COBALT SUBSTITUTION FOR ZINC IN MARINE PHYTOPLANKTON

by

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Abstract

Cobalt is a trace metal which behaves as a nutrient in some marine phytoplankton species. Although cobalt concentrations in the open ocean are typically at least an order of magnitude less than those of zinc, in some regions of the ocean where zinc is extremely depleted cobalt concentrations approach those of zinc.

Laboratory cultures of marine phytoplankton demonstrate that cobalt additions to culture media can alleviate zinc limitation of growth. This substitution can restore growth to near maximum rates in zinc-limited oceanic species, but is less effective in coastal phytoplankton. The effectiveness of cobalt substitution is correlated to the concentrations of zinc at which species become zinc-limited: the lower the zinc concentration necessary for growth, the more effective the cobalt substitution. Analysis of cellular metal contents show that oceanic species have relatively high cobalt to zinc ratios, and thus a lesser preference for zinc over cobalt than do coastal species.

One mechanism by which the substitution of cobalt for zinc occurs in the coastal diatom *Thalassiosira weissflogii* is by direct metal substitution in a soluble form of the zinc enzyme carbonic anhydrase, which is used in acquiring inorganic carbon. A similar soluble carbonic anhydrase was found in an oceanic diatom as well. The function of both zinc and cobalt in carbonic anhydrase of *T. weissflogii* is apparent from the alleviation of carbon limitation in cultures under low partial pressures of CO_2 by addition of either zinc or cobalt to the culture medium.

Assays of soluble proteins in other phytoplankton indicate that another function in which cobalt may substitute for zinc is in the enzyme superoxide dismutase. In contrast, cobalt does not appear to substitute for zinc in RNA and DNA polymerases. Despite the lack of direct substitution of cobalt for zinc in the nucleic acid fraction of zinc-limited cells, cobalt additions cause redistribution of cellular zinc from the insoluble to the soluble and nucleic acid fractions of a cell. This effect, as well as the presence of a large part of total cellular zinc in the insoluble fraction of many phytoplankton, suggest that a major role of cobalt substitution for zinc occurs in proteins found in membranes. This insoluble protein may be a form of carbonic anhydrase, as a positive response to metal addition under low CO_2 is found some phytoplankton species when zinc and cobalt are added, despite assays showing no carbonic anhydrase activity in the soluble fraction of these algae. Despite the fact that cobalt substitution for zinc may occur chiefly in membrane-bound proteins, nearly half the cellular cobalt is always found in an unknown low molecular weight compound. This compound may be a phytochelatin complex which acts as an internal cobalt buffer.

The effect of zinc and cobalt in alleviating carbon limitation may be reflected in the distributions of these metals in the surface oceans: the concentration of cobalt in surface waters follows nutrient-like vertical profiles where zinc is very depleted and surface temperature is high; the decreased solubility of CO_2 warm waters may require phytoplankton in these regions to produce carbonic anhydrase to enhance inorganic carbon uptake and thus utilize cobalt in the absence of sufficient zinc.

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Abbreviations and terms

CA	carbonic anhydrase
SOD	superoxide dismutase
M'	inorganic metal concentration
pM	10^{-12} mole liter $^{-1}$
PAGE	polyacrylamide gel electrophoresis
P_{CO2}	carbon dioxide partial pressure
amol	10^{-18} mole
M quota	metal concentration per cell
REf	relative enrichment factor, ratio of molar $(Zn:Co)_x$ to (Zn': Co') where x is some fraction of the cell (eg, whole cell, membranes, etc.)
NA	nucleic acids

Chapter 1. Introduction

Zinc is an essential trace metal nutrient found in many enzymes in all varieties of life. The primary documented biological role of cobalt is in vitamin B_{12}, but cobalt may substitute for zinc in some enzymes. In the oceans, zinc typically exhibits a depth concentration profile similar to those of other nutrients; within and near the photic zone, where the bulk of the biological activity is concentrated, the concentration of dissolved zinc is depleted relative to its concentration at depth. In contrast, the concentration profiles of dissolved cobalt vary only slightly with depth in some parts of the oceans but exhibit profiles typical of nutrients in other areas. Where cobalt concentrations are depleted at the surface, one might infer that it is a quantitatively important or even limiting nutrient for the ambient biota. Cobalt depletion at the surface appears to coincide with regions where zinc is extremely depleted. The substitution of cobalt for zinc in functional enzymes of marine micro-organisms might be one reason for this correlation.

Background

Biological functions of zinc and cobalt- The best known biological function of cobalt in eukaryotic phytoplankton, as in all organisms, is in B_{12} (1). Known biological functions of B_{12} (and thus of cobalt) include the following reactions: 1) reduction of ribose to deoxyribose; 2) rearrangement of diols; 3) rearrangement of malonyl to succinyl; 4) transfer of methyl groups. However, as discussed below, there also appears to be a metabolic role for cobalt as a substitute for zinc, which is not involved in redox chemistry.

The primary role of zinc in enzymes is as a Lewis acid rather than as a redox reagent. If we consider the list of zinc metalloenzymes and proteins known for a range of organisms shown in Table 1-1 (2), most of the reactions listed do not require a change in oxidation state. Even when the enzyme is an oxidoreductase, such as alcohol dehydrogenase or superoxide

dismutase, zinc is not directly involved in electron transfer, which is instead performed by NAD and copper in these enzymes, respectively.

Of the transition metals, cadmium is the most similar to zinc chemically (because of a similar outer orbital electron configuration) and is a likely candidate for substitution. Cadmium has been examined previously (3, 4) and has been shown to substitute for zinc in marine phytoplankton when zinc is limited, but only in some species under a narrow range of conditions. If we consider other divalent metal cations as possible substitutes for zinc, the most likely candidates are other transition metals, for reasons of reactivity and size. In the Irving-Williams series, the strength of these metal cations as Lewis acids are: $Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+}$. Nickel and copper, like cadmium, can be toxic to marine phytoplankton (5), and thus cobalt may be the best substitute for zinc in enzymes in spite of being somewhat inferior to copper and nickel as a Lewis acid.

Of the zinc enzymes listed in Table 1-1, some enzymes that have already been found in plants are also likely to be found in phytoplankton: carbonic anhydrase (CA), RNA polymerase, and superoxide dismutase (SOD). Most studies of cobalt substitution for zinc have been performed on enzyme systems in vitro; after removal of zinc, cobalt restores function to these enzymes (6, 7, 8). In some proteins like bovine CA, the cobalt-substituted enzyme is less effective (6), while in others such as bovine carboxypeptidase A (8), the cobalt-substituted enzyme has a higher catalytic rate than the native zinc-containing enzyme. Such substitution does not only occur in vitro however. In *Escherichia coli* grown in medium with low zinc and high cobalt concentrations, cobalt is incorporated into a functional RNA polymerase (9) , although the bacteria show no signs of zinc limitation.

Carbonic anhydrase is induced by growth in low CO_2 media for a variety of microalgae. Within these algae, CA is found in a variety of cellular compartments, ranging from inside the chloroplast in *Chlorella vulgaris* (10), to the periplasmic space in *Dunaliella tertiolecta* (11), to outside the cell, in *Chlamydomonas reinhardtii* (12, 13). The high pH and high total dissolved

inorganic carbon (but low CO_2) environments of marine phytoplankton are conditions where the catalysis of bicarbonate to CO_2 by CA might be especially beneficial to photosynthetic organisms.

Mechanisms for combating oxidizing radicals are also likely to be important in photosynthetic organisms such as algae which grow in oxic environments with high light fluxes. All the species of green algae examined in one study were revealed to possess enzymes with superoxide dismutase (SOD) activity (14). There are three forms of SOD, containing iron, or manganese, or copper and zinc (Cu-Zn SOD)(14), and some algae have a Cu-Zn form of SOD. In vitro studies on metal substitution in bovine Cu-Zn SOD indicate that copper is the redox active metal ion in this enzyme, whereas substitutions of zinc by a number of different metals (including cobalt) result in nearly full activity (15). The non-specificity of the metal in the zinc site suggests that it is one function in which cobalt might easily substitute for zinc in phytoplankton in vivo.

The DNA and RNA polymerases are also important zinc enzymes because of their ubiquity and their relatively large cellular concentrations. All known forms of life require these polymerases to replicate their genetic material, and the known forms in most organisms are zinc enzymes. Work by Vallee and co-workers on the eukaryote *Euglena gracilis* demonstrated large impacts on the reproduction and morphology of this organism during zinc limitation (16, 17, 18); DNA content per cell doubles, while RNA and protein expression decline dramatically. The in vivo substitution of cobalt for zinc in RNA polymerase of *Escherichia coli* (9) suggests that a role for cobalt in polymerases might also exist for eukaryotes under zinc-limited conditions. Zinc associated with nucleic material has been found also in zinc finger proteins, which have no known catalytic role, but appear to stabilize (and perhaps regulate) gene expression (19, 20, 21). Such a non-catalytic role might also be one in which cobalt can readily substitute for zinc in vivo.

Cobalt substitution for zinc in phytoplankton- There is indirect in vivo evidence for substitution of cobalt for zinc in the enzymes of marine phytoplankton. Price and Morel (22) found that the addition of inorganic cobalt to growth media that already contains B_{12} partially alleviates zinc limitation in cultures of the marine diatom *Thalassiosira weissflogii*. In cultures of this phytoplankter not limited by zinc, the addition of cobalt has little or no effect, demonstrating that its impact on growth is not an alleviation of a specific cobalt deficiency.

Similarly, Sunda and Huntsman (23) found that cobalt is able to alleviate zinc limitation in two diatoms and a coccolithophore. In the two diatoms they studied, *Thalassiosira oceanica* and *Thalassiosira pseudonana*, they found that cobalt partially restores growth to cultures limited by zinc. In contrast, for a strain of the coccolithophore *Emiliania huxleyi* isolated from the Sargasso Sea, the maximum growth rate with cobalt but no added zinc is higher than the growth rate with zinc but no added cobalt. In that same study, they found that the prokaryote *Synechococcus bacillus* has an absolute requirement for cobalt which cannot be replaced by zinc; without added cobalt, cultures do not grow regardless of the concentrations of zinc. In all the eukaryotic phytoplankton, including *E. huxleyi*, increasing zinc from low and limiting conditions to higher but not toxic concentrations ($Zn^{2+} < 10^{-10}$ M) inhibits cobalt uptake. In contrast, zinc uptake is not significantly inhibited in any of the phytoplankton by increasing cobalt except at very high concentrations ($Co^{2+} > 10^{-10.5}$ M). On the basis of these data, Sunda and Huntsman hypothesized that the coccolithophores, which may have evolved when conditions in the ocean were less oxic and Co concentrations were higher, might retain a vestigial preference for cobalt over zinc.

Although studies of phytoplankton growth and metal uptake provide information on the occurrence and extent of cobalt substitution for zinc, they do not address the mechanisms by which substitution occurs. The discovery of the zinc enzyme carbonic anhydrase in the diatom *T. weissflogii* indicated one means by which substitution of cobalt for zinc might occur. Carbonic anhydrase production in *T. weissflogii* is induced by low partial pressures of CO_2 in

growth medium, but this response is inhibited by zinc limitation of cultures. A large fraction of the soluble cellular zinc (about two-thirds of the soluble zinc, or one-third of the total cellular zinc) is contained in proteins which exhibit CA activity (24). The large fraction of the total cellular zinc that is in the CA of zinc-replete cells and the large differences in CA production between zinc-limited and replete cultures provide other clues that this is one enzyme in which we might find cobalt substitution under zinc-limiting conditions. Additions of either cadmium, another metal which demonstrates an ability to substitute for zinc as a nutrient (4, 22), or cobalt restore CA activities, although not to the levels found in zinc-replete cells.

Cobalt and zinc in the oceans- Cobalt substitution for zinc in laboratory cultures of marine phytoplankton suggests that substitution might also occur in the field. Cobalt concentrations at depth are typically two orders of magnitude lower than those of zinc at the same depths in the open ocean. In some regions however, zinc concentrations near the surface may be one or two orders of magnitude lower than zinc concentrations at depth, while cobalt concentrations remain invariant over the same depths. Thus the concentrations of cobalt at the surface in these regions may be similar to those of zinc. The low zinc and relatively high cobalt concentrations in these areas parallel conditions in laboratory studies under which cobalt substitutes for zinc.

