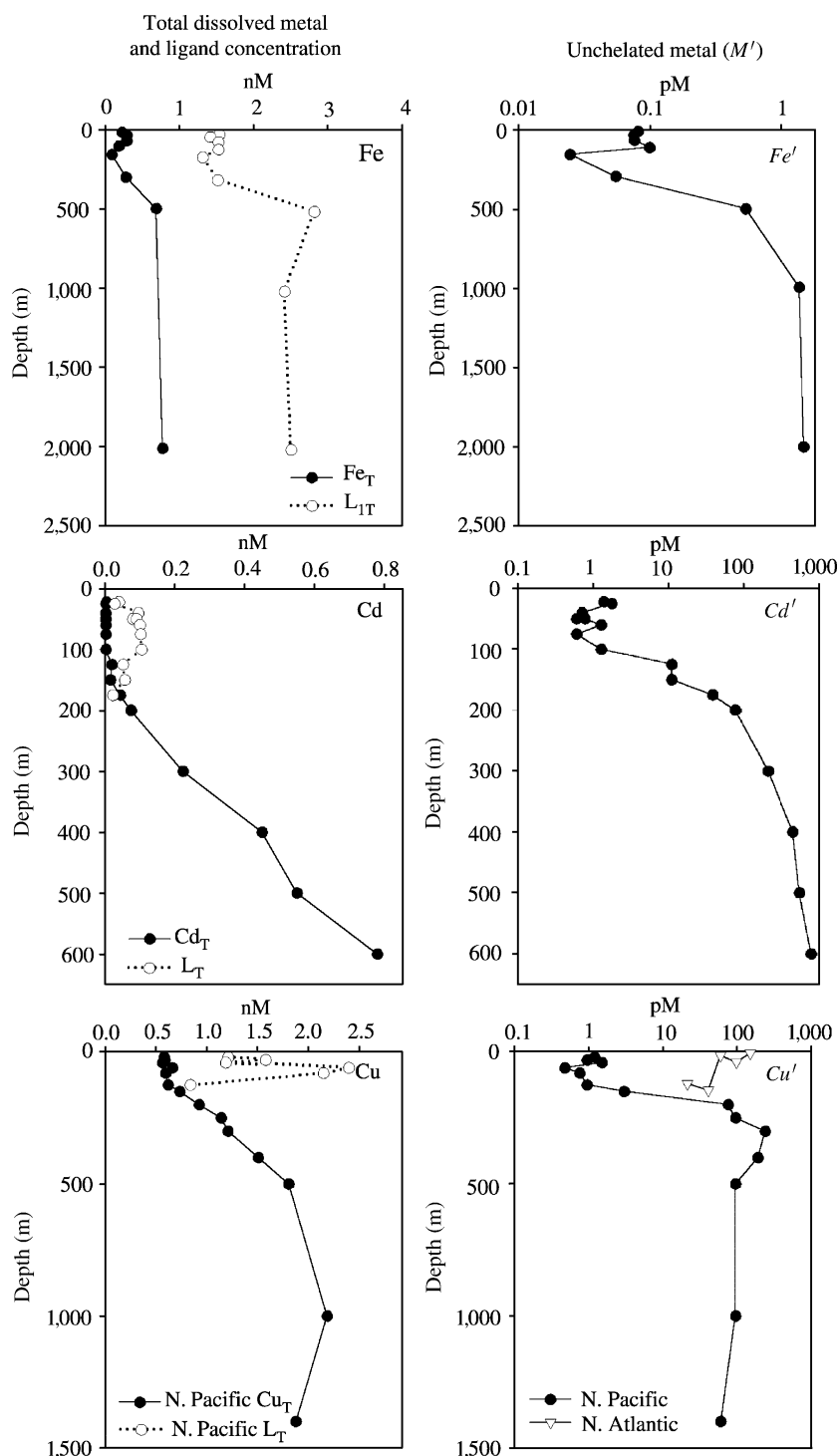
Figure 3 Concentration profiles of total dissolved metals (M_T), organic ligands specific to those metals (L_T), and uncomplexed metal as the sum of unchelated chemical species (M'). Fe data from central North Pacific (Rue and Bruland, 1995); Cd data from the central North Pacific (Bruland, 1989); Cu data from North Pacific (Coale and Bruland, 1988) and overlaid Sargasso Sea free Cu which is seasonally high (Moffett, 1995); Zn data from the central North Pacific (Bruland, 1989); Co data from the Sargasso Sea (Saito and Moffett, 2001a), Ni data from the Mediterranean Sea (Achterberg and van den Berg, 1997), total Mn data from Northeast Pacific (Martin and Gordon, 1988). Limited Mn speciation data suggests that Mn has negligible organic complexation (Sunda, 1984), thus it is assumed that $Mn' \sim Mn_T$ here. Data reported as free metal ion (e.g. M^{2+}) has been recalculated as M' (sum of all inorganic species) for the purposes of this chapter (using inorganic side reaction coefficients: $M'/M^{2+} = 35$ for Cd, 24 for Cu, 1.52 for Zn, and 1.41 for Co).
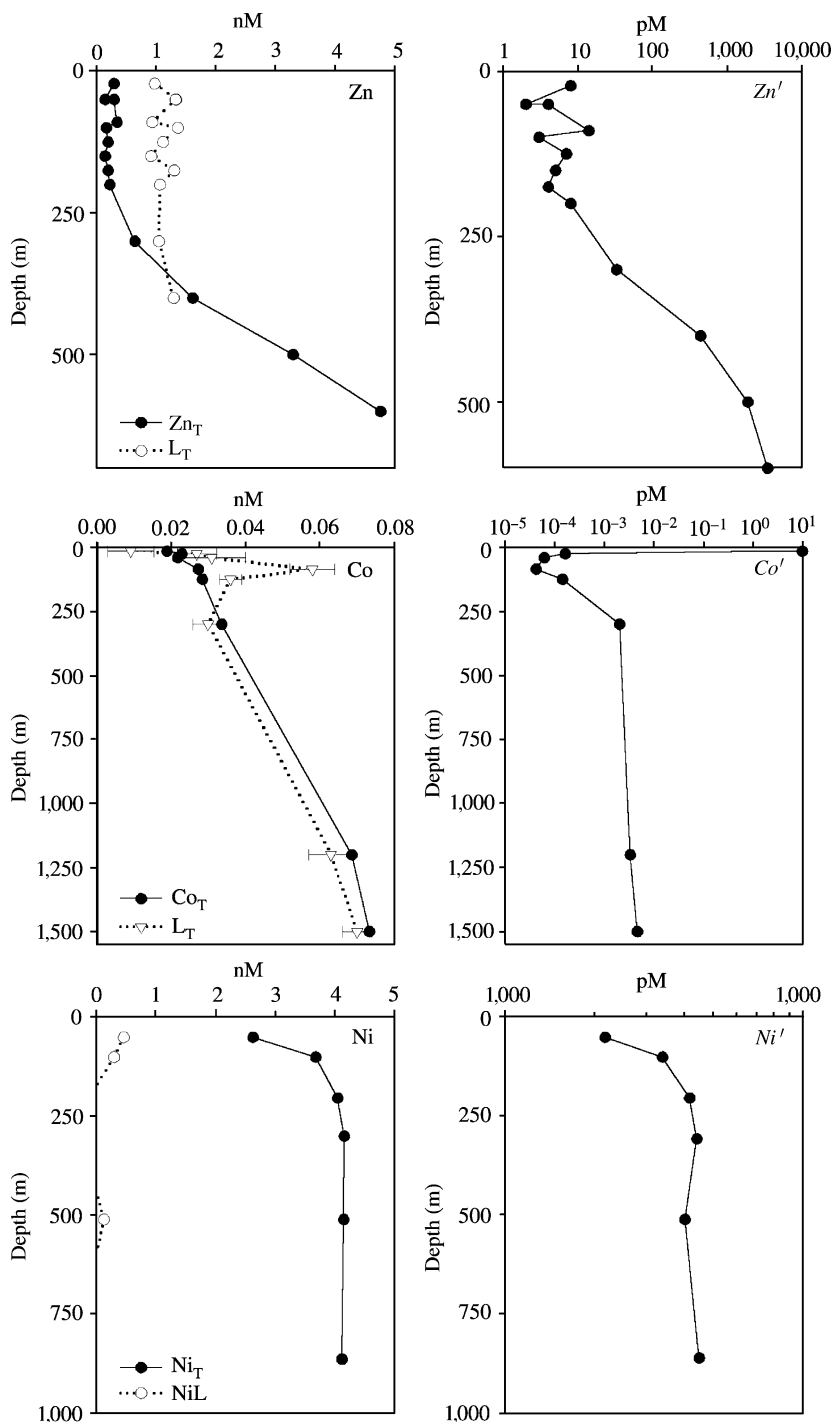
Figure 3   (continued).

To maintain sufficient concentrations of such trace elements and grow at rates of ~0.2–2.0 d^{-1}, marine microorganisms must possess particularly effective uptake systems. These systems are not generally known at the molecular level as few transport proteins have been characterized or even identified. Nonetheless, the available data, based chiefly on uptake kinetics in laboratory cultures, show that the mechanisms of uptake of trace elements of marine microorganisms are similar to those that have been characterized in model bacteria and yeasts. In most cases the uptake systems involve transmembrane transport proteins whose expressions are regulated as a function of the state of deficiency or sufficiency of the organism for the corresponding element. Figure 4 presents an example of the kinetics of (short-term) uptake of iron as a function of the
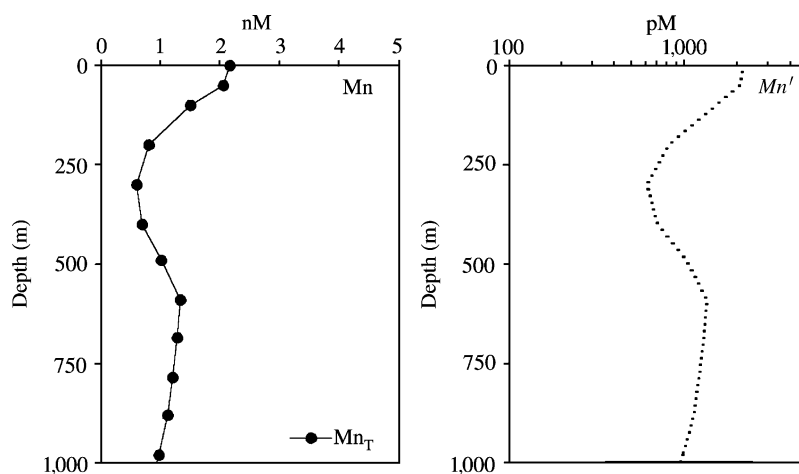
Figure 3 (continued).
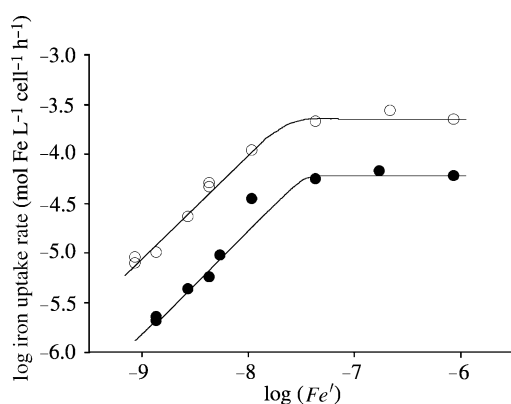
Figure 4 Short term (3 h) iron uptake rates in the diatom *Thalassiosira weissflogii* as a function of log (*Fe'*) (unchelated iron concentration) for high iron grown (closed symbols) and low iron grown (upon symbols) (after Harrison and Morel, 1986).

unchelated iron concentration, *Fe'*, and of the previous extent of iron sufficiency or deficiency in cultures of the diatom *T. weissflogii* (Harrison and Morel, 1986). The uptake rate follows typical Michaelis–Menten saturating kinetics and the maximum uptake rate increases several-fold when the organisms have been starved for iron. As for the growth data shown in Figure 2(b), short-term uptake kinetic data for trace metals are obtained in buffered media containing high concentrations of artificial chelators such as EDTA. By varying the nature or concentration of these chelators, one can verify that the uptake kinetics of the trace metal (i.e., its bioavailability) are determined by its unchelated concentration.

The term "bioavailability" is often used in an absolute sense when a particular form of a nutrient is either available or not available to an organism. In the case of trace-metal complexes, which

dissociate over a finite timescale, the concept of bioavailability must necessarily be tied to kinetics. A particular form of a metal is more or less available to a (particular) organism depending on the rate at which it can be taken up. For example, if a metal chelate, MY, is not directly taken up, its slow dissociation will eventually make the unchelated metal, *M'*, available to the organism (although the dissociation of MY may be too slow to allow the organism to grow).

The question arises as to whether the situation obtained in buffered laboratory cultures is applicable to the surface oceans. We know that, in surface seawater as in culture media, a major fraction of the trace metals of interest is present as complexes with strong organic ligands (Figure 3). But do these natural ligands control trace-metal availability in the same way as that EDTA does in artificial growth media? The bioavailability of chelated metals—or more precisely the mechanisms and kinetics of their uptake—must clearly depend on the nature of the chelate and the uptake system of the organism. When uptake is simply effected by transport proteins in the membrane, the kinetics of metal exchange between the chelator in the medium and the binding ligands on the proteins determine the uptake rate (the turnover rate of the transporter being rarely limiting). Unless the extracellular chelator has particular properties that allow the formation of a ternary complex chelator–metal–protein (in which case the chelator is effectively a "metallophore"; see below), this metal exchange requires release of the metal in seawater and the uptake kinetics should indeed be governed by the unchelated metal concentration in the medium (Hudson and Morel, 1993).