Cobalt utilization may also be reflected in the characteristics of cobalt concentration profiles in the field. Using the data of Martin and co-workers from various stations in the Northeast Pacific, Sunda and Huntsman found that in waters only slightly depleted in zinc at the surface (greater than 0.3 nM dissolved Zn), cobalt concentrations are fairly invariant with depth. Only in those areas with very low zinc concentrations are surface concentrations of cobalt depleted (Figure 1-1) (23). However, for some of the stations of Martin and co-workers' studies, cobalt concentrations vary only slightly with depth in spite of very low surface concentrations of zinc. Thus a factor other than low zinc concentration appears important in determining the use of cobalt as an algal nutrient.

This other factor might be temperature. If we plot temperature versus dissolved cobalt using the data of Martin and co-workers (25, 26), we find that low surface concentrations of dissolved cobalt are indeed correlated to high temperatures where dissolved zinc values are also low (Figure 1-2). Although temperature may influence a number of other factors, this effect is not likely to be an effect of less mixing in warm regions; dissolved cobalt is normalized to salinity (35 *‰*) in this plot, and dilution effects are thus excluded. Decreased CO_2 solubility will be discussed in later chapters as a possible cause for this temperature effect .

Speciation of cobalt and zinc - Because trace metal uptake in phytoplankton is generally controlled by the concentrations of labile aqueous species (hydrated metal ions and inorganic complexes)(27, 28) rather than by total dissolved metal concentrations, the speciation of zinc and cobalt in seawater is important. Thus although surface concentrations of total dissolved zinc in the North Pacific have been measured at ~0.1 nM (25, 29), complexation by organic ligands results in inorganic zinc species concentrations (Zn') of around 2 pM (29). Likewise, 50 to 90 % of the dissolved cobalt is also strongly bound by organic ligands in natural seawater samples (30, 31). In computer calculations (MINEQL(32)) of metal speciation in the artificial seawater medium Aquil (in which the concentrations of the major inorganic anions are those of natural seawater), the major inorganic species of zinc and cobalt are the hydrated metal ions (82 % and 60 % of Zn' and Co' respectively), followed by the sulfate (11 % and 13 %), chloride (5 % and 25 %), and hydroxide (2 % and 3 %) complexes. It is therefore important to consider concentrations of inorganic species (where the speciation data are available), rather than total metal concentrations, in comparing laboratory results to conditions in the field, or between different locations in the field.

Overview

The work described previously (on the characteristics of zinc and cobalt concentrations in the marine environments, the parallel effects of these metals on growth of marine

phytoplankton, and the evidence for direct substitution in enzymes of cobalt for zinc) provide the groundwork and motivation for our investigation of cobalt substitution for zinc in marine phytoplankton. Our findings are addressed briefly below.

In **Chapter 2**, we examine the growth of diatoms and coccolithophores at varying zinc and cobalt concentrations to determine the extent of zinc limitation and cobalt substitution in these species. By growing large coastal and both large and small oceanic species of phytoplankton, the differences between phylogenetic, physical, and environmental contributions to the evolution of nutrient metal responses may be more easily identified. In accord with trends found previously, oceanic species are less easily limited by zinc deficiency (33) and their zinc requirements can be more completely satisfied by cobalt substitution (23).

The laboratory data indicate that both zinc and cobalt are nutrients for all the species tested. These eukaryotic phytoplankton accumulate zinc more readily than cobalt, but this is perhaps not surprising given that dissolved zinc concentrations are typically nearly two orders of magnitude higher than those of cobalt in surface seawater. Organisms in the natural environment requiring large amounts of cobalt would generally be easily limited by cobalt deficiency and be outcompeted by organisms that could substitute zinc in the metal sites of cobalt enzymes (or replace cobalt enzymes by equivalent zinc enzymes). Exceptions might be found, such as the prokaryote *S. bacillus,* which has low cobalt requirements and may have an inherent inability to substitute cobalt with zinc or substitute cobalt enzymes with functionally equivalent zinc proteins (23).

The large amount of CA found previously in *T. weissflogii* (24) suggests that this enzyme is a likely target for cobalt substitution in zinc-limited cells. Restoration of CA activity by the addition of cobalt in that work does not in itself prove a substitution for zinc in CA. However, autoradiograms of zinc in cellular proteins separated by polyacrylamide gel electrophoresis (PAGE) provide indirect evidence of this; the addition of cobalt and increase in CA activity correspond to a decrease in zinc in the band where the majority of CA activity is

found (24). Combined with previous work in the literature which demonstrate substitution of zinc by cobalt in functioning CA of another model organism (6), the circumstantial evidence for this substitution occurring in a functioning CA of *T. weissflogii* is very strong.

The missing link in this chain of evidence is that a decrease in zinc and increase in CA activity do not prove that cobalt substitutes for zinc directly in this enzyme; it might for example result from a series of substitutions: cobalt substituting for another metal which, in turn, substitutes for zinc in CA. We address this question in **Chapter 3** with experiments measuring the quantity and mobility of proteins with cobalt and carbonic anhydrase activity in *T. weissflogii*. We find that similar to zinc, a large fraction of the soluble cobalt is contained in the fraction of soluble cell proteins with CA activity. Because the fraction which contains both CA activity and cobalt can be separated from other soluble cell proteins using both affinity column chromatography and PAGE sequentially, the results of these experiments establish as directly as is possible (short of protein isolation and in-vitro substitution) that cobalt indeed substitutes for zinc in the CA of *T. weissflogii*.

The functional cobalt-containing CA of *T. weissflogii* and the presence of CA in other algae (10, 11, 12, 34, 35, 36) suggest that cobalt substitution in carbonic anhydrase might be a common mechanism for cobalt alleviation of zinc limitation in other phytoplankton. **Chapter 4** explores this possibility for several species of marine diatoms and coccolithophores. Using the same methods we used previously for finding CA in *T. weissflogii*, we find measurable activity in only one other species, the diatom *Thalassiosira partheneia*, out of the 6 species we tested.

Observations of physiological responses to zinc and cobalt limitation provide additional insight into the biochemical mechanisms by which zinc is limiting and cobalt is substituting. We therefore also attempt to directly establish a role for zinc and cobalt in carbon acquisition in some of these species. Cultures of *T. oceanica* and *P. carterae* were grown under different partial pressures of CO_2 under zinc and cobalt -limited and replete conditions. Like *T.*

weissflogii , whose growth is limited by low P_{CO2} only at low zinc and cobalt concentrations, both *P. carterae* and perhaps *T. oceanica* are able to alleviate, if not overcome, carbon limitation in metal replete conditions.

Most of the species tested for CA activity exhibit some cobalt alleviation of zinc limitation in their growth, despite the lack of measurable CA activity in polyacrylamide gel assays. Therefore, the question of exactly how cobalt substitutes for zinc remains unanswered. Other likely candidates for this substitution are superoxide dismutase (SOD) and the DNA and RNA polymerases. The oxidizing conditions under which phytoplankton are typically found suggest a widespread necessity for mechanisms such as SOD activity to combat oxidative cell damage, and reproduction and protein expression in all living things require polymerases.

In **Chapter 5**, we examine the distribution of zinc and cobalt in the SOD and nucleic acid polymerases and other proteins for a set of test organisms. When nucleic material is precipitated from solutions of cell homogenates, a fraction of the soluble cellular zinc (ranging from 10 to 30 % of the total soluble zinc) precipitates with it. The amount of zinc in this fraction varies among the four species of algae studied, and within each species, between cultures grown at high and low zinc and cobalt concentrations. A similar association of cobalt with nucleic material is not apparent; the amount of cobalt which is removed by precipitation of nucleic acids is much lower, ranging from 3 to 10 % of the soluble cellular cobalt, and the variations within each species for cultures grown under different zinc and cobalt conditions are small. We also examined zinc and cobalt distribution among other cell proteins by measuring the distribution of these metals after electrophoresis on polyacrylamide gels (PAGE) and in crude fractionation of cell homogenates by centrifugation. For all the species, relatively more of the total cellular cobalt is found in the soluble fractions than for zinc, which is found primarily in the insoluble cell material for most of the species. Within the soluble fractions, the major distributions of cobalt and zinc also differ. The pattern that emerges is that other than in the soluble CA in *T. weissflogii,* and in SOD in some other species, cobalt and zinc appear in

different cellular proteins; typically Co does not directly substitute in the metal centers of soluble zinc proteins.

We consider our laboratory results in the context of previous laboratory work and field data in **Chapter 6.** In spite of some differences with previous findings, our results are largely consistent with the body of existing work on cobalt substitution for zinc. Similarly, the conditions in which these biological responses are found coincide with ranges of zinc and cobalt concentrations which we might expect to find in the field. Our results on the intracellular distribution of zinc and cobalt indicate that the mechanisms of substitution may not be the same for *T. weissflogii* and other phytoplankton. Directions for future work arising from these findings are considered.

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Table 2 Zinc Metalloenzymes 1982

Name	Number	Source	Role[a]
Class. I. Oxidoreductases			
Alcohol Dehydrogenase	9	Vertebrates, plants	A, D
Alcohol Dehydrogenase	1	Yeast	A
D-Lactate Dehydrogenase	1	Barnacle	?
D-Lactate Cytochrome Reductase	1	Yeast	?
Superoxide Dismutase	12	Vertebrates, plants, fungi, bacteria	(A)[b], D
Class II. Transferases			
Aspartate Transcarbamylase	1	*Escherichia coli*	B
Transcarboxylase	1	*Penicillium shermanii*	?
Phosphoglucomutase	1	Yeast	?
RNA Polymerase	10	Wheat germ, bacteria, viruses	A
DNA Polymerase	2	Sea urchin, T_4 phage	A
Reverse Transcriptase	3	Oncogenic viruses	A
Terminal dNT Transferase	1	Calf thymus	A
Nuclear poly (A) Polymerase	2	Rat liver, virus	A
Mercaptopyruvate Sulfur transferase	1	*E. coli*	?
Class III. Hydrolases			
Alkaline Phosphatase	8	Mammals, bacteria	A, (C)[b], D
Frutose-1,6-Biphosphatase	2	Mammals	C
Phosphodiesterase (exonuclease)	1	Snake venom	A
Phospholipase C	1	*Bacillus cereus*	A
Nuclease P_1	1	*Penicillium cirtrinum*	?
α-Amylase	1	*Bacillus subtilis*	B
α-D-Mannosidase	1	Jack bean	?
Aminopeptidase	10	Mammals, fungi, bacteria	A, C
Aminotripeptidase	1	Rabbit intestine	A
DD-Carboxypeptidase	1	*S. albus*	A
Procarboxypeptidase A	2	Pancreas	A
Procarboxypeptidase B	1	Pancreas	A
Carboxypeptidase A	4	Vertebrates, crustacea	A
Carboxypeptidase B	4	Mammals, crustacea	A
Carboxypeptidase (Other)	5	Mammals, crustacea, bacteria	A
Dipeptidase	3	Mammals, bacteria	A
Angiotensin-converting enzyme	3	Mammals	A
Neutral protease	16	Vertebrates, fungi, bacteria	A, (B)[b]
Collagenase	4	Mammals, bacteria	A
Elastase	1	*Pseudomonas aeruginosa*	?
Aminocylase	1	Pig kidney	?
β-Lactamase II	1	*B. cereus*	A
Creatininase	1	*P. putida*	?
Dihydropyrimidine aminohydrolase	1	Bovine liver	?
AMP deaminase	1	Rabbit muscle	?
Nucleotide pyrophosphatase	1	Yeast	A
Class IV. Lyases			
Fructose-1,6-biphosphate Aldolase	4	Yeast, bacteria	A
L-Rhammnulose-1-phosphate Aldolase	1	*E. coli*	A
Carbonic anhydrase	22	Animals, plants	A
δ-Aminolevulinic acid Dehydratase	2	Mammalian liver, erythrocytes	A
Glyoxalase I	4	Mammals, yeast	A
Class V. Isomerases			
Phosphomannose isomerase	1	Yeast	?
Class VI. Ligases			
t-RNA synthetase	3	*E. coli, Bacillus stearothermophilus*	A
Pyruvate carboxylase	2	Yeast, bacteria	?
Total	162*		

[a] A denotes a catalytic role, B a structural, C a regulatory, and D an undefined role. The ? indicates that available information is insufficient to make an assignment. See the text for further details.

[b] Letters in parentheses refers to roles fulfilled by metals other than zinc.

*Since this chapter was written, the total number of zinc enzymes has increased to beyond 200.