In some cases, however, the organism is able to accelerate the metal exchange by promoting the

release of the metal from the chelator. For example, marine diatoms possess an extracellular reductase that promotes the reduction of Fe(III) to Fe(II) in siderophore complexes, resulting in the release of Fe from the ligand (Maldonado *et al.*, 1999; Maldonado and Price, 2001). The system appears similar to that characterized in yeast (Figure 5) (Eide, 1998) which, in addition to a reductase, utilizes a multi-copper oxidase that functions in series with an Fe(III) transporter. Uptake of iron by this mechanism thus involves consecutive reduction and reoxidation of the iron. It is likely that some marine organisms have evolved ability to release iron from other types of biogenic iron compounds (Hutchins *et al.*, 1999a) or perhaps even from some iron minerals (Nodwell and Price, 2001).

Some trace-metal transport systems are even more complex than the one described in Figure 5 and involve the release of "metallophores" into the medium. The archetypes of these—and the only ones characterized so far—are the siderophores produced by various species of marine bacteria to acquire iron. In the model organisms in which they have been characterized, the mechanisms of uptake are quite varied and complex, often involving intermediate siderophores in the periplasmic space and several transport proteins (Neilands, 1981). The effect of such siderophores on iron bioavailability is clearly not the same as that of EDTA. While complexation by a siderophore makes iron directly available to the bacteria which take up the complex (and whose rate of iron uptake is proportional to FeY), it drastically reduces the bioavailability of iron to most other organisms (whose rate of iron uptake is proportional to Fe'). For organisms which are able to promote the release of iron from the siderophore, e.g., by reduction of Fe(III), the effect of complexation is a less drastic decrease in iron

uptake rate. Thus the release of siderophores into the medium results in a sort of economic warfare over iron—the organisms that have the necessary FeY transporter, including those that "cheat" by synthesizing the transporter but not releasing Y into the medium, obtain almost exclusive access to the chelated iron.

A number of siderophores from marine bacteria have been isolated from cultures with their structures elucidated. Some are similar to those of terrestrial organisms but two major families—viz., the marinobactins (isolated from *Halomonas marina*) and the aquachelins (isolated from *Marinobacter* sp.)—are characterized by fatty acid tails of variable lengths (Figure 6; Martinez *et al.*, 2000). Both have a peptidic head group that contains two hydroxamates and one α-hydroxy acid to complex iron. The amphiphilic nature of these siderophores is likely to be a key to their modus operandi in the extremely dilute medium of the oceans. Of great interest also to the oceanic cycling of iron are the photochemical properties of the iron complexes of marinobactins and aquachelins. Upon illumination, the α-hydroxy acid is cleaved along with the hydrophobic tail and Fe(III) is reduced to Fe(II) (Barbeau *et al.*, 2001). While lower than that of its parent compound, the affinity of the oxidized siderophore for Fe(III) is still quite high and may be important in the marine geochemistry of iron.
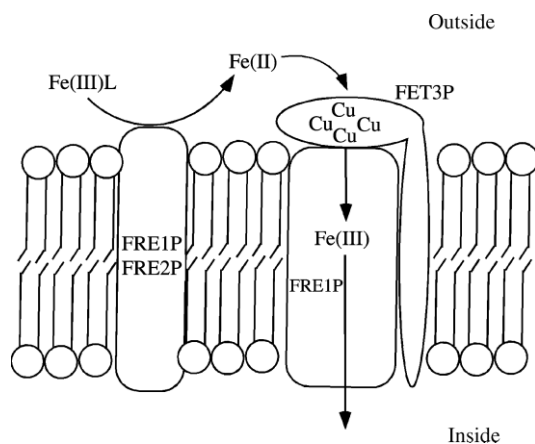

Figure 5 The iron reductase and high affinity uptake system in yeast. Fre1,2P, iron reductase proteins; Fet3P, multi-copper oxidase; FtrP, high affinity transport protein (after Eide, 1998).
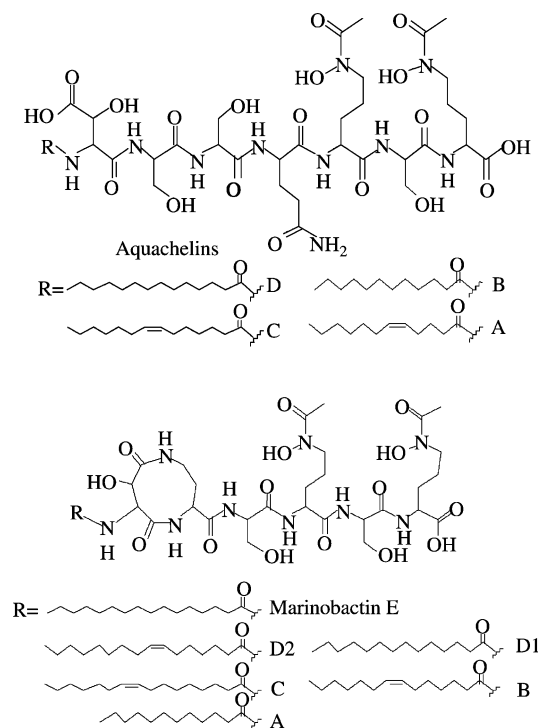

Figure 6 Two examples of ampiphilic siderophores produced by marine heterotrophic bacteria (after Martinez *et al.*, 2000).

Seawater is the most extreme environment on Earth in terms of its paucity of essential trace elements. Marine microorganisms have thus evolved some of the most efficient uptake systems possible and take up trace metals at rates near the maximum allowed by physics and chemistry. One can calculate absolute limits to the cellular uptake rates of metals by considering the simple case of uptake via transmembrane proteins (Hudson and Morel, 1990).

One limit is posed by the diffusion from the bulk medium to the cell surface. An unchelated bulk concentration of 2 pM (in the low range seen for metals such as zinc in surface seawater; see Figure 3) can supply by molecular diffusion at the most 10 amol d^{-1} to a (spherical) cell of radius $R = 4$ μm. (That is, 10^{-17} mol d^{-1}; this calculation assumes the concentration of M at the surface of the cell to be 1 pM, half that of the bulk, and neglects the supply of metal from the dissociation of chelates.) Thus, for example, the diffusive supply of unchelated zinc from surface seawater to several of the organisms listed in Figure 1 (which have about the right size and contain on average ~75 amol Zn/cell) would barely allow them to grow at a rate of 0.13 d^{-1}. Clearly, the supply of trace nutrients by diffusion causes a severe limitation to the size of marine phytoplankton since the diffusive supply increases as R and the need roughly as R^3.

Another limitation to the rate of cellular uptake of a trace metal is posed by the chemical kinetics of reaction with the ligands of the transport proteins. This rate is proportional to the intrinsic second order reaction rate, k, the concentration of unchelated metal at the surface, and the concentration of uptake ligands on the membrane (Hudson and Morel, 1990). For a fast reacting metal with a second order rate constant of $10^7 M^{-1} s^{-1}$ present at 1 pM at the cell surface, a rate of uptake of 10 amol d^{-1} requires 10 amol $cell^{-1}$ of surface ligands—as much as the metal quota itself. In a cell of 4 μm radius, this represents a surface density of uptake proteins of 3×10^4 molecules per $μm^2$; at such high densities, proteins with effective radii in excess of 1 nm would occupy most or all of the membrane surface area. This limit posed by chemical kinetics is less severe for fast reacting metals such as Cu^{2+} and more severe for slow reacting metals such as Ni^{2+}. It is also compounded by the need to simultaneously transport several essential elements that are all present at very low concentrations in surface seawater. The kinetic limit posed by crowding of uptake molecules also becomes increasingly severe for larger cells as the available surface area increases as R^2 and the necessary metal flux as R^3. In a competition for rare nutrients, including trace elements, size is clearly a critical factor for marine microorganisms.

We note that the production of metallophores to complex trace metals in the medium may increase the bulk dissolved concentration of target metals (e.g., by dissolving some solid species) but, depending on the specifics of the metallophore uptake systems, it does not automatically resolve the problems posed by diffusion and by the kinetics of reaction with transport proteins (Völker and Wolf-Gladrow, 1999).

6.05.2.3 Trace Element Storage

Little is known of the intracellular chemistry of trace metals in marine microorganisms. In model organisms *E. coli* and *S. cerevisiae*, it has been shown that there is no cellular pool of free ions such as Zn^{2+} or Cu^{2+} (Outten and O'Halloran, 2001; Rae *et al.*, 1999). The total cellular concentrations of these metals (~mM) are either in the form of metalloproteins or bound by various chaperones or storage molecules. The same is very likely to be true for all trace metals in all type of cells, including marine microorganisms. The best known metal storage proteins are the ferritins, which are extraordinarily effective at storing iron as ferrihydrate and found in most organisms (Andrews, 1998; Grossman *et al.*, 1992). Genomic information indicates that some cyanobacteria are likely to possess certain type of bacterio-ferritin (M. Castruita, personal communication), although no such molecule has so far been isolated from marine microorganisms.

Like other plants, eukaryotic marine phytoplankton synthesize small polypeptides known as phytochelatins (Ahner *et al.*, 1994) to bind some metals intracellularly. These molecules, which have the general formula γ-(glu-cys)$_n$-gly Figure 7, bind metals through the thiol ligands of the cysteines and have thus a high affinity for soft metals such as Cd^{2+} and Cu^{2+}. Indeed these two metals have been found to be particularly effective at triggering the synthesis of phytochelatins (chiefly the dimer; see Figure 7) in marine microalgae. Phytochelatins (and perhaps other cysteine rich peptides; Ahner, personal communication) provide a rapid detoxification response in the presence of elevated concentrations of such metals. Kinetic evidence for the export of the cadmium phytochelatin complex from marine diatoms into the medium show that these organisms likely possess a metal export system similar to that characterized for similar complexes into the vacuole of yeast (Oritz *et al.*, 1995). But in diatom cultures, phytochelatins are also synthesized in the presence of the lowest concentrations of metals achievable, and their production even increases when Zn^l is decreased to very low levels (Ahner *et al.*, 1998). Since these organisms are known to substitute cadmium for zinc when starved for zinc, it has been postulated that
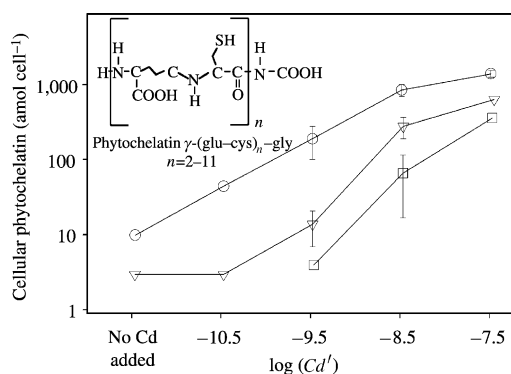
Figure 7 Phytochelatin concentrations in the marine diatom *Thalassiosira weissflogii* as a function of log (Cd') (unchelated cadmium concentration). Chain lengths for each phytochelatin dimer (circles) trimer (triangles), and tetramer (squares) (after Ahner *et al.*, 1994).

phytochelatins may serve to store cadmium as well as to detoxify metals (Ahner and Morel, 1999).

Over the course of a light–dark cycle, many metalloproteins that are involved in various aspects of nutrient acquisition, photosynthesis and respiration are in turn synthesized and degraded. This is the case, for example, of carbonic anhydrase in diatoms (Lane and Morel, 2000b), and of nitrogenase in the cyanobacterium *Trichodesmium* (Chen *et al.*, 1998)—both enzymes account for a significant fraction of the corresponding zinc and iron content of the organisms. Thus, it appears that intracellular trace metals are subject to dynamic trafficking as the cell goes through its various stages of division during the light–dark cycle. It may be so that in the metal-poor ocean, most of the storage of essential metals is in the form of functional proteins and that the metals are recycled among the metalloproteins as the need arises over the course of their growth and division.

6.05.3 THE BIOCHEMICAL FUNCTIONS OF TRACE ELEMENTS IN THE UPTAKE AND TRANSFORMATIONS OF NUTRIENTS

Not all biochemical uses of trace elements hold the same interest for oceanographers. The biochemical functions that utilize the largest fraction of a given metal in the cell are of most interest. A low metal concentration will limit the growth rate of the organism principally through impairment of these biochemical functions. Moreover, all environmental conditions that modify the need for these particular functions will also change the requirements of the organism for the metal.

Table 1 Known biochemical functions of selected trace elements that are known to account for a sizeable fraction of cellular metals in marine microorganisms.

Metal	Function
Mn	Oxygen evolving complex of PS II
	Superoxide dismutase
Fe	FeS centers (e.g., aconitase, ferredoxin)
	Cytochromes
	Superoxide dismutase
	Nitrate reductase (assimilatory and respiratory)
	Nitrite reductase (assimilatory and respiratory)
Co	Carbonic anhydrase
Ni	Urease
Cu	Plastocyanin
	Ferrous oxidase
	Amine oxidase
Zn	Carbonic anhydrase
Cd	Carbonic anhydrase
	Alkaline phosphatase?

Although we have limited quantitative information regarding enzymatic processes in cells and the corresponding trace element requirements, Table 1 provides a list of a few biochemical functions that are thought to correspond to major trace-metal requirements in marine phytoplankton. This list is quite short, reflecting in part our limited quantitative knowledge of biochemistry, particularly in marine microorganisms. This section of the chapter and the next are devoted in large part to justify the entries in Table 1. The trace-metal requirements of various biochemical functions have been quantified on the basis of theoretical calculations and empirical data. For example, in photosynthetic organisms, metal use efficiencies—mol C assimilated per unit time and per mole catalytic metal—can be calculated on the basis of the metal content of the catalyst, its specific reaction rate and the requirement for the products of that catalyst to fix carbon at a given rate (Raven, 1988, 1990). In a few experiments, the metal concentrations associated with particular enzymes have been measured directly. In others, the predicted effect of varying environmental conditions (e.g., light or nutrient concentrations) on the requirement for a particular metal and/or the effect of varying the metal concentration on enzyme activity have been tested.