Table 1-1. List of zinc metalloenzymes, from B. L. Vallee, in *Zinc Enzymes* T. G. Spiro, Ed. (Wiley, New York, 1983)

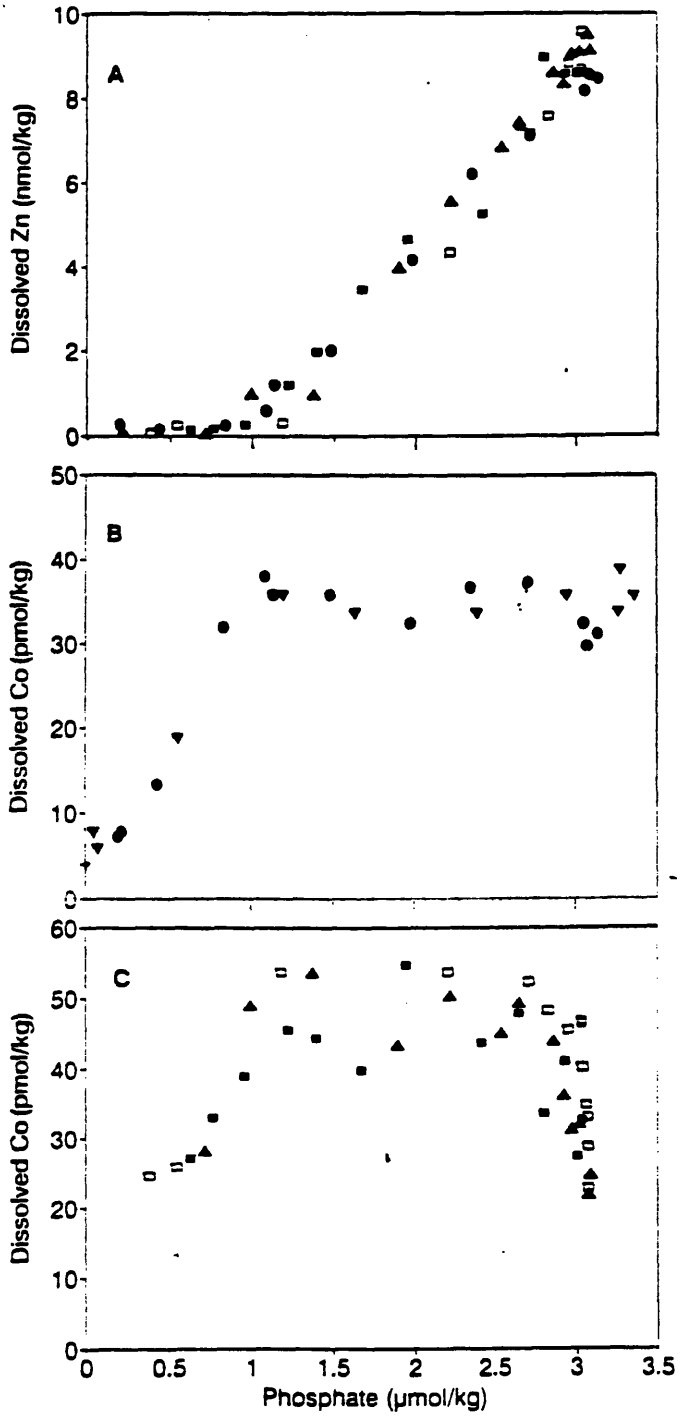
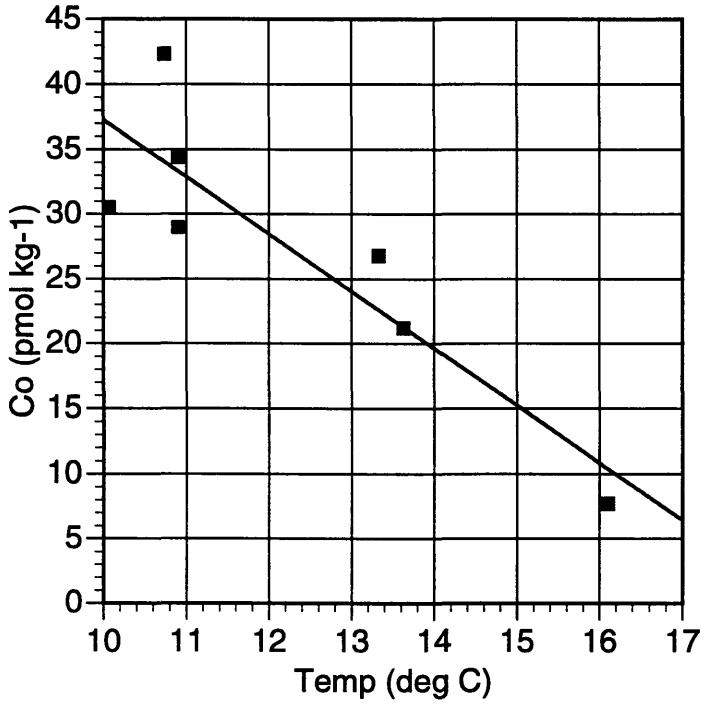


Figure 1-1. Correlation between dissolved zinc and cobalt vs. phosphate, from W. G. Sunda, S. A. Huntsman, *Limnology and Oceanography* **40**, 1404-1417 (1995) using the data of Martin et al. (1989) and Martin and Gordon (1988).

Plots of dissolved Zn and Co vs. phosphate for data from the North Pacific: Station T-4 (▼; 33.3°N, 139.1°W), T-5 (●; 39.6°N, 140.8°W), T-6 (■; 45.0°N, 142.9°W), T-7 (▲; 50.0°N, 145.0°W), and T-8 (□; 55.5°N, 147.5°W). Co data for station T-4 are taken from Martin and Gordon (1988) (no Zn data were published for this station). Zn and Co data from the remaining four stations are taken from Martin et al. (1989).

Figure 1-2. Correlation between temperature (as a proxy for CO_2) and surface dissolved cobalt in the Northeast Pacific and North Atlantic, from data of Martin et al. (25, 26), normalized to salinity of 35 ‰



f(x) = -4.41E+0*x + 8.14E+1
R^2 = 7.81E-1

Chapter 2. Effects of cobalt substitution for zinc on the growth of diatoms and coccolithophores

Introduction

Previous work has demonstrated the ability of marine phytoplankton to substitute other metals such as cobalt for the trace metal nutrient zinc (1, 2). In coastal waters and upwelling regions, the concentrations of cobalt are typically lower than those of zinc by an order of magnitude or more and do not exhibit nutrient-like profiles. In open ocean surface waters, when zinc is extremely depleted, the surface concentration of cobalt approaches that of zinc (2, 3, 4, 5), and its vertical concentration profile may show surface depletion typical of nutrients.

Price and Morel (1) demonstrated the ability of zinc-limited cells of the coastal marine diatom *T. weissflogii* to partially substitute both cobalt and cadmium for their usual zinc requirements. Although the substitution of zinc by cadmium was able to restore growth to maximal levels, cobalt was only able to partially alleviate zinc limitation in this diatom. Assays for activity of the zinc-containing enzyme carbonic anhydrase (CA) in electrophoresis gels revealed that cobalt might alleviate zinc limitation by direct substitution in this enzyme (6).

Sunda and Huntsman (2) demonstrated via effects on growth rates, uptake rates, and cellular metal to carbon ratios that the substitution of zinc by cobalt in marine phytoplankton is not unique to *T. weissflogii* . In fact, the presence of cobalt in the medium had made it appear that some species were not limitable by zinc deficiency in some previous experiments (7) . In agreement with the results of Price and Morel for *T. weissflogii*, Sunda & Huntsman generally found that in many species, cobalt substitutes imperfectly for zinc in cultures of phytoplankton limited by zinc.

The exception they found was an oligotrophic strain of the coccolithophore, *Emiliania huxleyi* from the Sargasso Sea, which exhibits a requirement for cobalt only partially alleviated by zinc. Sunda and Huntsman speculated that this might be a vestigial preference of *E. huxleyi's* phylogenetic lineage. Having arisen at an earlier time in a less oxic ocean, when cobalt was more

soluble and thus more available, *E. huxleyi* perhaps would have evolved and maintained a set of cobalt enzymes, much as some modern prokaryotes have (8).

If we consider this evolutionary argument, it implies that close relatives of *E. huxleyi*, i.e. other coccolithophores, would be most likely to possess similar requirements for cobalt. This possibility was suggested but not examined by Sunda and Huntsman. Another factor which may determine the responses to different metals and preferences for these metals in phytoplankton is the environmental niche that they occupy. The degree to which these phylogenetic and environmental factors manifest themselves may vary, depending upon the selective pressures on the evolution of a particular species.

Here we examine the differences in the abilities of several species of marine phytoplankton to substitute cobalt for zinc, and hope to illuminate the reasons for these differences. The strength and the limitation of the work of Sunda and Huntsman was its breadth; they examined a wider variety of phytoplankton taxa, and were thus only able to look at one or two species from each of four classes. Here we limit our examinations to species within only two classes of plankton, diatoms and coccolithophores, the dominant eukaryotic species in much of the open oceans. By constraining our study to fewer but more closely related species and even including two isolates of one species, *E. huxleyi*, we hope to uncover phylogenetic and/or enviromental patterns in the zinc limitation of these phytoplankton and their ability to alleviate such limitation by cobalt substitution .

Materials and Methods

We grew sets of cultures, co-varying zinc and cobalt concentrations, for three coccolithophores and three diatoms. The coccolithophores chosen were a coastal species from Woods Hole, MA, *Pleurochrysis carterae* (Cocco II, CCMP 645), and two strains of *E. huxleyi*, one from the Gulf of Maine (CCMP 1516), and the other from the Sargasso Sea (BT6, CCMP 373). The latter is the same strain grown by Sunda and Huntsman (2) For comparison, three diatoms, *Thalassiosira weissflogii* (Actin, CCMP 1336), from Great South Bay, NY, *Thalassiosira*

oceanica (13-1, CCMP 1005), from the Sargasso Sea, and *Thalassiosira subtilus* (50AiT) from the equatorial Pacific, were also grown.

Polycarbonate centrifuge tubes (28 ml, Nalgene) were prepared by soaking in 1 N HCl overnight, then rinsing with Milli-Q ultra-pure water. Tubes filled with Milli-Q water were sterilized by microwaving on high power for 15 minutes. Aquil medium was prepared with several modifications to the standard recipe (9) ; no additions of cobalt or zinc were made initially, and one-tenth of the usual nutrient mix (phosphate, nitrate, and silicate) was used for tubes growing *E. huxleyi* to avoid nitrate inhibition of coccolith formation and photosynthesis found by some researchers (10).

Sterile media was dispensed into the cleaned and sterilized culture tubes, and zinc and cobalt concentrations of 100, 20, 4, or 0 percent of the full Aquil recipe (16 and 22 pM inorganic* , equivalent to 80 and 50 nM total zinc and cobalt, respectively) were added to each tube. Stock cultures of each species were transferred, at a dilution of 1:10, into tubes containing sterile Aquil lacking EDTA or trace metals. Cells were allowed to grow for several days to nearly (> 60 % of) their previous maximum density in order to reduce carryover metal contamination and assure that the cells were growing before the start of the experiment. Experimental tubes were then inoculated with cells (ca. 100-1000 cells ml^{-1}) and grown at 22°C under continuous fluorescent lighting at an intensity of ~150 μEinsteins m^2 sec^{-1}.

In vivo fluorescence of each tube was measured daily using a Turner Model 10/005 fluorometer. Growth rates were determined by a linear least squares fit of the natural log of fluorescence versus time in days. Statistical significance of differences in growth rates was tested with a standard t-test with respect to the null hypothesis of zero effect.

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* Inorganic zinc and cobalt concentrations (Zn' and Co') are the sum of concentrations of the hydrated metal ions and complexes with inorganic ligands (e.g. chloride, sulfate, hydroxide) in the solution. In a medium such as Aquil or seawater where the concentrations of these inorganic ligands are essentially constant, the inorganic species are nearly directly proportional to the free metal activities. In the case of zinc, greater than 80 % of the inorganic zinc consists of free zinc ion, with the rest primarily sulfate, chloride, and hydroxide species. About 60 % of the inorganic cobalt is free cobalt, with the remainder being chloride, sulfate, and hydroxide species.

Results

For all the strains of phytoplankton tested, cultures with additions of either zinc or cobalt grow faster than the cultures with neither metal added, indicating growth limitation by zinc and cobalt. In all cultures, trace contaminating amounts of nutrient metals in culture media and tube surfaces as well as in carryover from stock cultures are sufficient to allow low phytoplankton growth rates in the absence of added zinc or cobalt. The contamination level is less than 3.2 nM total (0.64 pM inorganic) zinc, and less than 2 nM total (0.88 pM inorganic) cobalt, since there are generally significant differences (ranging from 60 to 95 % significance levels, depending on species) between the growth rates of cultures at these concentrations (4 percent of the Aquil concentrations) and those of cultures with neither metal added .