The list given in Table 1 will undoubtedly be longer as we learn more about the biochemical functions that are significant for biogeochemistry and require relatively large concentrations of particular trace elements. But this list is also notable by the absence of some "obvious" metalloproteins. For example, many well-known zinc proteins, including RNA- and DNA-polymerases, seem to represent only minute fractions of cellular zinc. In the same way, present evidence indicates that Cobalamin

(i.e., vitamin B_{12})-containing enzymes account for only a small fraction of the cobalt quota of marine phytoplankton (Sunda and Huntsman, 1995b; Wilhelm, 1995). Many such metalloenzymes are quite interesting in their own right but they probably do not represent a critical link between the geochemical cycles of trace metals and of major nutrients.

6.05.3.1 Trace Metals and the Marine Carbon Cycle

How fast marine organisms fix inorganic carbon and what controls that rate—are topics of central interest to oceanographers. So far, the only trace element that has been shown beyond doubt to limit primary production in some regions of the oceans is iron. This is perhaps not surprising in view of the extremely low concentration of iron in some surface waters (see Figure 3(a)) and of the relatively large iron requirement of marine phytoplankton (Figure 1). To better understand how various trace elements affect the marine carbon cycle we need to review briefly some of the biochemistry involved in the photosynthetic fixation of inorganic carbon and the respiration of organic compounds.

6.05.3.1.1 Light reaction of photosynthesis

The first series of steps in photosynthesis, known as the light reaction, involve the absorption of photons, the evolution of O_2, and the formation of high energy compounds (ATP) and reductants (NADPH). The absorption of photons and the transfer of electrons from H_2O to NADPH are carried out by two photosynthetic systems, PS II and PS I, working in series (Figure 8). The pigments that harvest light in each photosynthetic system and transfer the energy to the respective PS II and PS I reaction centers vary somewhat among families of algae but contain no trace elements. The formation of oxidants and reductants in the two reaction centers, as well as the transfer of electrons between them and upstream and downstream from them, involve a large number of redox intermediates such as quinones, Fe−S centers, cytochromes and ferredoxin, most of which contain Fe. An overall tally reveals 3 and 12 iron atoms per PS II and PS I reaction centers, respectively, and 8 in the electron transport chain (Raven, 1990). Depending on the relative proportions of these various components (e.g., cyanobacteria have a relatively high proportion of iron laden PS I reaction centers) and the reaction kinetics of the slowest electron transfer step, one can calculate the iron requirement for a given rate of photosynthesis (Raven, 1990). The results uniformly show that the photosynthetic apparatus contributes the largest iron requirement for oxygenic photosynthetic organisms, typically about two-third of the total. Some of the photosynthetic iron can be economized by replacement of redox intermediates: soluble cytochrome b553 can be replaced by copper-containing plastocyanin in cyanobacteria and green algae; and, in many species, ferredoxin can be replaced by flavodoxin, a flavin which contains no metal (Doucette *et al.*, 1996; La Roche *et al.*, 1993). These economies do not appear to be very large, however, they amount to ~10% of the total iron requirement.

On the oxidative side of PS II, the oxidation of water to O_2 also involves four manganese atoms,

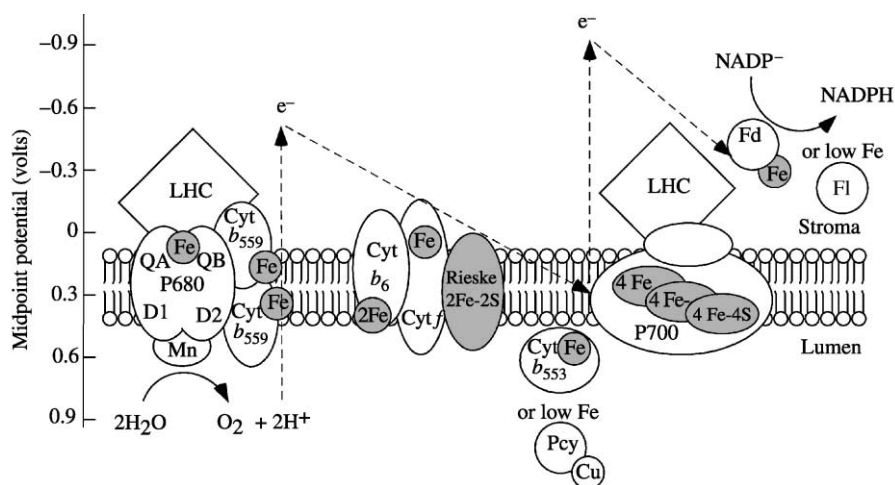**Figure 8** A diagram of the photosynthetic apparatus of oxygenic phototrophs, illustrating the proteins that contain iron (Fe) with the potential of electrons at each step superimposed. Abbreviations: LHC, light harvesting complex; Cyt, cytochrome; b, f with subscripts refer to the spectroscopic type of cytochrome; P680, the reaction center of photosystem II (PSII); P700 the reaction center of photosystem I (PSI).

each exchanging one electron. This allows four one-electron-transfer steps to yield one four-electron-transfer reaction. There is no known replacement of manganese in the oxygen evolving complex which represents a major pool of cellular manganese. All photons that are absorbed by phytoplankton do not have beneficial (photosynthetic) or benign (heat or fluorescence) consequences. Some result in the formation of extremely noxious compounds, particularly active oxygen species such as the superoxide radical, O_2^-. Organisms protect themselves from injury by these harmful species by catalyzing their reactions into less noxious ones. In particular, the dismutation of O_2^- into O_2 and H_2O_2 is catalyzed by the enzyme superoxide dismutase (SOD). In marine phytoplankton, SOD is a manganese, or perhaps, an iron enzyme (Peers and Price, personal communication), while a Cu–Zn SOD is found in other organisms.

6.05.3.1.2   Dark reaction of photosynthesis

The second series of biochemical steps in photosynthesis, known as the dark reaction, involves the use of the reductant and the energy produced in the light reaction to reduce inorganic carbon to organic carbon through a process known as the Calvin cycle. In the first step, catalyzed by the enzyme Rubisco (Ribulose 1-5-bisphosphate carboxylase oxygenase), CO_2 reacts with Rubp (Ribulose bisphosphate), a C_5 compound, to form two PGAs (phosphoglyceric acid, a C_3 compound). Subsequent phosphorylation reactions with ATP and reduction by NADPH end up providing glyceraldehyde 3P from which various compounds are synthesized including glucose. None of the nine enzymes involved in the Calvin cycle are metalloenzymes, although Rubisco is known to be activated by either manganese or magnesium (Jensen, 1990). But metals are nonetheless involved in the dark reaction of photosynthesis because of the inefficacy of Rubisco. This ancient enzyme (which presumably evolved early in Earth's life when CO_2 was plentiful and O_2 rare) requires CO_2, not HCO_3^-, as a substrate, has a low affinity for CO_2 ($K_{1/2} = 20$–100 μM) (Badger *et al.*, 1998) and, as indicated by its name, is subject to a competitive reaction with O_2. The net result is that CO_2 must somehow be concentrated near Rubisco to allow efficient carbon fixation in marine microalgae whose medium contains 2 mM HCO_3^-, and only 10 μM CO_2.

6.05.3.1.3   Carbon concentrating mechanisms

The best known carbon concentrating mechanism (CCM), is that of freshwater cyanobacteria
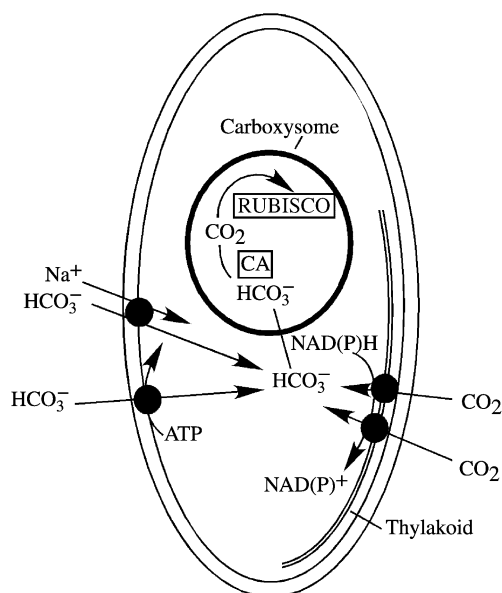
Figure 9 A model of the cyanobacterial carbon concentrating mechanism from *Synechococcus* PCC 7942, a freshwater species. Solid circles represent transporters located on the plasma membrane and interior to the cell wall. Boxes represent the catalyzing enzymes CA, Carbonic Anhydrase; RUBISCO, Ribulose 1-5 bisphosphate Carboxylase Oxygenase. The carboxysome is the site of carbon fixation (dark reactions) and the thylakoid is the site of the light reactions of photosynthesis (after Badger *et al.*, 2002).

which is depicted in Figure 9. This system consists of two HCO_3^- transporters (one powered by the inward Na^+ flux, the other by ATP), and two CO_2 pumps which appear to work by diffusion of CO_2 through the membrane down a gradient maintained by a yet to be elucidated NADPH-dependent transformation of CO_2 into HCO_3^- on the inner side of the membrane (Ohkawa *et al.*, 2002; Price *et al.*, 2002). Under normal conditions (i.e., atmospheric CO_2), the transport of HCO_3^- dominates over that of CO_2 (Price *et al.*, 1994; Yu *et al.*, 1994). The net result of the cyanobacterial CCM is the accumulation in the cytosol of a concentration of HCO_3^- far in excess of its equilibrium with CO_2. This is possible because the uncatalyzed hydration/dehydration reaction of HCO_3^-/CO_2 is relatively slow, its half-life being ~50 s, compared, for example, to a molecular diffusion time of few milliseconds in cells. The provision of CO_2 to Rusbisco in the carboxyzome is then enabled by a carbonic anhydrase (CA), a zinc enzyme that catalyzes the HCO_3^-/CO_2 reaction (Price and Badger, 1989).

With various modifications, the CCM of freshwater cyanobacteria is also generally accepted as the underlying scheme for the CCM of marine cyanobacteria and eukaryotic phytoplankters. In particular, the CCM of the model

chlorophyte, *Chlamydomonas*, appears to involve similar HCO_3^- and CO_2 transporters through the plasmalemma. Often eukaryotes possess a CA in their periplasmic space. By maintaining equilibrium between HCO_3^- and CO_2, this enzyme avoids the depletion of CO_2 that would occur at the surface of these large cells as a result of CO_2 pumping and slow diffusion from the bulk medium. (In seawater, the HCO_3^- concentration is too high (2 mM) to be significantly depleted at the surface of photosynthesizing cells.)

Diatoms have been shown to accumulate inorganic carbon via a mechanism that is markedly different from the CCM of cyanobacteria and more akin to the C_4 mechanism found in some higher plants (Figure 10) (Reinfelder *et al.*, 2000). (The characterization of the inorganic carbon acquisition system of diatoms as a unicellular C_4 pathway is controversial. The central point of contention is whether a majority of the inorganic carbon—or a significant fraction of it, depending on conditions—goes through a C_4 compound before being fixed, or if the formation of C_4 compounds is chiefly an anaplerotic process and their decarboxylation an unimportant side reaction.) Inorganic carbon is transported across the plasmalemma by diffusion of CO_2 which may be supplemented by active transport of HCO_3^-, depending on concentrations in the medium. Equilibrium between CO_2 and HCO_3^- is maintained at the cell surface by a periplasmic CA whose activity is enabled by the proton buffering of the silicon frustule (Milligan and Morel, 2002). A cytoplasmic CA maintains HCO_3^-/CO_2 equilibrium on the other side of the membrane. As we shall see, the cytoplasmic CA of diatoms and, likely, their periplasmic CA, can use zinc, cobalt,

or cadmium as their metal centers. Under atmospheric p_{CO_2} conditions, the dehydration of HCO_3^- to CO_2 in the periplasm and the subsequent diffusion of CO_2 into the cell appear to constitute the main source of inorganic carbon to diatoms (Tortell and Morel, 2002). Cytoplasmic HCO_3^- serves as the substrate for the enzymatic formation of a C_4 compound, oxaloacetate then malate, which is eventually decarboxylated in the chloroplast to feed CO_2 to Rubisco. The main enzymes catalyzing the carboxylation and decarboxylation reactions appear to be PEPC, which, like Rubisco can be activated by either manganese or magnesium, and PEPCK, which can be activated only by manganese.

6.05.3.1.4 Respiration

The remineralization of organic matter into CO_2, which closes the organic carbon cycle, is carried out by both the phytoplankton themselves, to produce necessary energy, and by heterotrophic bacteria which make a living from it. During the day, phytoplankton respire about a quarter of their fixed carbon and again about as much during the night (Raven, 1988). The net result is that nearly half of the carbon fixed photosynthetically is respired by the photosynthesizers themselves. Most of the rest is eventually respired by bacteria, only a small fraction of which is exported to the deep sea (Honjo, 1996).

In oxygenated waters, the respiration of sugars for the production of ATP occurs chiefly through the citric acid cycle. As in photosynthesis, the electron carrying intermediates of respiration—which include aconitase with an Fe_4S_4 center and cytochromes—are rich in iron. In phytoplankton
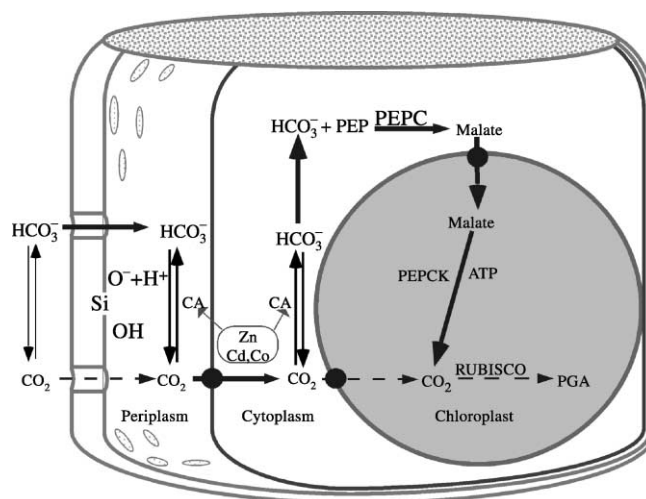

Figure 10 A hypothetical model of carbon acquisition in the marine diatom *Thalassiosira weissflogii*. Solid circles represent transporters. Catalyzing enzymes CA, carbonic anhydrase; PEPC, phosphoenol pyruvate carboxylase; PEPCK; phospoenol pyruvate carboxykinase; RUBISCO, Ribulose 1-5 bisphosphate Carboxylase Oxygenase (after Morel *et al.*, 2002).

the iron requirement for respiration is estimated to account for about one-third of the total (the bulk of the rest being involved in photosynthesis as described above; Raven, 1988).

Like phytoplankton, heterotrophic bacteria require the iron contained in the electron carriers of respiration. Various species of bacteria also express enzymes that are specialized for the degradation of particular classes of organic compounds. Many of these enzymes contain iron or zinc (a few contain copper) to carry out the additional redox reactions. The net result: most of the cellular iron in heterotrophic bacteria is directly involved in respiration and that, normalized to carbon or cellular mass, the iron requirement of these heterotrophs is predicted to be smaller by about three times than that of phytoplankton (Raven, 1988).

6.05.3.2 Trace Metals and the Nitrogen Cycle

Nitrogen, after carbon and oxygen and on par with hydrogen, constitutes a large fraction of biomass. It is generally thought to be the principal nutrient limiting the primary production of the oceans (Dugdale and Goering, 1967; Glibert and McCarthy, 1984). The question of nitrogen limitation can be considered on two scales. (i) From the point of view of a given phytoplankter in surface seawater, what are the available nitrogen species and how can the organism utilize these sources of nitrogen? (ii) At the scale of the whole ocean or ocean basins, what determines the overall mass of nitrogen available for plant growth, i.e., what controls the balance between N_2 fixation and denitrification? Most of the processes involved in the acquisition of nitrogen by phytoplankton and the overall cycling of nitrogen in seawater involve metalloenzymes and are thus of prime interest to marine bioinorganic chemists.

6.05.3.2.1 Acquisition of fixed nitrogen by phytoplankton

As depicted in Figure 11, phytoplankton can, in principle, acquire nitrogen from many different compounds, including ammonium, nitrite, nitrate, urea, aminoacids, amines, etc. In addition some species of cyanobacteria are able to fix dinitrogen, N_2, and are thus able to grow even in the absence of "fixed" nitrogen thereby increasing the oceanic pool of useable N in the process. Regardless of the original source of N, its final assimilation into aminoacids follows a unique pathway, the glutamine synthetase, glutamate oxoglutarate aminotransferase (GS-GOGAT) pathway (Zehr and Falkowski, 1988): NH_4^+ reacts with glutamate to form glutamine, followed by the transfer of an amine group from glutamine to α-ketoglutarate to form two molecules of glutamate. The enzyme GOGAT contains an Fe–S center.

Upstream from the GOGAT system, specialized enzymatic transport and transformation pathways enable the production of internal NH_4^+ from a variety of external nitrogen-containing species. Those depicted in Figure 12 are not exhaustive of all the possibilities. Vice versa, not all phytoplankton species have the enzymatic machinery to obtain nitrogen from all types of sources; on the contrary, the ability to acquire and assimilate
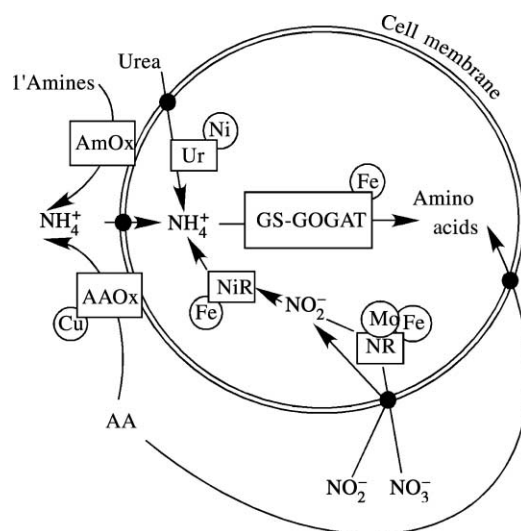
Figure 11 Nitrogen sources and metabolic pathways in marine phytoplankton. Solid circles are transporters. Boxes are the catalytic enzymes and open circles are metals associated with each enzyme. Ur, Urease; NR, Nitrate Reductase; NiR, Nitrite Reductase; AAOx, amino acid oxidase; AmOx, amine oxidase; GS-GOGAT, Glutamine Synthetase- Glutamate oxyglutarate aminotransferase (or glutamate synthase).
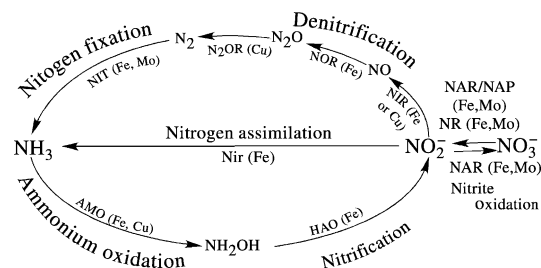
Figure 12 A diagram of the nitrogen cycle with catalyzing enzymes and metal requirements of each step. NIT, nitrogenase; AMO, ammonium mono-oxygenase; HAO, hydroxylamine oxidoreductase; NAR, membrane-bound respiratory nitrate reductase; NAP, periplasmic respiratory nitrate reductase; NR, assimilatory nitrate reductase; NIR, respiratory nitrite reductase; NiR, assimilatory nitrite reductase; NOR, nitric oxide reductase; N₂OR, nitrous oxide reductase.

nitrogen from particular sources likely represents an important ecological specialization for an organism.

Practically, all phytoplankton species are able to take up NH_4^+ actively from the external medium (Wright and Syrett, 1983). Both nitrite (NO_2^-) and nitrate (NO_3^-) are also taken up by a majority of marine phytoplankton species by what is thought to be a single transporter that does not differentiate between the two oxidation states (Cresswell and Syrett, 1982; Galvan and Fernandez, 2001). The transport mechanisms for NH_4^+, NO_2^-, and NO_3^- in marine phytoplankton are thought to be similar to those of vascular plants and involve both high and low affinity systems.

In all photoautotrophs, reduction of NO_3^- to NH_4^+ is achieved in two distinct enzymatic steps (Campbell, 2001). First, assimilatory nitrate reductase (NR) catalyzes the two electron reduction from NO_3^- to NO_2^-. NR is a large soluble cytoplasmic enzyme with FAD (flavin adinine dinucleotide), an iron-containing cytochrome and molybdopterin prosthetic groups, and requires NADH and/or NADPH as an electron donor (Guerrero *et al.*, 1981). Functional NR is in the form of a homodimer and therefore requires two atoms of iron per enzyme. Following transport into the chloroplast, NO_2^- undergoes a 6 e^- reduction to NH_4^+ via assimilatory nitrite reductase (NiR). NiR, a soluble chloroplastic enzyme, contains five iron atoms per active enzyme molecule, and requires photosynthetically reduced ferredoxin as an electron donor (Guerrero *et al.*, 1981).

For an average N : C ratio of 1 : 6, the reduction of NO_3^- to NH_4^+ necessitates eight electrons compared to the 24 for carbon reduction. It is thus not surprising that the utilization of NO_3^- as a source of nitrogen should represent a sizeable cost of both energy and iron supply and algae growing on nitrate are calculated to require 60% more iron than algae growing on ammonium (Raven *et al.*, 1992).

Many small organic molecules potentially constitute an excellent source of nitrogen for marine phytoplankton, particularly in oligotrophic waters where the concentrations of NH_4^+, NO_2^-, and NO_3^- are vanishingly low. For example, urea, $CO(NH_2)_2$, which is the most abundant form of low molecular mass organic nitrogen in the sea, has been shown to account for up to 25–50% of the nitrogen taken up by phytoplankton in various areas of the oceans (McCarthy, 1972; Varela and Harrison, 1999). In most species, urea is metabolized by hydrolysis to ammonium, catalyzed by the nickel enzyme, urease. In some classes of the Chlorophyceae (Bekheet and Syrett, 1977; Leftley and Syrett, 1973), assimilation of urea is effected by a two-step process involving the enzyme urea amidolyase.

Despite their low concentrations in seawater ($<1\ \mu M$), amino acids can account for a sizeable fraction of the nitrogen uptake of phytoplankton in the sea (up to 10–50%) (Mulholland *et al.*, 1998, 2002; Pantoja and Lee, 1994). Many species of marine phytoplankton, including representative of all major classes, are able to take up actively a variety of free amino acids and to use them as nitrogen sources (Antia *et al.*, 1991). In some species of coccolithophores and dinoflagellates, a mechanism involving external cleavage of NH_4^+ has been demonstrated (Palenik and Morel, 1990a,b). The deamination reaction is catalyzed via a periplasmic L-amino acid oxidase and results in the formation of hydrogen peroxide and an α-keto acid. Under appropriate conditions, some phytoplankton species can also obtain NH_4^+ released extracellularly from amines (Palenik *et al.*, 1991). A periplasmic amine oxidase oxidizes the amine to an aldehyde, resulting in the release of H_2O_2 and NH_4^+ for uptake. As in higher plants where they have been characterized, the amine oxidases of marine phytoplankton appear to be copper metalloenzymes.

6.05.3.2.2 N_2 fixation and the nitrogen cycle

The overall pool of fixed nitrogen in the oceans is determined by a balance between N_2 fixation and denitrification, two of the major processes involved in the nitrogen cycle. The concentration of nitrate and nitrite that can be denitrified is maintained by nitrification of ammonium. As shown in the diagram of Figure 12, trace elements are involved in every step in the nitrogen cycle. Nitrification is carried out by two specialized classes of aerobic chemoautotrophs: first ammonia oxidizing nitrifiers use copper- and iron-containing ammonia monooxygenase (AMO) to oxidize NH_4^+ to NH_2OH and iron-containing hydroxylamine oxidoreductase (HAO) to oxidize NH_2OH to NO_2^-; then nitrite oxidizing nitrifiers oxidize NO_2^- to NO_3^- via the Fe/Mo-enzyme nitrite oxidoreductase. Denitrification is a form of anaerobic respiration utilized by a variety of heterotrophs that requires four separate types of metalloenzymes: respiratory nitrate reductase (periplasmic NAP; or membrane-bound NAR) contains Fe and Mo; respiratory nitrite reductase (NIR) contains either copper or iron; nitric oxide reductase (NOR) contains iron; and nitrous oxide reductase (N_2OR) contains copper.

In nitrogen fixation, the difficult reduction of N_2 to NH_4^+ is effected by the nitrogenase enzyme, which contains iron and molybdenum. An iron + vanadium form of nitrogenase and an iron-only form are also known, but their presence in marine phytoplankton has not yet been established

(perhaps because molybdenum is the most abundant trace metal in the oceans). Diazotrophic growth is calculated to require $\sim 7-11$ times more iron than growth on ammonium (Kustka *et al.*, 2003a). It is generally thought that the colony-forming cyanobacterium *Trichodesmium* is responsible for the bulk of N_2 fixation in the oceans. Many other species of cyanobacteria also possess the ability to fix nitrogen, however, and the question of how much N_2 is fixed by what organisms is presently contentious (Zehr *et al.*, 2001).

6.05.3.3 Phosphorus Uptake

Phosphate transporters have been characterized in many model organisms, though relatively little mechanistic work has been done in marine phytoplankton. Phosphate transport is effected by high and low affinity transporters and dependent on ATP, Na^+, and Mg^{2+} in several diatoms (Cembella *et al.*, 1984). These observations are found to be consistent with the well known active transport system of yeast (Raghothama, 1999). The dependence of phosphate transport on Mg^{2+} in diatoms and yeast suggests that eukaryotes may transport an uncharged cation phosphate complex ($MeHPO_4$, where Me may be Ca^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}) as has been observed in heterotrophic bacteria (van Veen, 1997).

As is the case for nitrogen, organic compounds constitute a significant source of phosphorus for some species of marine phytoplankton, particularly in oligotrophic waters. In most organisms, the principal enzyme involved in cleaving the phosphate group from organophosphates is the zinc enzyme alkaline phosphatase. This enzyme has indeed been found in a number of species of marine phytoplankton, usually as a periplasmic enzyme.

6.05.3.4 Silicon Uptake

Because of the importance of diatoms in oceanic productivity, silicon is an important algal nutrient in seawater. A transporter of $Si(OH)_4$ has been isolated and sequenced (Hildebrand *et al.*, 1998; Hildebrand *et al.*, 1997) and the physiology of silicon uptake has been well studied (Martin-Jezequel *et al.*, 2000). Nonetheless, the molecular mechanism of $Si(OH)_4$ transport and silica frustule formation in diatoms are still largely mysterious. From indirect evidence, it appears possible that the $Si(OH)_4$ transporter may contain zinc, coordinated to cysteines, as a metal center in the portion of the protein exposed to the outside of the cell (Hildebrand, 2000; Rueter and Morel, 1981). If true, this would be an unusual example of a transport protein functioning with a metal center.