For *T. weissflogii* (Table 2-1, Figure 2-1), zinc additions are markedly better than cobalt additions in stimulating growth, in agreement with previous results (1) for the same diatom. With sufficient zinc, additions of cobalt have virtually no effect on the growth rate. However, even with full Aquil concentrations of cobalt, low concentrations of zinc result in a decrease in growth rate to about 80 percent of the maximum. When neither metal is added, growth is greatly reduced, to about 40 percent of the maximum.

Zinc has a similar effect on the growth of the coccolithophore *Pleurochrysis carterae* (Table 2-2, Figure 2-2). With sufficient zinc, growth is independent of cobalt concentrations. In the absence of added zinc, the cultures with maximum concentration of cobalt grow at about 80 percent of the maximum rate, again demonstrating the inability of cobalt substitution to completely alleviate zinc limitation. In the case where neither metal is added, the growth rate is only about 30 percent of the maximum.

The differences between the effects of zinc and cobalt on the growth of *T. oceanica* are smaller, although the patterns observed in this oceanic diatom are similar to those found in *T. weissflogii* (Table 2-3, Figure 2-3). With the normal Aquil zinc concentration, growth is largely

independent of cobalt concentration. Cobalt added without zinc allows growth of about 90 percent of the maximum, slightly better than for the coastal organisms. Growth with neither metal added is very low, near 10 percent of the maximum rate.

For the BT6 strain of *E. huxleyi* , growth is not decreased by lower zinc or cobalt (Table 2-4, Figure 2-4) except if neither metal is added to the medium, which causes growth at about 70 percent of the maximum rate. The other *E. huxleyi*, CCMP 1516, appears unaffected by decreasing cobalt when full zinc is present, and is very slightly or not at all affected by decreasing zinc with full cobalt (Table 2-5, Figure 2-5). Growth occurs near the maximal rate for nearly all the cultures except for those with either no zinc or no cobalt. In accord with all the other species, the growth rate is lowest in the culture with neither metal added, reaching about 60 percent of the maximum rate.

Growth rates are also shown for the large oceanic diatom *T. subtilus* (Table 2-6, Figure 2-6). At normal Aquil concentrations of zinc and cobalt, it appears that these metals may be slightly toxic to the organism, because the growth rate is lowest when both metals are at their maximum, and growth improves slightly with decreasing zinc at maximum cobalt or with decreasing cobalt at maximum zinc. When only one of these metals is added, decreases in growth with decreasing zinc or cobalt concentrations are within the errors of growth rate calculations, except for the case of no addition of either zinc or cobalt, which grows at about 65 percent of the maximum rate for this species.

Discussion

According to our results and those of Sunda and Huntsman (2), the replacement of zinc by cobalt found by Price and Morel (1) in *T. weissflogii* appears to occur generally in eukaryotic marine phytoplankton. Remarkably, for all six species of phytoplankton we tested, growth limitation caused by low zinc concentrations is significantly alleviated ($p \leq 0.1$) by addition of cobalt; when neither zinc nor cobalt is added to cultures , the resulting growth rates are markedly

depressed, ranging from 10 to 80 percent of the maximum rates, but the addition of cobalt restores the growth rates to approximately 75 to 100 percent of their maximum values. That the effects of cobalt are not simply manifestations of a specific cobalt limitation is evident from the lack of any influence of changing cobalt concentrations in the presence of high and constant zinc concentrations for most of the cultures. This however does not mean that there may not be a specific requirement for cobalt that is met by the very low background cobalt concentrations that could not be removed from our culture media and containers. Furthermore, in the case of the BT6 strain of *E. huxleyi* , the data of Sunda and Huntsman suggest that there may be a requirement or preference for cobalt that is not completely met by additions of zinc.

E. huxleyi BT6 is one of the two isolates studied both by Lee and Morel (11) and Sunda and Huntsman (2) that are also included in this study (the other strain that is common to these studies is *T. oceanica*, which will be discussed later). Our data, contrary to Sunda and Huntsman's, do not indicate a requirement or preference for cobalt in this *E. huxleyi* strain. Unlike these authors, we find that growth is not limited by decreasing Co' with high Zn' (inorganic metal concentrations, Zn' and Co', are defined in methods, p.28) in the culture medium. In our study growth is not significantly limited unless neither metal is added (Zn' < 0.6 pM, Co' < 0.9 pM). Lee and Morel also studied the same strain of *E. huxleyi* and were able to limit growth at low zinc concentrations (Zn' = 0.2 pM) when no cobalt or cadmium was added.

Although there are some differences in the growth rates of the same strain (BT6) at low zinc and cobalt concentrations among these studies, for all the cases, limitation consistently occurs at inorganic concentrations of zinc and/or cobalt near 1pM (below $[Zn^{2+}]$ or $[Co^{2+}]$ of $10^{-12.5}$). This occurs despite differences in light levels (~500 $\mu E\ m^{-2}\ sec^{-1}$ for Sunda and Hunstman, 150 $\mu E\ m^{-2}\ sec^{-1}$ for this work, and 100 $\mu E\ m^{-2}\ sec^{-1}$ for Lee and Morel), light cycles (14 h light : 10h dark cycle for Sunda and Huntsman, continuous for Lee and Morel and this work), and differences in the medium (filtered seawater supplemented with chelator and metals for Sunda and Huntsman, artificial medium (Aquil) for Lee and Morel and this work). The higher maximum growth rates in

Sunda and Huntsman's cultures (1.2 day^{-1}, versus 0.9 day^{-1} here), which received more light, might be one reason they find cobalt limitation even with high zinc concentrations whereas we do not.

The widespread ability of zinc-limited phytoplankton to grow faster in media with added cobalt stands in contrast to the results obtained with cadmium, the other metal found by Price and Morel to alleviate zinc limitation in *T. weissflogii*. For several species of phytoplankton, Lee and Morel (11) found that cadmium is able to act as a nutrient substitute for zinc under mildly limiting (Zn' = 3.2 pM) conditions when cadmium is added at a comparable (Cd' = 4.6 pM) concentration. At severely limiting conditions of low zinc (Zn' = 0.16 pM) however, addition of the same concentration of cadmium is toxic to most of the phytoplankters. In contrast, cobalt is not toxic to zinc-limited cultures at similar concentrations. Without exception, cobalt benefits phytoplankton growth in zinc-limiting conditions (Zn' < 0.64 pM) when inorganic cobalt concentrations ranging from 0.88 to 22 pM are added to the culture medium.

The primary differences that we find among the species examined are in their degrees of preference for zinc versus cobalt and in the concentrations at which these metals become limiting. These characteristics may be shaped by a number of factors: phylogeny, because genetics determines what metabolic pathways an organism possesses; physical characteristics, which constrain the interactions between a cell's metabolism and its environment; and environmental pressures, which determine the survival and distribution of genetic characteristics. By studying only two classes of phytoplankton but including both large and small species as well as large species from different environments, we provide a context from which we might make generalizations about the characteristics and distribution of zinc and cobalt interreplacement in phytoplankton in particular, and from that, inferences about metal substitution in phytoplankton in general.

One reason for the differences and similarities in the biochemistry and physiology of species is their phylogenetic relatedness. Sunda and Huntsman found that the prokaryote *Synechococcus*

bacillus has an absolute requirement for cobalt which cannot be substituted by zinc. In their results, the BT6 strain of *Emiliania huxleyi* also exhibits a slight preference for growth on cobalt, even though it grows nearly as well on zinc. These observations led the authors to speculate that the coccolithophores might have retained a vestigial requirement for cobalt because they had evolved earlier in earth's history than the diatoms, the most modern algae in their experiments.

This appears to be a reasonable hypothesis, because many bacteria such as *Synechococcus* , which evolved even earlier than the coccolithophores, have retained requirements for cobalt long after the biosphere became oxic (8) . However, our data are not consistent with a greater preference for cobalt in coccolithophores. With high concentrations of zinc, growth of the coastal coccolithophore, *P. carterae*, remains unchanged whether or not any cobalt is added to the medium. Likewise, a decrease in growth rate appears at Zn' of 3.2 pM in this coccolithophore, much like in the coastal diatom *T. weissflogii*. This stands in contrast to the oceanic coccolithophore, *E. huxleyi* BT6, which grows equally well with either only zinc or only cobalt added to the medium, and which does not become significantly zinc limited unless Zn' is less than 0.6 pM.

Thus it appears that phylogeny does not determine the responses of eukaryotic phytoplankton to zinc limitation and their substitution of cobalt for zinc, leaving the physical and environmental factors to be examined. Others have indicated possible connections between cell size or environmental origin to responses of phytoplankton to zinc limitation (12). Calculations indicate that larger phytoplankton should be more nutrient-limited than small ones, all other factors being equal, which may explain the typically smaller size of oceanic phytoplankton; reducing cell size has the dual effect of reducing the per-cell requirement for nutrients (by decreasing biomass per cell) as well as increasing the cell's surface area to volume ratio. In this way, small phytoplankton might decrease the degree of zinc deficiency caused by diffusion limitation of uptake and thus decrease the effect of low external zinc concentrations. However, Lee and Morel (11) found that size differences do not explain the trends observed in the zinc limitation of phytoplankton for two of the species they studied. Although the coastal diatom *Thalassiosira pseudonana* and coccolithophore *Pavlova*

lutheri, are both very small, approximately the same size as the oceanic diatom *T. oceanica*, they are quite sensitive to zinc deficiency, growing at only 60 and 40 percent of their maximum rates, respectively, when inorganic zinc in the medium is decreased five-fold to only 3.2 pM. In our study, we find yet another exception to the putative correlation between cell size and zinc requirements: the large oceanic diatom *T. subtilus* grows at or near its maximum rate at low zinc and cobalt concentrations and grows equally well with zinc or cobalt, much like the smaller oceanic species.

Since neither phylogeny nor size differences sufficiently explain differences between the species in responses to zinc limitation and cobalt substitution, we are compelled to consider environmental selection as the most important characteristic in determining individual phytoplankter responses to low metal conditions. Brand and co-workers (13) examined the responses of 21 species of neritic (coastal) and oceanic phytoplankton to zinc limitation . They concluded that oceanic species are less sensitive to low concentrations of zinc than neritic varieties. Nonetheless exceptions are found within habitat groups. For example, the neritic diatoms *Skeletonema costatum* and *Streptotheca tamesis* are only limited to 75 and 85 percent of their maximum growth rates, respectively at the lowest zinc activities ($pZn^{2+} \sim 13$, or $Zn' \sim 0.2$ pM). In contrast, other coastal species typically grow at less than 50 percent of the maximum rates at those same zinc concentrations. However, cobalt was added to all the culture medium in these experiments (10 nM total cobalt into seawater buffered with 100μM EDTA, yielding inorganic cobalt concentrations of approximately 4.4 pM, 20 percent of the usual concentrations found in Aquil). Sunda and Huntsman's and our findings on the replacement of zinc by cobalt in many if not all phytoplankton may therefore explain the relatively high growth rates observed at low zinc concentrations for two of the neritic species in the study of Brand and co-workers. With 4.4 pM Co' added to medium without zinc, even the most easily zinc-limited (i.e. the coastal species) phytoplankters that we test here, *T. weissflogii* and *P. carterae*, grow at approximately 50 and 60 percent of their maximum

rates respectively. Similar additions of cobalt to the medium for oceanic species result in near maximal growth rates.

The data available on zinc limitation (separate from cobalt substitution) in marine phytoplankton are summarized in Table 2-7 for the species in the studies of by Brand and co-workers, and in Table 2-8 for the studies of Sunda and Huntsman (2), Lee and Morel (11), and this work. Species of phytoplankton in these studies are compiled and grouped by the inorganic zinc concentrations at which growth (μ) is estimated to be 80 percent of the maximum rate (Zn_{80}'). Within groups, species are listed in alphabetical order. The selection of Zn_{80}' as an indicator of zinc limitation was not arbitrary: for most of the growth data, differences in rates of that magnitude (with maximum standard errors of ~10 % of the calculated growth rates) could be distinguished with greater than 80 % confidence (t-test). Both linearized curve fitting (Lineweaver-Burke) and fully non-linear curve fits (to a Monod model) did not result in good correlation to the data. Therefore, linear interpolation was used to calculate Zn_{80}'.