6.05.4 EFFECTS OF TRACE METALS ON MARINE BIOGEOCHEMICAL CYCLES

In the preceding section, we have seen how trace metals are involved in some of the biochemical mechanisms responsible for the uptake and transformations of carbon, nitrogen, phosphorus, and silicon by marine organisms, with a focus on phytoplankton growth and productivity. Using this information about their functions, we now examine the extent to which trace metals affect the marine biogeochemical cycles of major algal nutrients. The relevant information is sparse and often indirect. For each trace metal, we review the laboratory data that indicate the biochemical functions—and, when known, what particular metalloproteins—utilize a major fraction of the cellular quota of nutrients and identify the principal effects of metal limitation in cultures of marine microorganisms. We also review the scant field data that shed light on the role of trace metals in the acquisition and processing of nutrients by marine phytoplankton and their growth.

6.05.4.1 Iron

6.05.4.1.1 *Iron and growth rates*

We have seen that iron plays a key role in biochemical electron transfer processes, including the light reaction of photosynthesis and the respiration of organic carbon, and that, according to calculations, the numerous iron-containing redox intermediates involved in these processes account for the bulk of the relatively high iron requirement of both phytoplankton and heterotrophic bacteria. As a result it has been relatively easy to demonstrate iron limitation in cultures of various species of marine phytoplankton (Anderson and Morel, 1982; Hudson and Morel, 1990; Sunda and Huntsman, 1995b, 1997). For example, as illustrated in Figure 13, the growth rates of the diatom *T. weissflogii* and of the dinoflagellate *Prorocentrum minimum* become limited when the unchelated iron concentration falls below 100 pM.

It has also been relatively easy to demonstrate the iron limitation in phytoplankton growth in the field. Numerous incubation experiments and a few mesoscale experiments (involving patches of tens of square kilometers of surface ocean) have consistently shown that iron addition promotes phytoplankton growth in high nutrient–low chlorophyll (HNLC) regions of the oceans, including the northern Pacific (Coale *et al.*, 1998; Martin *et al.*, 1989), the equatorial Pacific (Coale *et al.*, 1998; Martin *et al.*, 1994; Price *et al.*, 1991, 1994), the Southern Ocean (Boyd *et al.*, 2000), and some
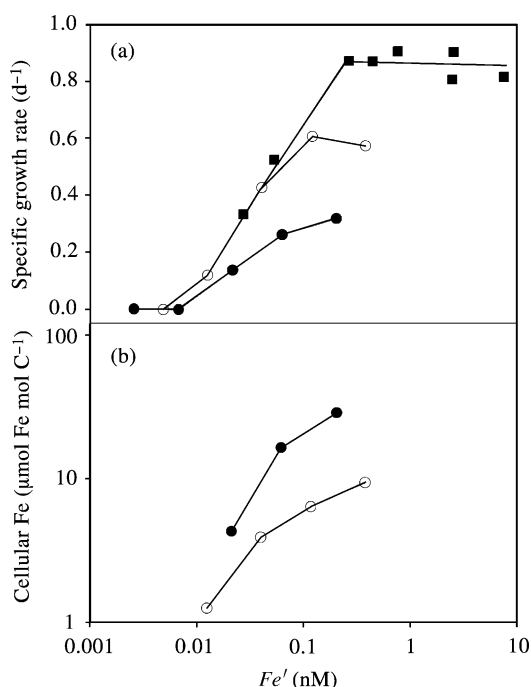
Figure 13 (a) Growth rate as a function of log(*Fe′*) (unchelated iron concentration) for the marine diatom *Thalassiosira weissflogii* (squares) and the marine dinoflagellate *Prorocentrum minimum* (circles). (b) Intracellular Fe : C as a function of log(*Fe′*) and irradiance in *P. minimum*. Open symbols 500 μmol quanta $m^{-2} s^{-1}$. Closed symbols 50 μmol quanta $m^{-2} s^{-1}$ (after Sunda and Huntsman, 1997).

upwelling coastal regions (Hutchins *et al.*, 1998, 2002). For example, over the seven days following the second iron addition in the IRONEX-II experiment in the equatorial Pacific, the concentration of pennate diatoms in the iron-enriched patch increased by ~100 times compared to that outside the patch (Cavender-Bares *et al.*, 1999) (Figure 14).

There are interesting differences in how major phytoplankton taxa in HLNC regions of the oceans respond to iron additions. As exemplified in Figure 14, in a majority of experiments, the most dramatic difference between the +Fe incubations and the controls is the rapid growth of relatively large diatoms which become dominant. This is not unexpected since large phytoplankters should be particularly sensitive to nutrient limitation owing to the relatively low surface area to volume ratio which, on a per mass basis, decreases the diffusion rate of nutrients from the bulk and the availability of membrane area to anchor necessary transporters (vide supra). Addition of iron promotes faster growth of diatoms which can then outpace large grazers such as copepods, particularly under conditions where some of them may not have been representatively included in the sample being incubated (Banse, 1990).

A different response is usually seen for cyanobacteria. As seen in Figure 14, there is little change in the standing crop of *Prochlorococcus* or *Synechococcus* upon iron addition (Cavender-Bares *et al.*, 1999). But the mean fluorescence per cell of these phytoplankters increases markedly, indicating a higher chlorophyll concentration and, presumably, a faster rate of photosynthesis. An increase in the growth rate of cyanobacteria upon iron addition has indeed been confirmed by demonstrating an increase in the frequency of dividing cells in the population in the +Fe patch (Mann and Chisholm, 2000). The reason the standing crop of these faster growing cyanobacteria shows little response to iron addition is simply that their micrograzers (unlike the large zooplankters that feed on diatoms) are able to keep pace with them. In view of their small size, it is not clear why these organisms should be particularly limited under ambient iron conditions. The answer seems to be that they proportionally require more iron than eukaryotes (Brand, 1991; Raven, 1988, 1990), and that they are in fact somewhat less Fe-limited than diatoms, e.g., 60% instead of 20% of μ_{max} (Mann and Chisholm, 2000; Martin *et al.*, 1994).

The coccolithophores, which constitute the third family of phytoplankters that are most commonly found in the open oceans including some HNLC regions, do not appear to respond much to iron additions (Lam *et al.*, 2001). Because these organisms are responsible for the bulk of the precipitation of $CaCO_3$ in the open oceans, the net effect of iron addition is to increase the ratio of inorganic carbon fixed into organic biomass to that precipitated as $CaCO_3$. The absence of response of coccolithophores to iron additions presumably indicates that they are not limited by iron availability, even in very low-Fe environments. In fact, some culture studies show that oceanic coccolithophores have particularly low-Fe requirements (Brand, 1991; Brand *et al.*, 1983; Sunda and Huntsman, 1995a). In addition *Emiliana huxleyi* (the most abundant coccolithophore species) is able to take up iron at a faster rate per unit area of cell surface in heavily chelated medium than diatoms and dinoflagellates (Sunda and Huntsman, 1995a), and the maximum growth rate of coccolithophores is often low, reflecting their adaptation to low-nutrient (including iron) conditions in open-ocean environments. Thus, it is perhaps not surprising that the growth of coccolithophores should not be noticeably stimulated by iron additions.

6.05.4.1.2 *Iron uptake*

A question arises when the laboratory and field data are compared quantitatively. As is typical, the laboratory data of Figure 13(a) shows that little
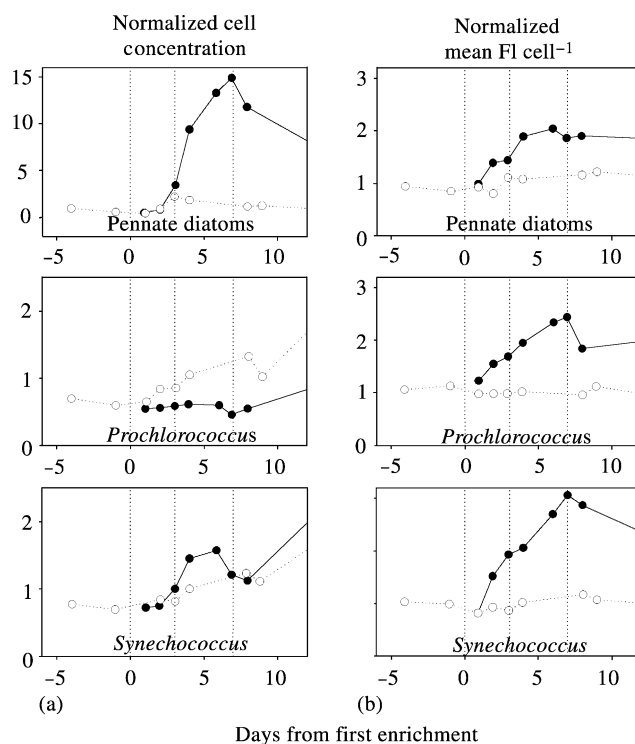
Figure 14 Response of different phytoplankton groups in the iron fertilized patch (closed symbols) relative to outside the patch (open symbols) of IronEx II in the equatorial Pacific Ocean. Normalized mean pigment fluorescence per cell is orange fluorescence for *Synechococcus* and red for *Prochlorococcus* and pennate diatoms. Fluorescence and cell abundance are normalized to values outside the patch (after Cavender-Bares *et al.*, 1999).

growth of coastal diatoms and dinoflagellates should occur below $Fe' = 50$ pM. Data for open-ocean species show growth limitation at a few pM. In the field, it has been shown by electrochemical measurements that 99% of the total dissolved iron concentration is present as strong organic complex. Thus, for example, the ambient diatom population in the equatorial Pacific, which is known to grow at about 0.3 day^{-1}, does so at ambient $Fe' \sim 0.015$ pM (Rue and Bruland, 1997). How can diatoms in the field grow so fast at such low unchelated iron concentrations that do not allow laboratory cultures to grow and the rate of diffusion to the cell surface to be sufficient for the organism? Clearly, other pools of iron must be accessible to these diatoms. Field experiments have confirmed that iron complexed to some siderophores can be taken up by the ambient flora, presumably by the mechanism illustrated in Figure 5 (Maldonado *et al.*, 1999). But additions of siderophores to field samples have also resulted in a decrease in iron uptake by phytoplankton (Hutchins *et al.*, 1999b), implying that iron–siderophore complexes may not be the only source of iron to primary producers, and that other, perhaps dominant, modes of iron acquisition are to be discovered.

6.05.4.1.3 *Iron and electron transfer*

In view of the dominant role of iron in the electron transport chain of photosynthesis, one can presume that a decrease in photosynthetic rate is the main effect of iron limitation on phytoplankton. This is confirmed indirectly in laboratory cultures where low iron availability can be partly compensated by high light intensity: the same specific growth rate can be achieved by the dinoflagellate *Prorocentrum minimum* at a lower ambient unchelated iron concentration when the light intensity is higher (Sunda and Huntsman, 1997) (Figure 13(a)). The photosynthetic units of cells growing at higher light intensity require less chlorophyll *a* and less iron, and this is reflected in a lower overall cellular iron quota (Figure 13(b)). In cyanobacteria, severe iron limitation causes cells to freeze, wherever they are in the cell cycle, consistent with a reduction in electron transport efficiency (Saito, 2001). The prediction that heterotrophic bacteria should require less iron than photosynthetic organisms appears also to be borne out by laboratory data. Under iron-limiting and replete conditions, the range for heterotrophs is 0.1– 12 μmol Fe (mol C)$^{-1}$ (Granger and Price, 1999) and that for eukaryotic phytoplankton

is 7–100 μmol Fe (mol C)$^{-1}$ (Sunda and Huntsman, 1995a). The scant available field data are also consistent with the prediction that iron stress should be reflected in the nature and the concentration of cellular electron transport carriers: in a transect from Vancouver Island to the subarctic Pacific gyre, it has been observed that the ratio of flavodoxin to ferredoxin in field populations increased as the iron concentration decreased (La Roche *et al.*, 1999).

6.05.4.1.4 *Iron and nitrogen acquisition*

We have seen that besides its central role in photosynthesis and respiration, iron also plays an important role in the acquisition and cycling of nitrogen. As predicted from calculations, laboratory cultures growing on NO_3^- require ~60% more iron than those growing on NH_4^+ (Maldonado and Price, 1996). However, laboratory experiments have not usually shown a difference in the growth of iron-limited cells grown on NO_3^- or NH_4^+ with the one exception of severely iron-depleted *T. oceanica* cultures (Henley and Yin, 1998; Kudo and Harrison, 1997; Maldonado and Price, 1996). In the field, it appears that the effect of low iron concentrations on NO_3^- uptake and assimilation is a significant part of diatom limitation (Price *et al.*, 1994). While small prokaryotes can acquire sufficient nitrogen from the low ambient NH_4^+ concentration, large diatoms cannot and must rely on NO_3^-, which requires additional iron for assimilation. At low iron concentrations, these organisms are thus co-limited by iron and nitrogen, as can be shown by the increase in growth rate resulting from either iron or NH_4^+ addition.

Because of the high-Fe content of nitrogenase and the additional energy requirements, the effect of iron on N_2 fixation should be even greater than that on NO_3^- uptake and assimilation. Data on relevant marine organisms have been difficult to obtain as *Trichodesmium*, the organism thought to be responsible for the bulk of N_2 fixation in the oceans, is notoriously fussy in culture. None-theless, laboratory studies have shown that *Trichodesmium* requires 5 times more iron when grown on N_2 than it does when grown on NH_4^+ (similar to the theoretical Fe factor of 7–11 times) (Kustka *et al.*, 2003b) (Figure 15(a)). Nitrogenase activity (measured by acetylene reduction) shows a precipitous drop below a threshold iron quota of ~35 μmol Fe (mol C)$^{-1}$ (Berman-Frank *et al.*, 2001) (Figure 15(b)). The results of such studies have been used, together with models of iron inputs to surface seawater, to predict that N_2 fixation in most of the oceans is actually limited by iron availability.

6.05.4.2 Manganese

We have seen that of all the trace metal requirements of phytoplankton, manganese is second only to iron. It is indeed also easy to limit the growth of diatoms such as *T. pseudonana* by lowering the Mn' concentration in the medium (Sunda and Huntsman, 1996) (Figure 16). But in the open ocean, the dissolved concentration of manganese in surface waters—in contrast to those of most other trace elements—is higher than that in deep waters (Figure 3) and usually far in excess of those that limit the growth of laboratory cultures. The high ambient Mn^{2+} concentration
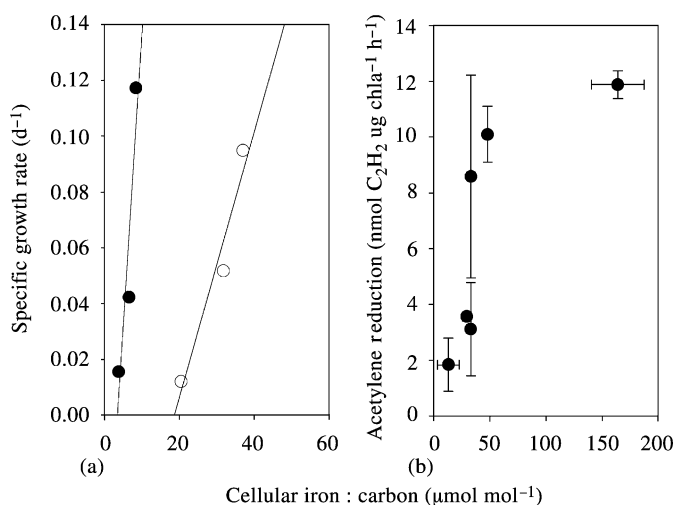

Figure 15 (a) Intracellular iron normalized to carbon in laboratory grown *Trichodesmium* sp. (a marine nitrogen-fixing cyanobacterium) with NH_4^+ (closed symbols) and N_2 (open symbols) as nitrogen sources (after Kustka *et al.*, 2003b). (b) Acetylene reduction rates (a proxy for N_2 fixation rates) as a function of cellular iron (after Berman-Frank *et al.*, 2001).
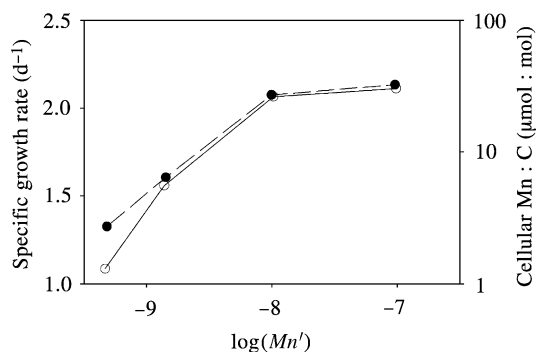
Figure 16 Specific growth rate (open symbols) and cellular Mn : C ratio (closed symbols) of the marine diatom *Thalassiosira pseudonana* as a function of $\log(Mn')$ (unchelated manganese concentration) (after Sunda and Huntsman, 1996).

is mirrored by a relatively low affinity for manganese uptake in marine phytoplankton (Sunda and Huntsman, 1998a). Zinc, copper, and cadmium have in fact a higher affinity for the manganese uptake system of model species than manganese itself. The result is that in some polluted coastal waters, a major effect of high metal concentrations is to inhibit manganese uptake by phytoplankton (Sunda and Huntsman, 1998a). Most of the manganese requirement of phytoplankton is in the water oxidizing complex of PS II. This can be seen in the slopes of specific growth rate versus Mn : C relationships obtained in laboratory cultures (Sunda and Huntsman, 1998c) which agree with those predicted from manganese use efficiency models based solely on the manganese requirement for O_2 evolution (Raven, 1990). The much greater availability of manganese than iron in surface seawater suggests that manganese might also replace iron in some other metalloenzymes in marine organisms. Indeed, manganese apparently provides a perfect replacement for iron in superoxide dismutase (SOD) in cultures of the diatom *T. pseudonana*. The cellular concentrations of manganese or iron used in SOD are sizeable fractions of the respective cellular quotas in this organism. Because the Mn- and Fe-SODs have the same molecular mass and migration patterns on non-denaturing protein gels, it has been suggested that the two metals may substitute for each other in the same enzyme (Peers and Price, personal communication).

From a bioinorganic perspective, one of the most interesting aspect of the marine geochemistry of manganese involves oxidation by bacteria. Although thermodynamically favorable, the chemical oxidation of Mn(II) to Mn(IV) by oxygen is exceedingly slow at the seawater pH. The oxidation of Mn(II) in the surface ocean is thus bacterially mediated (Sunda and Huntsman, 1988). In some cases, Mn(II) oxidation is effected

extracellularly by the bacteria themselves, in others, by their spores (Francis and Tebo, 2001, 2002). The enzymes responsible for catalyzing the oxidation of Mn(II) have been partly characterized. In all cases they contain a Cu-binding motif that is typical of multi-copper oxidases (Francis and Tebo, 2001, 2002) (Figure 17). It has been verified in cultures that the bacterially mediated oxidation of Mn(II) is indeed dependent on the presence of sufficient concentrations of copper in the medium (Brouwers *et al.*, 1999; van Waasbergen *et al.*, 1996). Photoinhibition of this bacterial oxidation increases the concentration and the residence time of the photoproduced Mn(II) in surface seawater (Sunda and Huntsman, 1988).

6.05.4.3 Zinc, Cobalt, and Cadmium

It is now becoming clear that the physiological importance of zinc rivals that of iron in biology. Although the cellular concentration of zinc is typically lower than that of iron (Figure 1), the number of known Zn-metalloproteins appears to be much larger than that of Fe-metalloproteins (Maret, 2002). In cultures of marine phytoplankton, it is commonly observed that zinc, cobalt, and cadmium can partially replace each, depending on the species. Thus these three metals often play similar biochemical roles in the marine environment. The distinct geochemistries of these elements and their different utilization by various taxa of phytoplankton likely influence the community composition of marine assemblages.

It appears that cobalt plays a particularly important role in the growth of cyanobacteria (Saito *et al.*, 2002; Sunda and Huntsman, 1995b). Both *Prochlorococcus* and *Synechococcus* show an absolute cobalt requirement that zinc cannot substitute for (Figure 18(a)). The growth rate of *Synechococcus* is little affected by low zinc concentrations, except in the presence of cadmium which then becomes extremely toxic (Saito *et al.*, personal communication). The biochemical processes responsible for the major cellular utilization of zinc and cobalt in marine cyanobacteria are unknown, however. These metals may be involved in carbonic anhydrase and/or other hydrolytic enzymes. Cobalamin (vitamin B_{12}) synthesis is a function of cobalt in these organisms, yet B_{12} quotas tend to be very small (on the order of only 0.01 μmol (mol C)$^{-1}$) and hence are not likely represent a significant portion of the cellular cobalt (Wilhelm and Trick, 1995).

The importance of cobalt in the physiology and ecology of cyanobacteria is underscored by evidence showing that they produce strong, specific cobalt chelators. Production of such cobalt
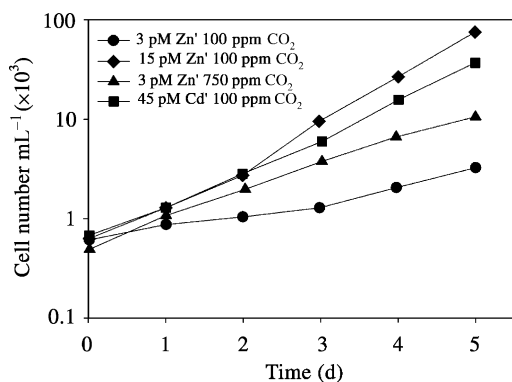
(a)

MnxG	527	M	H	I	H	F		
MofA	304	I	H	L	H	G		
CumA	95	I	H	W	H	G		
Asox	94	I	H	W	H	G		
Lacc	78	V	H	W	H	G		
Hcer	119	F	H	S	H	G		
Fet3	8	M	H	F	H	G		
CopA	99	I	H	W	H	G		
			2		3			

(b)

MnxG	572	F	F	H	D	H	(*Bacillus* sp. SG-1)
MofA	384	W	Y	H	D	H	(*Leptothrix discophora* SS-1)
CumA	136	W	Y	H	P	H	(*Pseudomonas putida* GB-1)
Asox	137	F	Y	H	G	H	(plants)
Lacc	121	W	Y	H	S	H	(fungi/plants)
Hcer	178	I	Y	H	S	H	(vertebrates)
Fet3	52	W	Y	H	S	H	(yeast)
CopA	140	W	Y	H	S	H	(*Pseudomonas syringae* PT23)
				3		3	

(c)

MnxG	281	H	V	F	H	Y	H	V	H
MofA	1174	H	P	V	H	F	H	L	L
CumA	391	H	P	I	H	L	H	G	M
Asox	480	H	P	W	H	L	H	G	H
Lacc	508	H	P	I	H	K	H	G	N
Hcer	994	H	T	V	H	F	H	G	H
Fet3	341	H	P	F	H	L	H	G	H
CopA	542	H	P	I	H	L	H	G	M
		1			2		3		

(d)

MnxG	334	H	C	H	L	Y	P	H	F	G	I	G	M
MofA	1279	H	C	H	I	L	G	H	E	E	N	D	F
CumA	442	H	C	H	V	I	D	H	M	E	T	G	L
Asox	542	H	C	H	I	E	P	H	L	H	M	G	M
Lacc	585	H	C	H	I	A	S	H	Q	M	G	G	M
Hcer	1039	H	C	H	V	T	D	H	I	H	A	G	M
Fet3	411	H	C	H	I	E	W	H	L	L	Q	G	L
CopA	590	H	C	H	L	L	Y	H	M	E	M	G	M
		3	1	3				1				1	

Figure 17 Copper-binding sites in multi-copper oxidases. Amino acid alignment of the copper-binding sites in MnxG, MofA, CumA, and other multi-copper oxidases. The letters A–D correspond to the 4 copper binding sites. Abbreviations: Asox, ascorbate oxidase (cucumber and squash); Lacc, laccase (fungi); Hcer, human ceruloplasmin; FET3, an Fe(II)-oxidizing protein in yeast; and CopA, a copper-resistance protein. The amino acids conserved among the different proteins are shaded and the copper-binding residues are numbered according to the spectroscopic type of copper they potentially coordinate.

organic complexes has been observed during a *Synechococcus* bloom in the equatorial Pacific (Saito and Moffett, 2001b) and uptake of organically complexed cobalt has been demonstrated in *Prochlorococcus* cultures (Saito *et al.*, 2002). These results have led to the hypothesis that cobalt ligands in surface seawater are produced by cyanobacteria and that they are "cobalophores" whose function in cobalt chelation and uptake is analogous to that of siderophores for iron.

Unlike marine cyanobacteria, coccolithophores are able to substitute cobalt and zinc for each other almost indifferently (Figure 18(c)), but, in some instances, have been observed to reach maximal growth rates only at high cobalt concentrations (Sunda and Huntsman, 1995b). Addition of cadmium to low zinc cultures of the ubiquitous species *Emiliana huxleyi* grown with organic phosphate is effective in restoring high growth rate and alkaline phosphatase activity (Xu, Ho, and Morel, unpublished data). This suggests that cadmium may substitute for zinc as a metal center in alkaline phosphatase.

In diatoms, it is generally possible to demonstrate an absolute zinc requirement, but above that threshold, addition of zinc, cobalt, or cadmium to the cultures are equally effective at promoting fast growth. This is illustrated for zinc and cadmium in Figure 19 which also shows that the growth rate can be accelerated by increasing the p_{CO_2} of the medium. Native protein gels from cultures labeled with ^{65}Zn, ^{57}Co, or ^{109}Cd demonstrate that a large fraction of the cellular zinc, cobalt, or cadmium (depending on medium composition) is associated

Figure 18 The effect of varying cobalt and zinc concentrations on growth rate in (a) the marine cyanobacterium *Prochlorococcus* (MED4-Ax) (source Saito *et al.*, 2002) and (b) the marine coccolithophorid *Emiliania huxleyi* (after Sunda and Huntsman, 1995b).

Figure 19 The effect of varying zinc, cadmium and CO_2 concentrations on growth rate of the marine diatom *Thalassiosira weissflogii*. Low Zn' (unchelated zinc concentration = 3 pM) reduces growth rate at 100 ppm CO_2. Increasing CO_2 to 750 ppm or adding Cd' (unchelated cadmium concentration = 45 pM) to the medium allows for higher growth rates (after Lane and Morel, 2000a).

with carbonic anhydrase (CA) (Morel *et al.*, 1994). There is thus little doubt that a primary role of zinc, cobalt, and cadmium in diatoms is to serve as a metal center in a CA that catalyzes inorganic carbon acquisition and that limiting these metals leads to a reduction in the rate of carbon uptake and fixation (Figure 11).