A linear interpolation between two points suffers from some limitations, namely a large uncertainty arising from the paucity of data points employed in such a calculation. However, it has an advantage of excluding growth data away from μ_{80}: when there is growth rate data bracketing μ_{80}, the calculated Zn_{80}' will also be bracketed by the Zn' of the corresponding higher and lower growth rates, thus providing reasonable bounds for Zn_{80}'.

What is apparent from both tables is that the pattern noted by Brand et al., that neritic species generally require larger amounts of zinc, largely holds true. The calculated values of Zn_{80}' are not the same between tables (because of the addition of cobalt in Table 2-7), and are also not exactly the same within the Table 2-8 for the same species in the different studies. As noted before, despite differences in growth rates of *E. huxleyi* BT6 among the different studies in Table 2-8, the same trends hold: this coccolithophore is limited at low zinc concentrations ($Zn' < 2$ pM). Likewise, *T. oceanica*, another species grown by Lee and Morel and also by Sunda and Huntsman, is also limited only at low zinc. In this study, *T. oceanica* is slightly more easily limited by zinc

deficiency than in either of those studies (perhaps because of less zinc or cobalt contamination), and the calculated Zn' at which limitation occurs falls into the next higher range (2-3 pM).

There are only two notable exceptions to the trend of lower zinc requirements in oceanic than neritic species. The coastal chlorophyte, *Dunaliella tertiolecta*, was found by Lee and Morel to be limited only at very low concentrations of zinc. We note also that in the study of Brand et al., growth of the oceanic dinoflagellate (the only dinoflagellate in that study) *Thoracosphaera heimii* is significantly limited at the minimum zinc concentration and may not fit the general pattern of Table 2-7 . In spite of these two exceptions, the ranges of concentration in which zinc becomes limiting are different for oceanic and neritic species, with only slight overlap.

If we assume that additions of cobalt to the medium can effectively substitute for zinc in all the cultures of Brand and co-workers, then Zn_{80}' will be underestimated for all species in Table 2-7. In our cultures, a Co' of 4.4 pM in medium without added zinc results in a growth rate in *T. weissflogii* approximately equal to an addition of Zn' ~ 2 pM (between 3.2 and 0.6 pM Zn'), and thus the actual Zn_{80}' for all the species may be about 2 pM higher than the calculated Zn_{80}' ranges in Table 2-7 . After this adjustment, most of the oceanic species in the study of Brand et al. would be limited around 2 pM Zn' (rather than near 0.1 pM), which is comparable to the ranges dividing neritic and oceanic species in the other studies (Table 2-8).

Perhaps not coincidentally, concentrations of 2 pM inorganic zinc in surface waters of the North Pacific were measured by Bruland (14), in areas with surface concentrations of 0.1 nM total dissolved zinc (similar to total dissolved zinc values found by Martin et al. (15) in the North Pacific). Thus it appears that the concentrations of Zn' at which oceanic species begin to show signs of zinc limitation in laboratory cultures are in an environmentally relevant range.

According to our data and those of Sunda and Huntsman, the other systematic difference between the coastal and oceanic species is in the extent to which they can substitute cobalt for zinc. A requirement for high zinc generally coincides with a poor ability to substitute cobalt for zinc. In Figure 2-7 we compare the susceptibility of phytoplankton to zinc limitation, as measured by Zn_{80}',

to the efficiency of the zinc and cobalt interreplacement, as measured by the ratio of the growth rates with only cobalt added to the growth rates with only zinc added (μ_{Comax}/μ_{Znmax}). The trend is apparent (> 99 % confidence that the slope of μ_{Comax}/μ_{Znmax} versus Zn_{80}' is < 0 when only data from this study (filled symbols) are included); species which become zinc-limited at relatively higher zinc concentrations are also less able to substitute effectively with cobalt. Whether neritic phytoplankton with apparently low requirements for zinc, such as *Skeletonema costatum* and *Streptotheca tamesis* (from Brand et al., and thus perhaps with higher zinc requirements than indicated by Table 2-7) might also have a better ability to substitute with cobalt is unknown.

In the open ocean, where surface zinc concentrations are generally low (3, 5, 14). phytoplankton enhance their survival by maintaining rapid growth at low concentrations of zinc. However, the ability to tolerate zinc limitation by decreasing quotas, increasing uptake rates, or some combination thereof cannot explain the beneficial effect of increasing external cobalt concentrations on zinc-limited phytoplankton. In fact, if cobalt had no metabolic function, then this metal would be detrimental to the cell as an antagonistic competitor for zinc uptake and enzyme binding sites (this is probably what happened with cadmium additions in many of the experiments of Lee and Morel (11)). Cobalt therefore clearly has some metabolic functions by which it alleviates zinc limitation. In vitro studies show that some zinc enzymes such as carbonic anhydrase can often function when cobalt is substituted for zinc; some cobalt enzymes (e.g. CA in particular) however may be less efficient than the native zinc-containing proteins (16). This may explain why a high absolute requirement for zinc correlates with a poor ability to substitute effectively with cobalt (as in Figure 2-7): higher demand for zinc leads to more substitution of (less effective) cobalt and a greater impact on the overall cell metabolism. In contrast, phytoplankton with low requirements for zinc would always be able to find sufficient or nearly sufficient zinc for their most critical functions. The impact of zinc-limitation, and therefore the extent and impact of inefficient cobalt-substituted enzymes, would be less in these organisms .

A complementary explanation for differences in the abilities of neritic and oceanic species to substitute cobalt for zinc might be found in their evolutionary development. In the open ocean zinc concentrations are depleted whereas cobalt concentrations (relative to zinc concentrations) are elevated. Oceanic species might therefore have evolved efficient mechanisms for the substitution of cobalt (or other metals) for zinc. Natural selection should then favor phytoplankters that optimize their biochemistry to use cobalt (or other metals) instead of zinc in many enzymes (perhaps even at the expense of the efficiency of the zinc form) . What exactly those enzymatic functions might be, we will address for *T. weissflogii* specifically in Chapters 3 and 5 and explore in Chapters 4 and 5 for other phytoplankton.

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Table 2-1. Growth rates (day^{-1}) of tube cultures of *Thalassiosira weissflogii,* co-varying cobalt and zinc concentrations. Standard error on slope of regression (ln(fluorescence) vs. days) given. Metal additions listed are fractions relative to the standard concentrations found in Aquil growth medium (80 and 50 nM total or 16 and 22 pM inorganic zinc and cobalt, respectively).

	Zn	Zn/5	Zn/25	no Zn
Co	0.99 ± .08	0.95 ± .05	0.87 ± .02	0.77 ± .04
Co/5	1.02 ± .01	0.74 ± .00	0.54 ± .03	0.50 ± .04
Co/25	1.01 ± .01	0.60 ± .02	0.34 ± .09	0.39 ± .07
no Co	1.02 ± .01	0.60 ± .02	0.35 ± .10	0.38 ± .06

Table 2-2. Growth rates (day^{-1}) of tube cultures of *Pleurochrysis carterae,* co-varying cobalt and zinc concentrations. Standard error on slope of regression (ln(fluorescence) vs. days) given. Metal of the growth medium listed are additions relative to the standard concentrations found in Aquil growth medium (80 and 50 nM total or 16 and 22 pM inorganic zinc and cobalt, respectively).

	Zn	Zn/5	Zn/25	no Zn
Co	0.58 ± .03	0.58 ± .04	0.46 ± .02	0.48 ± .02
Co/5	0.58 ± .03	0.55 ± .02	0.40 ± .01	0.36 ± .01
Co/25	0.56 ± .03	0.52 ± .02	0.30 ± .01	0.25 ± .02
no Co	0.57 ± .03	0.42 ± .01	0.27 ± .01	0.20 ± .02

Table 2-3. Growth rates (day^{-1}) for tube cultures of *Thalassiosira oceanica,* co-varying cobalt and zinc concentrations. Standard error on slope of regression (ln(fluorescence) vs. days) given. Metal additions listed are fractions relative to the standard concentrations found in Aquil growth medium (80 and 50 nM total or 16 and 22 pM inorganic zinc and cobalt, respectively).

	Zn	Zn/5	Zn/25	no Zn
Co	0.96 ± .09	0.94 ± .11	0.93 ± .10	0.88 ± .09
Co/5	0.92 ± .09	0.93 ± .10	0.83 ± .11	0.71 ± .09
Co/25	0.93 ± .10	0.84 ± .12	0.47 ± .07	0.11 ± .03
no Co	1.00 ± .09	0.94 ± .11	0.19 ± .03	0.11 + .02

Table 2-4. Growth rates (day^{-1}) of tube cultures of *Emiliania huxleyi (BT6),* co-varying cobalt and zinc concentrations. Standard error on slope of regression (ln(fluorescence) vs. days) given. Metal additions listed are fractions relative to the standard concentrations found in Aquil growth medium (80 and 50 nM total or 16 and 22 pM inorganic zinc and cobalt, respectively).

	Zn	Zn/5	Zn/25	no Zn
Co	0.91 ± .03	0.88 ± .03	0.86 ± .03	0.89 ± .02
Co/5	0.93 ± .03	0.92 ± .07	0.92 ± .04	0.91 ± .04
Co/25	1.03 ± .04	0.92 ± .06	0.86 ± .06	0.92 ± .05
no Co	0.92 ± .06	0.89 ± .07	0.84 ± .04	0.70 ± .06

Table 2-5. Growth rates (day^{-1}) of tube cultures of *Emiliania huxleyi (CCMP 1516),* co-varying cobalt and zinc concentrations. Standard error on slope of regression (ln(fluorescence) vs. days) given. Metal additions listed are fractions relative to the standard concentrations found in Aquil growth medium (80 and 50 nM total or 16 and 22 pM inorganic zinc and cobalt, respectively).

	Zn	Zn/5	Zn/25	no Zn
Co	0.63 ± .11	0.60 ± .09	0.62 ± .09	0.60 ± .09
Co/5	0.63 ± .09	0.64 ± .09	0.62 ± .09	0.58 ± .09
Co/25	0.63 ± .09	0.61 ± .09	0.57 ± .08	0.56 ± .08
no Co	0.60 ± .08	0.56 ± .09	0.41 ± .07	0.37 ± .05

Table 2-6. Growth rates (day^{-1}) of tube cultures of *Thalassiosira subtilus* (50AiT), co-varying cobalt and zinc concentrations. Standard error on slope of regression (ln(fluorescence) vs. days) given. Metal additions listed are fractions relative to the standard concentrations found in Aquil growth medium (80 and 50 nM total or 16 and 22 pM inorganic zinc and cobalt, respectively).

	Zn	Zn/5	Zn/25	no Zn
Co	0.48 ± .08	0.53 ± .07	0.57 ± .08	0.70 ± .06
Co/5	0.64 ± .07	0.63 ± .08	0.58 ± .06	0.65 ± .05
Co/25	0.61 ± .05	0.64 ± .06	0.64 ± .05	0.61 ± .04
no Co	0.72 ± .05	0.68 ± .03	0.64 ± .03	0.50 ± .02

Table 2-7. Phytoplankton species studied for zinc limitation, from Brand et al., grouped by inorganic zinc concentrations at which growth rates of cultures are 80% of the maximum rates (Zn_{80}'). Species are listed in alphabetical order within ranges.

Oceanic species	Zn_{80}' (pM)	*Neritic Species*	Source
Bacteriastrum delicatulum	< 0.1		B
Cyclococcolithina leptopora			B
Hemiaulus sinensis			B
Rhizosolenia setigera			B
Synechococcus sp.			B
Thalassiosira oceanica			B
Umbilicosphaera sibogae			B
	0.1 - 0.3	*Bacteriastrum hyalinum*	B
Emiliania huxleyi			B
Gephyrocapsa oceanica			B
Rhizosolenia stolterfothii			B
		Skeletonema costatum	B
		Streptotheca tamesis	B
Umbilicosphaera hulburtiania			B
	> 0.3	*Asterionella glacialis*	B
		Biddulphia mobiliensis	B
		Ditylum brightwellii	B
		Hymenomonas carterae	B
		Lithodesmium undulatum	B
		Thalassiosira pseudonana	B
Thoracosphaera heimii			B

Table 2-8. Phytoplankton species studied for zinc limitation, from Sunda and Huntsman (S), Lee and Morel (L), and this study (Y, in boldface), grouped by inorganic zinc concentrations at which growth rates of cultures are 80% of the maximum rates (Zn_{80}'). Species are listed in alphabetical order within ranges.