Intracellular CAs from the diatom *T. weissflogii* have been isolated and sequenced, and their activities have been monitored as a function of p_{CO_2}, Zn', Co', and Cd' in the culture medium (Lane and Morel, 2000b; Roberts *et al.*, 1997). As expected both the CA activity of cell extracts and the concentrations of the CAs increase when the p_{CO_2} of the medium is decreased (Figure 20). Low Zn' also limits CA activity and the concentration of a cytoplasmic CA, TWCA1, measured by quantitative Western analysis. The measured increase in CA activity and in TWCA1 concentration upon addition of either zinc or cobalt to low Zn' cultures show that TWCA1 can use zinc or cobalt indifferently as its metal center. In contrast, the increases in CA activity observed upon addition of cadmium to low-Zn' cultures result from the synthesis of a wholly distinct Cd enzyme, CdCA. This is the first native Cd enzyme discovered. X-ray spectroscopy (EXAFS) shows that, in TWCA1, zinc is coordinated to three imidazole nitrogens from histidines, similar to the zinc coordination in mammalian α-CAs (Cox *et al.*, 2000) (Figure 21). In contrast, XANES data indicate that, in CdCA, cadmium is likely to be coordinated to sulfur ligands from cysteins. These two CAs from diatoms have no homology to any other known CAs (or any other protein or to each other) and they thus constitute new classes, the δ-CAs and the ε-CAs
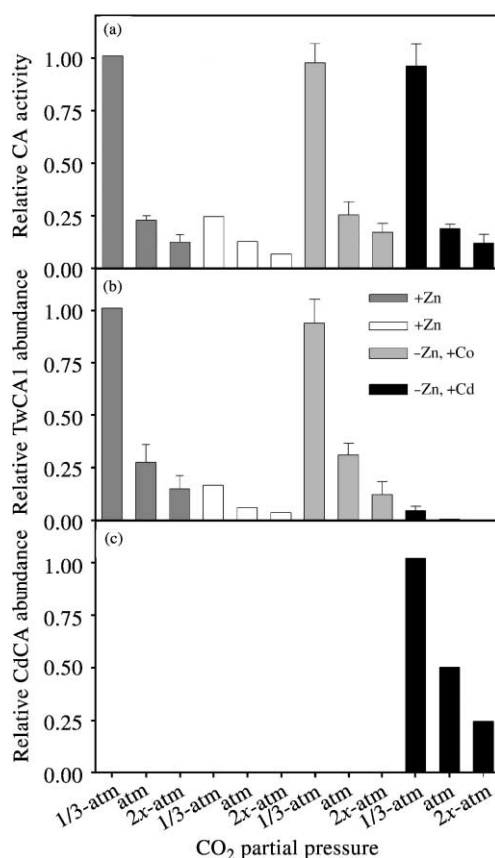

Figure 20 Relative levels of carbonic anhydrase (CA) activity (A), amounts of TWCA1 protein (a zinc containing CA) and amounts of CdCA protein (a cadmium containing CA) in the marine diatom *Thalassiosira weissflogii* as a function of metal treatment and CO_2 levels. $+Zn = 15$ pM Zn', $-Zn = 3$ pM Zn', $+Co = 21$ pM Co', $+Cd = 45$ pM Cd' (after Morel *et al.*, 2002).

(Smith and Ferry, 2000). The periplasmic CAs of diatoms have not yet been isolated or characterized. Whole cell CA activity assays under various conditions of p_{CO_2}, Zn', Co', and Cd' indicate that these periplasmic enzymes may also be able to utilize zinc, cobalt, or cadmium as their metal centers (Morel *et al.*, 2002).

The available field data show that the unchelated surface concentrations of zinc, cobalt, and cadmium are on the order of a few picomolar or even lower (Bruland, 1992, 1989; Ellwood and van den Berg, 2000, 2001; Saito and Moffett, 2001b). These are the ranges of unchelated metal concentrations over which limitation and replacement occurs in laboratory cultures of marine phytoplankton. The geographic distribution of the surface concentrations of these metals supports the idea that phytoplankton in the field take up cobalt and cadmium as a replacement for zinc. As seen in Figure 22, the disappearance of cobalt and

cadmium in surface waters of the north Pacific becomes correlated with that of P (which serves as a convenient measure of algal nutrient uptake) when the concentration of zinc is depleted
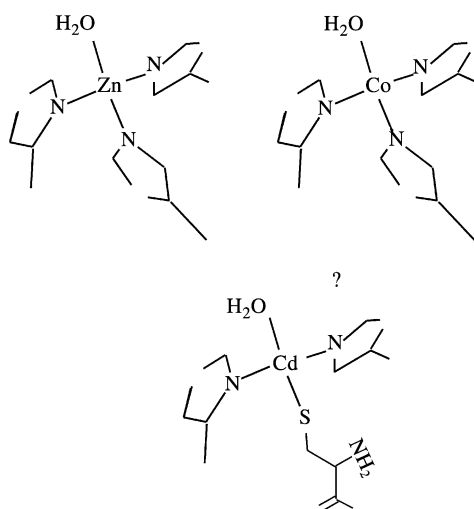

Figure 21 Active centers of carbonic anhydrase (CA) from the marine diatom *Thalassiosira weissflogii.* The zinc and cobalt centers are found in the protein TWCA1; (Cox *et al.*, 2000); Cox, unpublished data). The Cd center is found in the protein CdCA and is hypothetically based on unpublished XANES data showing sulfur binding and unpublished protein sequence data.

below approximately 1.2 nM (Martin and Gordon, 1988; Sunda and Huntsman, 1995b; Sunda and Huntsman, 2000). More directly, data on the concentration of cadmium in phytoplankton in the upwelled waters off Monterey, CA, whose flora is dominated by diatoms, show a clear increase in phytoplankton cadmium when zinc and CO_2 are low in concentrations (Cullen *et al.*, 1999) (Figure 23).

Despite the very low concentrations of zinc, cobalt, and cadmium in surface seawater and all the data showing that these three metals are taken up and used by phytoplankton, the evidence for limitation of primary production by any of these metals is only anecdotal (Coale, 1991; Fitzwater *et al.*, 2000). Several incubation experiments in which zinc, cobalt, or cadmium have been added have yielded negative results (although this is a difficult claim to document since most such negative results remain unpublished). One reason for these negative results may of course be the difficulty inherent in experiments where the controls must be free of zinc contamination. Another reason may simply be that in regions where both upwelling and aeolian inputs of essential trace metals are very low, iron is usually "most limiting." In this respect it is worth noting that a sizeable proportion of the few experiments that have shown increased phytoplankton growth upon zinc, cobalt, or cadmium addition were
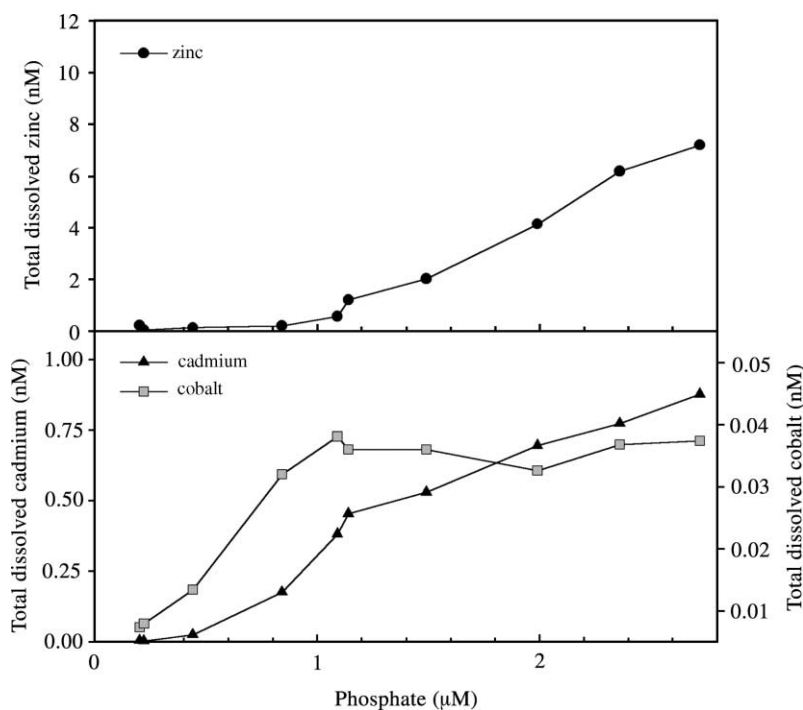

Figure 22 The sequential drawdown of zinc, cadmium, and cobalt in the north Pacific suggestive of biochemical substitution in the phytoplankton community. Metal versus phosphate concentrations are plotted from vertical profile T-5 (after Sunda and Huntsman, 1995b, 2000; Martin *et al.*, 1989).
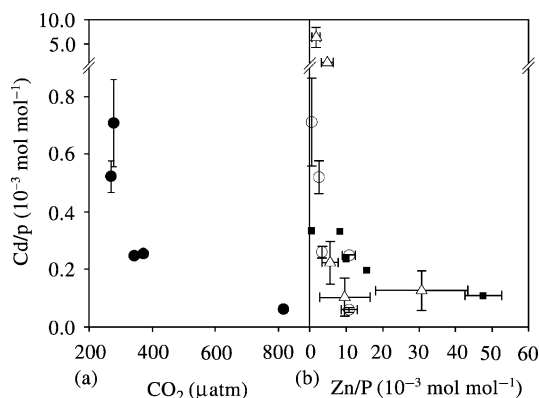
Figure 23 (a) Cd : P ratio as a function of the partial pressure of CO_2 of a natural phytoplankton assemblage from the coast of central California. (b) Cd : P versus Zn : P in different size fractions of the natural phytoplankton assemblage. Total particles $>0.45\,\mu m$ (circles) $5-53\,\mu m$ fraction (squares) $>53\,\mu m$ fraction (triangles) (after Cullen *et al.*, 1999).

performed in conjunction with iron addition. For example, Saito and Moffett (2001b) reported a significant increase in chlorophyll after two days upon Fe + Co addition compared to little effect with Fe- or Co-only additions.

Another, more biochemical, explanation may be that if Zn/Cd/Co limitation manifests itself primarily as an inability to synthesize carbonic anhydrase, phytoplankton starved of these metals may acquire inorganic carbon by transporting HCO_3^- (at some cost) or by relying on CO_2 diffusion. As a result growth might only be marginally slowed down, and the major effect of Zn/Co/Cd limitation may be a shift in phytoplankton species composition rather than a decrease in the photosynthetic rate of the community as seen under iron limitation. On the basis of the differences in the inorganic carbon acquisition systems discussed above, the effects of adding these metals are expected to be different for different species of phytoplankton. Indeed it has been demonstrated in the field that diatoms take up chiefly CO_2 (derived in part from dehydration of HCO_3^- catalyzed by extracellular CA) while cyanobacteria take up mostly HCO_3^- (Tortell and Morel, 2002). It has also been shown that modulation of the ambient p_{CO_2} changes the expression of CA in diatoms in the field. Thus the need for zinc and cobalt, and their uptake by diatoms should increase at low p_{CO_2} as has been observed for cadmium (Figure 23). In cyanobacteria and coccolithophores, the link between Zn/Co and Cd supply and inorganic carbon acquisition is likely to be much looser and the variations in requirements for Zn/Co and Cd cannot be predicted since their principal biochemical roles are presently unknown.

6.05.4.4 Copper

Laboratory data demonstrating copper limitation of marine phytoplankton are rare. It has generally been difficult to limit the growth rates of cultures by lowering the copper concentration in the medium, indicating a very low absolute copper requirement and/or a very effective uptake system in most species (Sunda and Huntsman, 1995c). If, as predicted, plastocyanin normally accounts for a major fraction of the copper quota of phytoplankton, a low-copper requirement would result from the known ability to replace plastocyanin by cytochrome *c* in the electron transport chain of the light reaction in a number of taxa.

Some experiments demonstrating copper limitation in marine phytoplankton apparently involve the utilization of external copper oxidases. Under conditions where ethalonamine is the sole nitrogen source in the culture medium, the coccolithophore *Pleurochrysis carterae* requires high copper concentration for growth (Palenik and Morel, 1991). This organism possesses an extracellular amine oxidase to cleave NH_4^+ from the amine and such enzymes normally contain copper (Figure 11). Under iron-limiting conditions, cultures of several species of diatoms and a coccolithophore required high copper concentrations for rapid iron uptake and growth (Price, Maldonado and Granger, personal communication). This is consistent with the involvement of a multi-copper oxidase in the uptake system for chelated iron (Figure 5). An apparent high copper requirement, independent of iron uptake, has also been observed in oceanic species of diatoms such as *Thalassiosira oceanica* (Price, Maldonado, and Granger, personal communication). Because such species are known to have extraordinarily low iron requirements, it has been hypothesized that copper may be substituting for iron in some important biochemical pathways.