Oceanic species	Zn_{80}' (pM)	*Neritic Species*	Source
	< 2	*Dunaliella tertiolecta*	L
Emiliania huxleyi BT6			L,S,Y
Thalassiosira oceanica			L,S
Thalassiosira subtilus			Y
Emiliania huxleyi 1516	2-3		Y
Thalassiosira oceanica			Y
	> 3	*Heterocapsa pygmaea*	L
		Pavlova lutheri	L
		Pleurochrysis carterae	L,Y
		Tetraselmis maculata	L
		Thalassiosira pseudonana	L,S
		Thalassiosira weissflogii	L,Y

Figure 2-1. Growth rates of *T. weissflogii* versus Zn' in Aquil with either high or no cobalt added to the medium (■) Co' = 22 pM (●) Co' ~ 0.2 pM

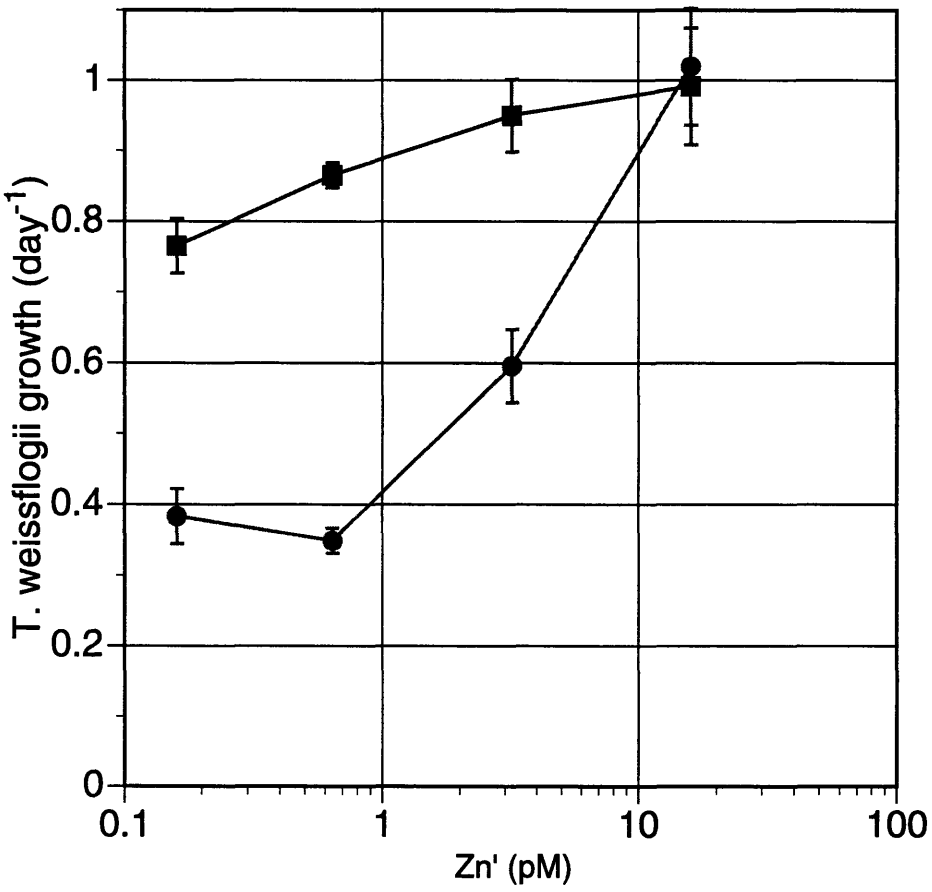


Figure 2-2. Growth rates of *P. carterae* versus Zn' in Aquil with either high or no cobalt added to the medium (■) Co' = 22 pM (●) Co' ~ 0.2 pM

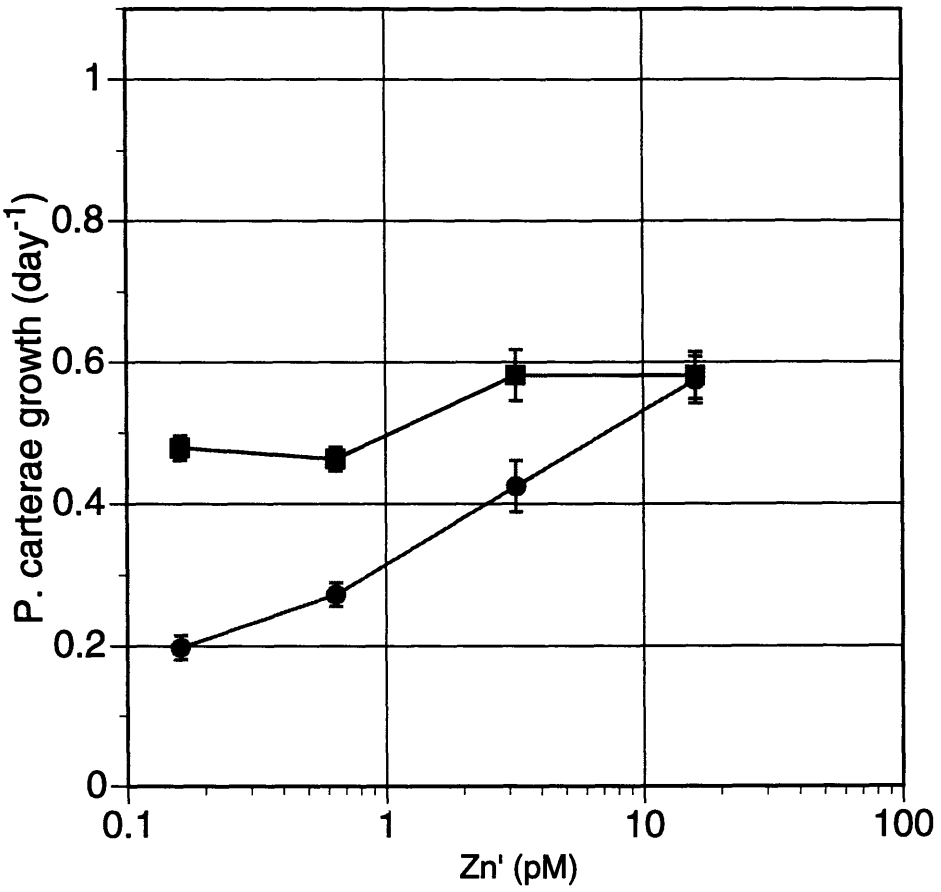


Figure 2-3. Growth rates of *T. oceanica* versus Zn' in Aquil with either high or no cobalt added to the medium (■) Co' = 22 pM (●) Co' ~ 0.2 pM

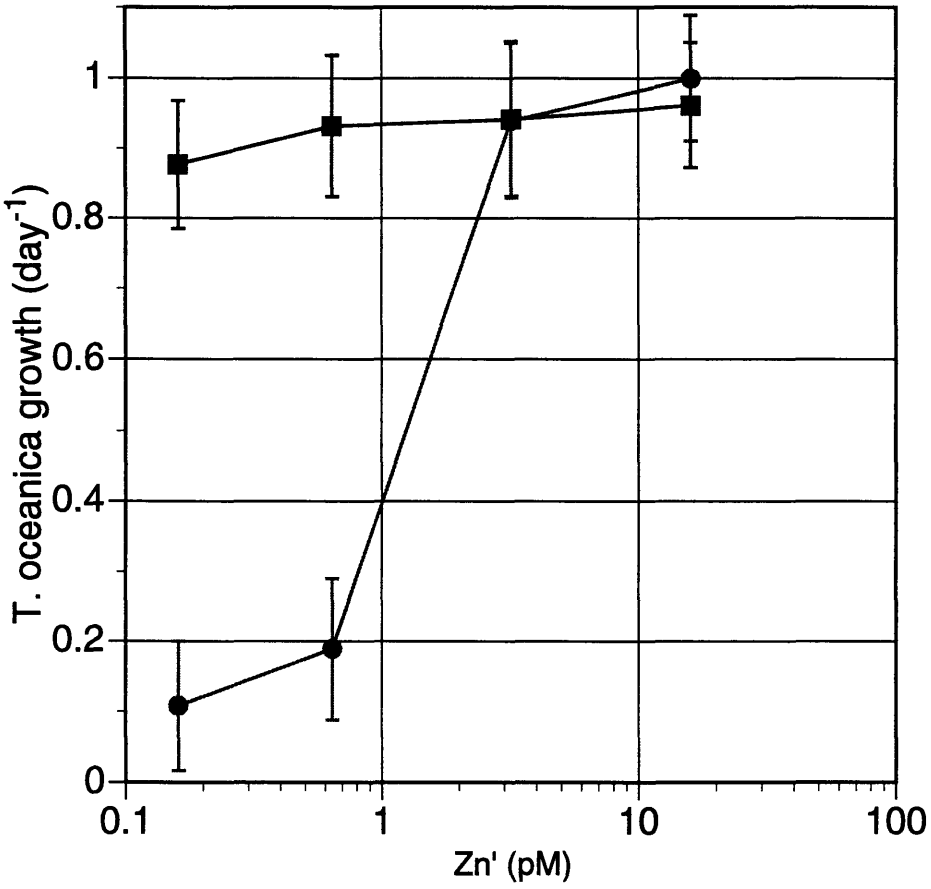


Figure 2-4. Growth rates of *E. huxleyi* BT6 versus Zn' in Aquil with either high or no cobalt added to the medium (■) Co' = 22 pM (●) Co' ~ 0.2 pM

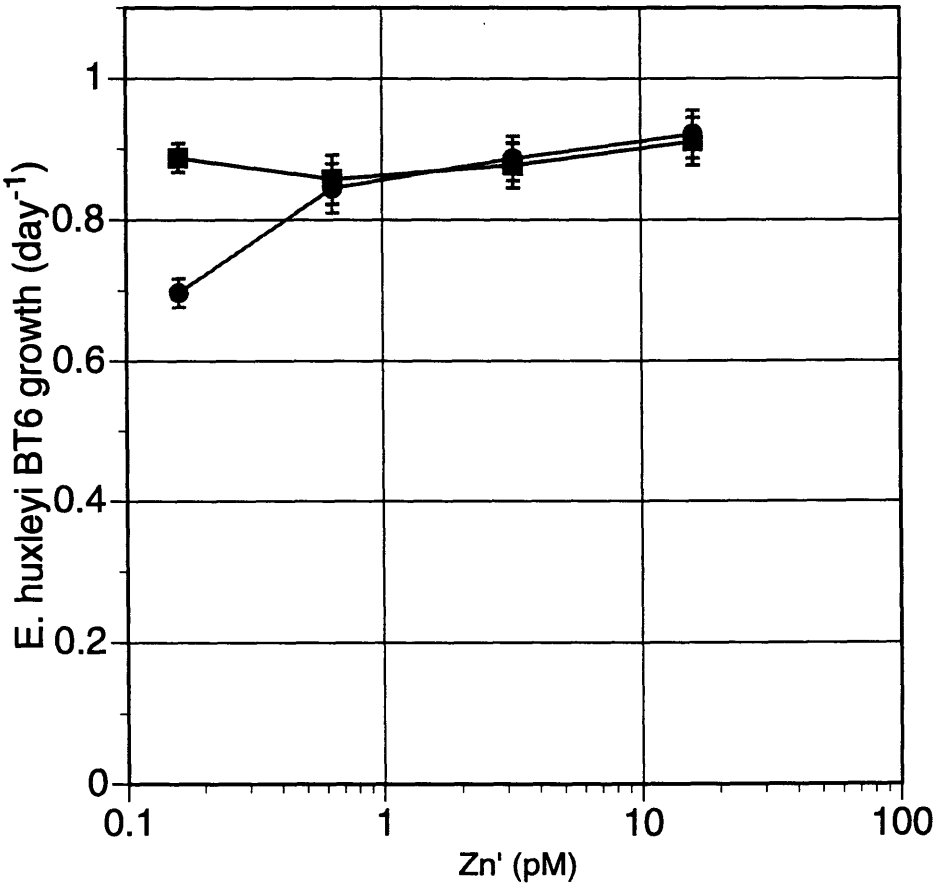


Figure 2-5. Growth rates of *E. huxleyi* CCMP1516 versus Zn' in Aquil with either high or no cobalt added to the medium (■) Co' = 22 pM (●) Co' ~ 0.2 pM