While copper limitation of marine phytoplankton has rarely been observed, many experiments have demonstrated that copper is extremely toxic to various species, often decreasing growth even at only a few pM unchelated concentrations (Anderson and Morel, 1978; Sunda and Guillard, 1976). When these data are compared to field data on copper concentration and speciation in seawater, it appears that Cu^I concentrations may be approaching values that are toxic to some species in coastal waters. At these levels the biological effects of copper are complex and often appear to involve interference with the uptake or assimilation of other essential trace metals such as zinc or manganese (Sunda and Huntsman, 1998a). An indication that copper concentrations may be marginally toxic to the ambient flora in coastal waters is provided by the analysis of phytochelatins in the algal biomass. As seen on Figure 24, in
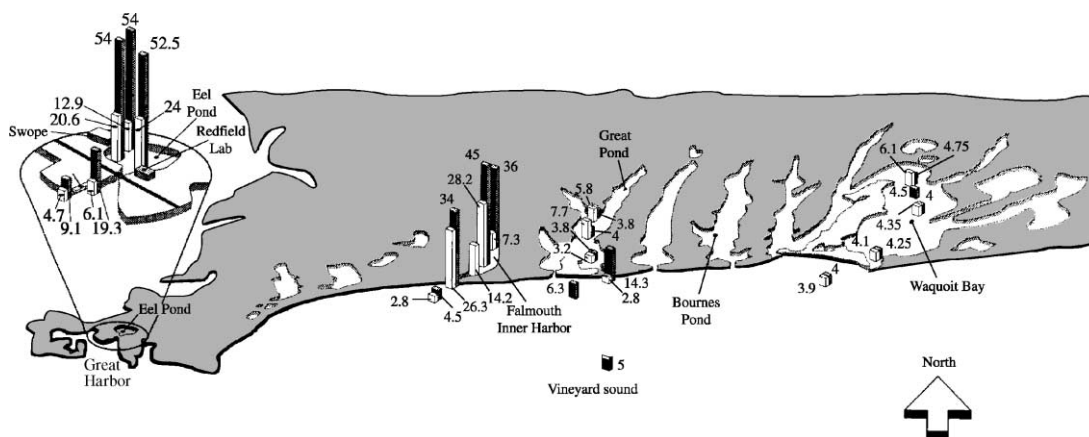
Figure 24 A map of western Cape Cod, MA, USA showing sample sites and concentrations of total dissolved copper and phytoplankton associated phytochelatin. Dark bars represent total copper (nM) and light bars are phytochelatin (μmol (g chl$a$)$^{-1}$) (after Ahner *et al.*, 1997).

embayments around Cape Cod, high phytochelatin concentrations correlate with high concentrations of unchelated copper (Ahner *et al.*, 1997). Incubation experiments confirm that copper is likely to be the major inducer of phytochelatins in these waters. In the open ocean, copper may also be toxic to some species of phytoplankton. For example, in the Sargasso Sea, it appears that the concentration of unchelated copper in the surface mixed layer is too high for the growth of the cyanobacterium *Prochlorococcus*, particularly the low-light adapted strain which thrives at great depths (Mann *et al.*, 2002). The molecular basis for the high sensitivity of *Prochlorococcus* to copper is unknown. It is worth noting that the strong copper chelators in seawater (which are still unidentified) seem to be released by another group of cyanobacteria, the *Synechococci*, which are less sensitive to copper toxicity (Moffett, 1995; Moffett *et al.*, 1990).

While most of the data on the effect of copper on marine phytoplankton has focused on copper toxicity, some data point to a potential limiting role of copper in other marine microorganisms. At very low unchelated copper concentrations, cultures of denitrifiers are unable to reduce N_2O to N_2—the last step in denitrification (Granger and Ward, 2002) (Figure 25). This inhibition results from insufficient supply of metal to the copper metalloenzyme, nitrous oxide reductase (N_2OR, see Figure 12). The denitrifiers (at least those in culture) have apparently not evolved an alternative for copper-containing N_2OR, nor are they able to reuse in N_2OR the copper contained in respiratory nitrite reductase (for many of them have the copper form of NIR). On the basis of these results, it has been hypothesized that copper availability may be an important factor, in addition to oxygen concentration, in regulating the marine production of nitrous oxide. Some
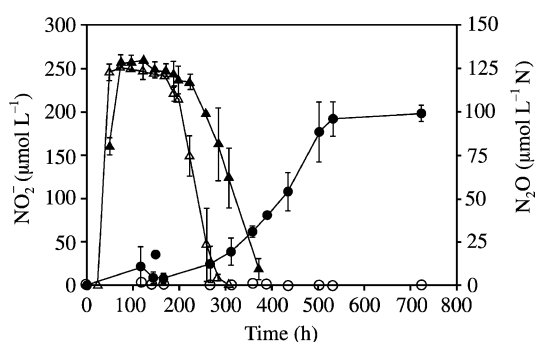

Figure 25 Effect of copper on nitrite (NO_2^-) consumption and nitrous oxide (N_2O) evolution in a culture of the denitrifying bacterium *Pseudomonas stutzeri*. Concentration of nitrite (triangles) and nitrous oxide (circles) in copper-replete (open symbols) and copper-deficient (closed symbols) medium (after Granger and Ward, 2002).

parts of the oceans such as the Arabian Sea are "hot spots" where high N_2O concentrations are accumulated at mid-depth and released to the atmosphere. As a result of advection of anoxic coastal water masses, this area is rich in sulfide which is apt to complex and precipitate copper and may result in copper limitation of nitrous oxide reductase activity.

6.05.4.5 Nickel

Of all the first row transition elements, nickel has received the least attention from oceanographers. Although the vertical profiles of nickel concentration in the oceans exhibit a surface depletion characteristic of nutrients, the surface values remain typically in the 1–5 nM range (Bruland *et al.*, 1994) (Figure 3), much in excess of the other elements discussed so far. Nonetheless, laboratory data demonstrate that
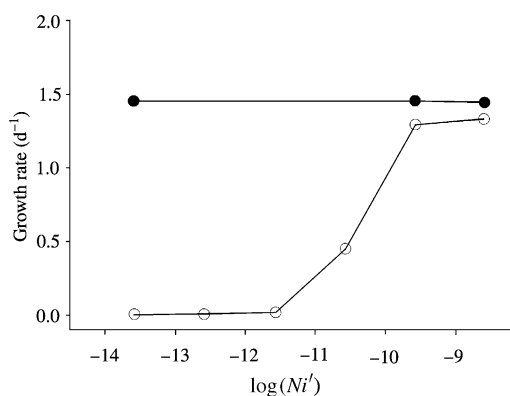
Figure 26 Growth of the marine diatom *Thalassiosira weissflogii* as a function of nickel concentration with ammonium (closed symbols) and urea (open symbols) as nitrogen sources (after Price and Morel, 1991).

nickel is necessary for the assimilation of urea. As shown in Figure 26, the diatom *T. weissflogii* grows equally well in the presence of $CO(NH_2)_2$ or NH_4^+, only when the unchelated nickel concentration is in excess of 0.2 nM (Price and Morel, 1991). Since the importance of urea as a nitrogen source for marine phytoplankton has been demonstrated in field studies, the high surface concentration of nickel in surface seawater is puzzling. As discussed above, it is possible that this high concentration reflects slow kinetics of uptake caused by the inertness of the Ni^{2+} ion. For example, the diatom *T. weissflogii* must take up roughly 15–30 amol of nickel per day to grow on urea at a specific growth rate of 1.5 day^{-1}. With a second order rate constant ~ 1–$3 \times 10^4\,M^{-1}\,s^{-1}$ for binding to an uptake ligand (reflecting the slow rate of loss of hydration water from the Ni^{2+} ion), and a nickel concentration of 1 nM, 1–10 amol of uptake proteins per cell would be necessary to achieve that uptake rate. As shown before, this is a very high density of membrane proteins. Despite their need for nickel to assimilate urea, marine phytoplankton may thus be unable to deplete the nickel concentration of surface seawater below a few nanomolars, simply because of the kinetic inertness of the Ni^{2+} ion (Hudson *et al.*, 1992). In view of the difficulty inherent in acquiring Ni^{2+} from seawater, the apparent ability of cobalt to replace nickel for urease activity in some phytoplankton species (Rees and Bekheet, 1982) may be oceanographically relevant.

6.05.5 EPILOGUE

6.05.5.1 Paleoceanographic Aspects

It is interesting to consider the variations in trace-metal availability in the oceans throughout the geologic history of the Earth relative to the trace-metal requirements of marine phytoplankton. Previous workers have postulated that trace-metal solubility was strongly influenced by the changing redox conditions of the oceans resulting from the activity of early oxygenic organisms, chiefly cyanobacteria (Williams and Frausto da Silva, 1996). It is generally agreed that Fe(II) and S(−II) at the Earth's surface have been oxidized over a period of some 2 billion years, before the build up of oxygen in the ocean and atmosphere (Holland, 1984). Thus life is thought to have originally evolved in an ocean rich in iron which would have become gradually depleted as a result of the formation of insoluble iron oxyhydroxides. Iron being in excess of sulfur in the Earth crust could not have been all precipitated as sulfides. While Fe is a trace element in the present day oceans it is the fifth most abundant element in continental rock. In parallel, trace metals, such as copper, zinc, cadmium, and molybdenum that form highly insoluble sulfides and were presumably rare in the early oceans, would have become gradually more abundant upon the oxidation of sulfide to sulfate. The concentrations of manganese and cobalt, whose sulfides are somewhat more soluble but which also form insoluble oxyhydroxides, would have, like that of iron, decreased over time. It is possible that the deep oceans may have been particularly high in sulfide for a period of time 2.5–0.5 billion years ago resulting in low iron, copper, zinc, cadmium, and molybdenum conditions (Anbar and Knoll, 2002).

As a result of selection pressure during the course of evolution, the changing concentrations of trace metals over geologic time should be somehow reflected in the trace-metal requirements of marine phytoplankton (Raven, 1988; Saito *et al.*, 2002; Scott, 1990; Sunda and Huntsman, 1995b). Indeed the trace-metal physiology of cyanobacteria is strikingly consistent with the presumed chemistry of the early oceans. Cyanobacteria have a relatively large iron quota (Raven, 1988), have an absolute cobalt requirement that zinc cannot substitute for (Saito *et al.*, 2002; Sunda and Huntsman, 1995b), and are extremely susceptible to copper and cadmium toxicity (Brand *et al.*, 1986; Mann *et al.*, 2002). In contrast, eukaryotic phytoplankton, particularly the diatoms and coccolithophores which evolved some 3.5 billion years later in an oxic and metal-poor ocean, have lower requirements for iron, lower sensitivity to copper and cadmium, and unusual enzymes with alternative metals, such as the cadmium carbonic anhydrase.

Why then haven't the cyanobacteria evolved in response to the large changes in trace-metal chemistry between an ancient reducing ocean and a modern oxidizing one? Why have they kept a biochemical apparatus that reflects early ocean

chemistry instead of evolving one more similar to that of the eukaryotes? While we have no answer to such questions, we can speculate that the adaptive response of cyanobacteria has been to release into seawater specific metal chelators for the acquisition of essential metals, e.g., siderophores and cobalophores for iron and cobalt, and for the detoxification of others, e.g., strong copper chelators (Mann *et al.*, 2002; Moffett and Brand, 1996). In this view, the oceans are a *milieu externe* whose chemistry is controlled by (and for) cyanobacteria (and other prokaryotes) and has been so for billions of years. The eukaryotic phytoplankton then learned to make a living in this "inhospitable" environment by adapting their biochemistry and controlling the chemistry of their intracellular space and immediate surroundings—their *milieu interne*. As we consider the co-evolution of marine life and geochemistry which is reflected in the modern biochemistry of marine microorganisms and the chemistry of seawater, we must take into account the different timescales over which various organisms have been adapting to their marine environment (and modifying it).

6.05.5.2 A View to the Future

Over the past several decades, the importance of trace elements in the marine environment has become apparent, with major regions of world's oceans now known to be affected by iron limitation, and with the first accurate measurements of incredibly scarce quantities of other essential metals. Yet, we understand little of how metal limitation manifests itself in the biochemistry of marine microorganisms or of the role of trace elements in the geochemistry of the oceans. But we now know enough to ask relevant questions at both the molecular and global scale, and are able to use novel experimental tools to help answer these questions.

It appears that the genomic revolution and the experimental tools associated with it should also revolutionize the field of oceanography; and marine bioinorganic chemistry should be at the heart of that revolution. The sequencing of complete genomes for a variety of marine microorganisms (e.g., at the time of this writing, four marine cyanobacteria and a marine diatom have been sequenced) will provide us with the ability to search for putative metalloproteins, allow genomic microarray experiments to be conducted, and facilitate proteomic work. These genomic and proteomic tools, together with others, will allow us to address a number of difficult but important questions about the interactions of trace elements and the biota in the marine environment.

The outstanding questions in marine bioinorganic chemistry can be organized around three principal topics. First are the questions related to the trace element requirements of marine microorganisms: how do open-ocean species manage with extraordinarily low metal concentrations? What metals are they able to substitute for each other? What unusual enzymes (perhaps with "exotic" metal centers) or enzymatic pathways are they using? What differences are there between the requirements of different organisms (prokaryotes versus eukaryotes; autotrophs versus heterotrophs; diatoms versus coccolithophores; etc.)? How does the scarcity of nutrients such as carbon, nitrogen, phosphorus, and iron affect the trace element requirements of phytoplankton?

Then there are wider questions about the possible limiting roles of various trace elements in key biogeochemical processes: Do trace elements other than iron limit phytoplankton growth and primary production? Is the composition of phytoplankton assemblages controlled by trace elements? Are various processes in the cycle of nitrogen limited by trace elements (e.g., N_2 fixation by iron; N_2O reduction by copper)? What are the links between trace elements and the reduced sulfur cycle in surface seawater?

Finally, we must somehow resolve the vexing problem of the chemical speciation of trace elements in seawater: what is the chemical nature of the various metal chelators whose existence has been demonstrated by electrochemistry? Is the chemistry of several metals in surface seawater really controlled by metallophores released by prokaryotes? Or are dissolved metals chiefly present as parts of metalloproteins in the process of remineralization? How do metal chelators affect the residence times of metals (in particular, scavenged elements such as iron and cobalt) and in turn how do those chelators influence the global carbon cycle via changes in marine primary productivity?

By focusing on a molecular elucidation of key biochemical processes in the marine biogeochemical cycles of elements, marine bioinorganic chemistry should help us understand the subtle and complex interdependence of marine life and ocean geochemistry, and how they have evolved together over the history of the Earth.

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