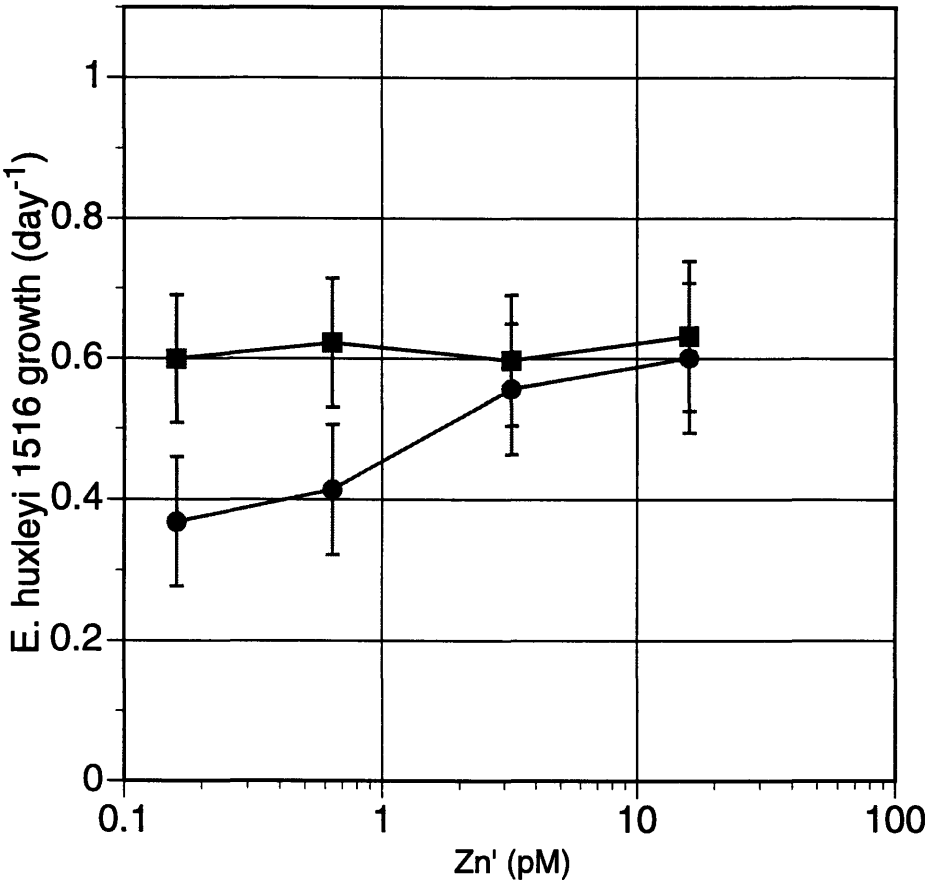


Figure 2-6. Growth rates of *T. subtilus* 50AiT versus Zn' in Aquil with either high or no cobalt added to the medium (■) Co' = 22 pM (●) Co' ~ 0.2 pM

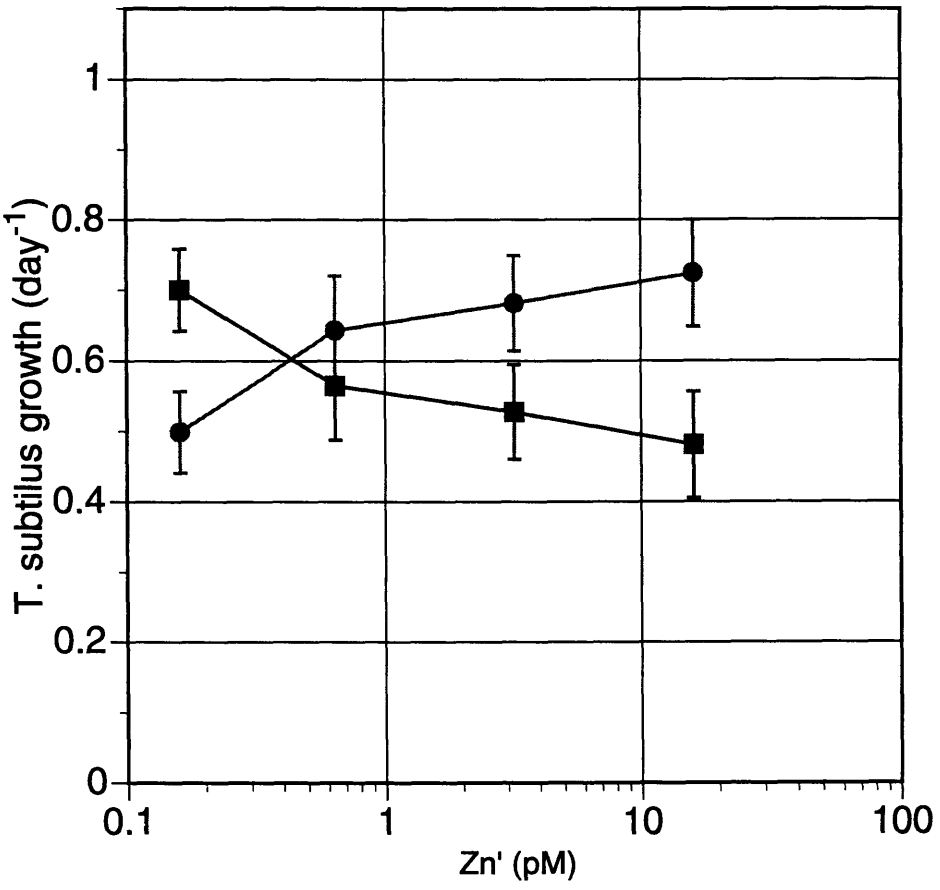
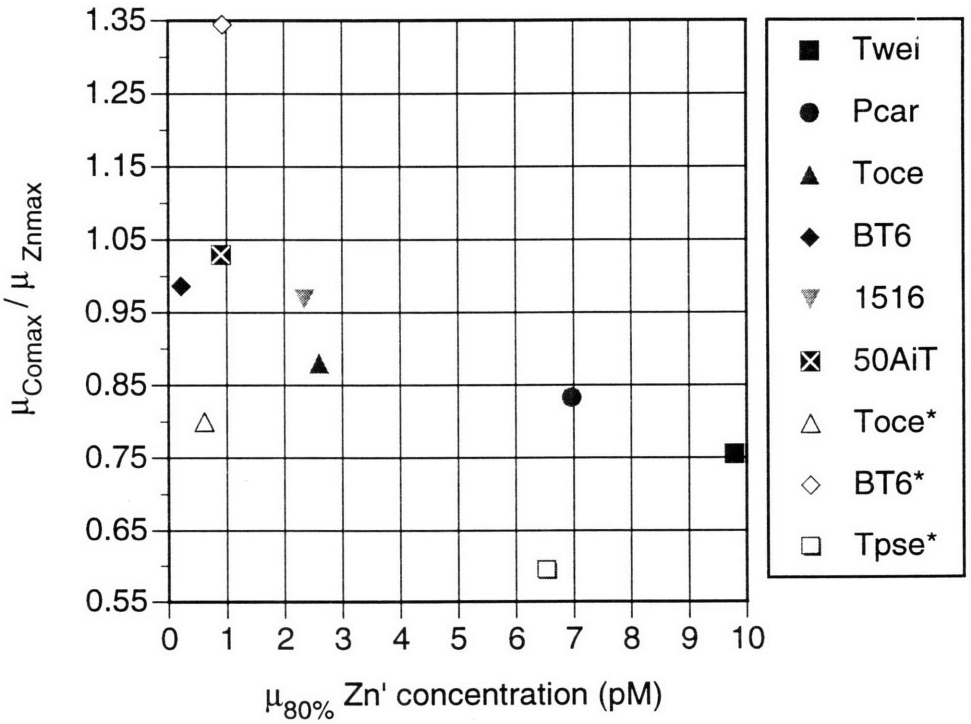


Figure 2-7. Ratio of maximum growth rates in Aquil medium with only cobalt added (μ_{Comax}) to maximum growth rates in medium with only zinc added (μ_{Znmax}) versus the Zn' concentration at which is growth is estimated to be 80% of μ_{Znmax} for of six species of coastal and oceanic coccolithophores and diatoms. Calculation of the 80% μ_{Znmax} Zn' concentrations made using linear interpolation between growth rates at the lowest zinc concentration with no limiting effect and the growth rate nearest 80% of the maximum at a lower zinc concentration. For purposes of the interpolation, no added zinc was assumed to be 1% of the Aquil concentration, 0.16 pM. Twei = *Thalassiosira weissflogii* (Actin), Pcar = *Pleurochrysis carterae*, Toce = *Thalassiosira oceanica* (13-1), BT6 = *Emiliania huxleyi* (BT6), 1516 = *E. huxleyi* (CCMP 1516), 50AiT = *Thalassiosira subtilus* (50AiT). Open symbols are from results of Sunda and Huntsman (2).



Chapter 3. In vivo substitution of zinc by cobalt in carbonic anhydrase of a marine diatom

Introduction

It has been shown that cobalt can substitute partially for the zinc requirement of marine diatoms (1, 2). The addition of cobalt in zinc-limited cultures of both coastal and oceanic diatoms results in partial restoration of growth. Recent work has demonstrated a link between zinc availability and carbon acquisition in *Thalassiosira weissflogii* (3). This is apparently a result of the function of carbonic anhydrase (CA), a major zinc-containing soluble intracellular protein. There is evidence that adding cobalt restores CA activity in zinc-limited *T. weissflogii* (3).

Here we demonstrate that the mechanism of cobalt replacement of zinc occurs by in vivo substitution for zinc in at least one of the carbonic anhydrases of *T. weissflogii*. This substitution restores some of the carbonic anhydrase activity lost by zinc limitation. The concurrent improvement in growth rate apparently results from an improvement in zinc-limited cells' ability to acquire carbon.

Materials and Methods

Cultures of *T. weissflogii* were grown in synthetic culture media based on the standard recipe for Aquil (4) containing reduced concentrations of EDTA (10 μM total, to allow a low total amount of radioactive and stable cobalt to achieve the desired available trace metal activities) and modified zinc and cobalt levels: the regular zinc and cobalt levels (16 and 22 pM inorganic metal, i.e. free metal and complexes with inorganic ligands,and 8 or 5 nM total metal, respectively), one-tenth the zinc, one-tenth the cobalt, or one-tenth both the zinc and cobalt. Radioactive cobalt (NEN/DuPont, 18.5 x 10^4 Bq ^{57}Co per liter culture, as $CoCl_2$) was added before inoculating the media with cells. Media were allowed to equilibrate chemically for at least twelve hours before use.

Batch cultures were maintained in polycarbonate bottles under continuous artificial illumination at an intensity of 900 $\mu E\ s^{-1}\ cm^{-2}$. Bottles were kept open to the atmosphere (loosely-capped) and unmixed at 20 °C. Cells were counted using a hemacytometer. Once cells grew to a density of approximately 10^5 cells ml^{-1}, they were collected on 3 μm pore polycarbonate filters (Poretics) and stored in liquid nitrogen. Cells were then resuspended in deionized water with 20 μM leupeptin and 0.5 mM PMSF (Sigma) added as protease inhibitors. Resuspended cells were broken via sonication (Branson Sonifier 250) on ice at 50% power and a 50% duty cycle for two 30-s sessions with a 15-s pause for cooling. Unbroken cells and insoluble cell fragments were removed by centrifugation at 16,000 x g for 20 min. An equal volume of soluble cell homogenate (all the cultures were grown to approximately the same cell density, and resuspended and lysed in the same volume) was loaded into each lane. Samples were separated using non-denaturing electrophoresis on 10% polyacrylamide gels (pH 8.8) with a 5% stack (pH 6.8) in a 25 mM Tris, 250 mM glycine buffer.

After electrophoresis, the presence of carbonic anhydrase activity was assayed by the method of Patterson et al. (5). The pH change from the catalyzed dissolution of CO_2 was observed by blowing gaseous CO_2 on the surface of a gel stained in a 25 mM Tris, 250 mM glycine buffer (pH 7.6) containing 0.1% (wt/vol) of the color pH indicator bromcrescol purple (Sigma). The gels were frozen on dry ice to stop the reaction, photographed under UV illumination, then dried on a heated vacuum gel dryer. Dried gels were exposed to X-ray film (Kodak X-omat AR) to determine the location of material containing radioactive cobalt.

Subsamples of the cell homogenate were also taken and purified on an affinity column for carbonic anhydrase at 4 °C (6). Each sample was loaded on the column and washed with 25 mM Tris, 22 mM Na_2SO_4 buffer at pH 8.2, followed by a wash with 25 mM Tris, 300 mM $NaClO_4$ buffer at pH 8.7. Samples were then eluted from the column with a solution of 25 mM Tris, 500 mM $NaClO_4$ at pH 5.6. Eluted fractions were collected and concentrated by ultrafiltration through filters with a nominal molecular weight cutoff of 10,000 Daltons (Millipore Ultrafree MC).

Purified samples were also separated by non-denaturing gel electrophoresis. A separate lane of bovine carbonic anhydrase (Sigma) was run on each gel as a control.

To examine whether cobalt is similar to zinc in alleviating growth limitation in cultures grown under low P_{CO2}, we grew *T. weissflogii* in Aquil with 10-fold lower zinc and either 10-fold lower or regular Aquil cobalt concentrations, and regular zinc with 10-fold lower cobalt. These cultures were aerated continuously with an artificial air source containing nitrogen and oxygen in atmospheric proportions, and 100, 300, or 1,000 ppm CO_2. Cultures were maintained at 22 °C and grown under artificial fluorescent light at an intensity of 900 $\mu E\ s^{-1}\ cm^{-2}$. Subsamples of the cultures were removed periodically and counted with a Coulter Counter. The final pH of the cultures varied depending upon the level of carbon dioxide supplied, ranging from approximately 8.5 in the 100 ppm cultures, to 8.0 in the 300 ppm, and 7.5 in the 1,000 ppm cultures.

Results and discussion

The results for growth of *T. weissflogii* in large batch cultures mirror those for small cultures of the diatom (from the previous chapter), even though the absolute values are not the same, mainly due to differences in the light conditions. Among cultures of *T. weissflogii* with varying inorganic zinc and cobalt concentrations, those containing the smallest amounts of both metals grow at the lowest rate (growth rate; $\mu = 0.4\ d^{-1}$). The addition of zinc alone to Zn' = 16 pM (the regular Aquil concentration of inorganic zinc) results in nearly the usual rate of growth ($\mu = 1.3\ d^{-1}$), while adding cobalt alone increases growth to a lesser extent ($\mu = 0.8\ d^{-1}$). Addition of both at regular concentrations (Co'= 22 pM and Zn'= 16 pM) results in the usual maximum growth rate ($\mu = 1.4\ d^{-1}$).

Assays for carbonic anhydrase show that both zinc and cobalt concentrations affect the appearance of CA activity. In the first lane of the PAGE gel in Figure 3-1a, a sample from a culture with low concentrations of both zinc and cobalt, CA can not be detected (a control of

bovine carbonic anhydrase was used to ensure that the assay was functioning in the event that none of the samples showed any activity. However it provides no additional information about protein size in a non-denaturing gel, and is therefore not shown). Regular Co' in the culture medium causes cells to produce detectable levels of CA, and cultures grown at regular Zn' (whether at low or regular Co') exhibit yet higher apparent activity. Thus cobalt addition promotes parallel effects on the appearance of CA activity and growth rates in low zinc cultures.

The production of carbonic anhydrase in zinc-limited cells by addition of cobalt may be due either to the displacement of zinc in other proteins, or to a direct functional substitution of cobalt for zinc in CA. The autoradiogram of a PAGE gel in Figure 3-1b from samples of ^{57}Co-labeled cultures shows the cobalt in bands where there is carbonic anhydrase activity in Figure 3-1a. The major cobalt band found in all the lanes corresponds to the location of the majority of carbonic anhydrase activity.

Because of the broadness of the bands in the gel, the co-elution of CA and ^{57}Co does not positively demonstrate that the cobalt is present in CA. To establish this, we purified the soluble material from ^{57}Co-labeled cultures on a CA affinity column. After purification on the CA affinity column, only one radioactive band remains in each lane (Figure 3-2b). The location of these bands corresponds to the location of CA (Figure 3-2a), and the relative amounts of radioactive cobalt found in each lane (lane 1>2>3>4) appear similar to those observed in the main CA bands in the unpurified samples (Figure 3-1b). Densitometry measurements of square areas around the radioactive bands confims this (Table 3-1). Thus cobalt is indeed substituting for zinc in CA.

Because the radioactive cultures were all grown with the same concentration of ^{57}Co (but not the same total cobalt or zinc), variations in the intensity and size of the ^{57}Co bands from lane to lane (which each contain material from approximately equal numbers of cells; although this does not mean the quantities of protein in each lane are equal, it is equivalent to a per cell comparison) provide insight into the cobalt replacement of zinc in *T. weissflogii*. In the main CA band, the amount of radioactive cobalt is greatly decreased by the presence of (cold) zinc (compare lanes 1

and 3 in Table 3-1). The addition of an equivalent concentration of cold cobalt rather than zinc results in much less displacement of the ^{57}Co (lanes 2 vs. 3). Likewise, additional cold cobalt in a zinc-sufficient culture (lane 4) effects only a slight decrease in the ^{57}Co activity of the main CA band. From the autoradiogram data, it appears that the carbonic anhydrase of *T. weissflogii* has a higher affinity for zinc than for cobalt. The assays for CA also show that cells with high zinc produce either more enzyme or a more active form than those with only high cobalt. However, when zinc is insufficiently available, cobalt can clearly substitute for zinc in CA in an active form of the enzyme.

As seen in the autoradiograms, under conditions of low zinc, significant amounts of cobalt appear in two unidentified protein bands which have no measurable CA activity and which are not retained by the CA affinity column (and therefore likely not another CA isoform). However, the largest fraction of the soluble cellular cobalt is in carbonic anhydrase. This parallels the distribution of zinc in *T. weissflogii* (3), in which a large fraction of the soluble zinc protein is carbonic anhydrase. If a major function of cobalt under low-zinc conditions is as a cofactor for CA (as zinc is normally), we expect that like zinc, it might be involved in carbon acquisition. We tested this by varying P_{CO2} along with zinc and cobalt in *T. weissflogii* cultures. Cultures with low zinc and cobalt aerated under different partial pressures of carbon dioxide exhibited large differences in growth rates (Figure 3-3a), an effect which disappeared at high zinc (Figure 3-3c). For each P_{CO2}, growth rate is significantly higher ($p < 0.01$) in each of the cultures provided with high cobalt concentrations than in the corresponding low metal culture (Figure 3-3d) . Thus in those cultures that are clearly carbon-limited (by the combination of low zinc and low partial pressures of CO_2), addition of cobalt is able to partially restore growth rates (Figure 3-3b), in parallel to its effect on CA. The failure of cobalt to eliminate the sensitivity of *T. weissflogii* to P_{CO2} at low zinc parallels its inability to fully restore growth in zinc-limited cultures at atmospheric CO_2 levels.

The positive effect of high P_{CO2} on the growth of zinc-limited (1.6 pM Zn') cultures is not a result of a change in metal speciation resulting from the change in pH. Calculations of the speciation of zinc and cobalt in the pH range 7.5 to 8.5 made using MINEQL (7) show only a 3 % change in the Zn' and Co' over that range, with similarly small changes in the inorganic speciation (still predominantly Zn^{2+} and Co^{2+}). In Chapter 2, an 80 % decrease in zinc causes less than a 50 % decrease in growth of *T. weissflogii* in the range of Zn' between 3.2 and 0.6 pM. We might expect that a decrease in metal solubility from pH 7.5 to 8.5 of only 3 % would have a proportionally smaller effect on growth, and thus the effect on growth rate of this change in metal speciation would be so small as to be unmeasurable. In contrast, the difference in growth rate for high versus low P_{CO2} is approximately 0.2 day^{-1}, which is nearly as large as the 0.25 day^{-1} difference in growth between cultures of *T. weissflogii* in medium with Zn' of 3.2 and 0.6 pM (with no added cobalt) shown in Chapter 2.

Our culture data show that growth limitation caused by decreased zinc concentrations in cultures of *T. weissflogii* is partially offset by cobalt addition. This result confirms previous data of Price and Morel (1) in the same organism and is similar to the results for other *Thalassiosira* species, namely, *T. pseudonana* and *T. oceanica* (2). Although all of our culture media, including those containing low inorganic cobalt (Co' = 2.2 pM, Co_{tot} = 0.5 nM), contain 0.4 nM vitamin B_{12}, this organic cobalt source is unable to satisfy the zinc and cobalt requirements (in 10μM EDTA, normally Co' = 22 pM, Co_{total} = 5 nM) of zinc-limited algae. It has been noted recently by Sunda and Huntsman (2) that the amount of cobalt in vitamin B_{12} does not satisfy the cobalt requirement in zinc-limited *T. pseudonana* and *E. huxleyi*. Clearly there is a role of cobalt in some phytoplankton independent of its function as a metal cofactor in vitamin B_{12}.

Our data from PAGE gels, showing the effect of zinc and cobalt concentrations on detectable CA, extend previous results demonstrating a partial restoration of high CA expression by cobalt addition in low-zinc cultures (3) and a disappearance of CA in the same band from which zinc is lost upon zinc limitation. The appearance of cobalt in the CA band (as shown by radio-

labeling) under low-zinc conditions indicates that the positive effect of cobalt on CA production is not merely the freeing of zinc from other functions, but rather a direct substitution of cobalt for zinc in this enzyme in vivo.

The apparent affinity for zinc in carbonic anhydrase of *T. weissflogii* is much higher than for Co, and either the activity or quantity of the cobalt-substituted carbonic anhydrase expressed is lower than for the native zinc-containing enzyme. These differences may explain why the addition of cobalt improves but does not eliminate the growth limitation caused by low zinc concentration.

A lower activity of the cobalt-substituted CA of *T. weissflogii* would be similar to results in the literature for the bovine erythrocyte carbonic anhydrase substituted with cobalt in vitro (8). We note this similarity in the apparent efficacy of the cobalt-substituted enzyme is seen despite the absence of any sequence homology between the bovine and *T. weissflogii* CAs (9) and also despite differences in the methods by which the substituted metal is introduced into the enzyme.

Under low-zinc conditions, a large fraction of the cobalt in soluble cellular proteins is found in the largest CA band (only one Zn-CA band appears in the figures presented here because the concentration of the cell extract was not sufficient to visualize the minor bands previously seen), where we previously found much of the zinc under ordinary culture conditions (3) . In contrast, cadmium, when added to low-zinc cultures, is found primarily in a different CA band (in the figures presented here, the Cd-CA does not appear because no Cd is added to any of the cultures) which migrates less in a non-denaturing PAGE (10). The appearance of cobalt and Cd in different bands indicates that they substitute in different CA isoforms, and this might explain the differences in the growth response of cells: unlike Co, Cd is able to restore nearly full growth in zinc-limited cells . Nonetheless, as expected from cofactors in CA isoforms, both metals are clearly involved in carbon acquisition (3, 11). The difference in function of the Co-CA and Cd-CA may simply arise from differences in the locations of the enzymes, for example, perhaps internal and external to the cell.

The utilization of cobalt in phytoplankton CA when zinc is limiting should be reflected in the distribution of cobalt concentrations in the surface oceans. Concentrations of cobalt in seawater typically range from about 5 to 50 pmol kg^{-1} (12, 13) and are often fairly invariant at depth, dropping noticeably only very near the surface. Sunda and Huntsman (2) have noted that this cobalt depletion in surface seawater coincides with regions where zinc concentrations are very low (< 0.3 nmol kg^{-1}). The depletion of cobalt primarily in regions of low zinc might be expected if cobalt is a replacement for zinc in phytoplankton. According to our data, cobalt utilization by phytoplankton, and hence its depletion in surface seawater, ought to be linked also to CO_2 availability. Unlike cobalt concentrations at depth, cobalt in the top 20 meters from the North Atlantic and North Pacific (where zinc concentrations are low) are quite variable. We compared these surface cobalt concentrations to the corresponding (saturated) CO_2 concentrations in the water (which depend on temperature). Consistent with our expectations, the results, shown in Figure 3-4, demonstrate a greater depletion in cobalt where CO_2 is lowest. The magnitude of decrease in soluble CO_2 under an assumed constant atmospheric P_{CO2} over this temperature range (a factor of two decrease) is similar to the decrease in soluble CO_2 at a constant temperature under different P_{CO2} which induces high CA expression in laboratory cultures (3).

Even though the situation may be complicated by the different metal requirements of the dominant phytoplankton species in different areas of the ocean, the depletion of cobalt from surface seawater appears to be chiefly determined by both low zinc and CO_2 concentrations. Conversely, biological CO_2 uptake and export in the open ocean is likely dependent on the availability of some key trace metals, including cobalt.

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Table 3-1. Averaged gray density on autoradiograms of ^{57}Co in region of main CA activity (0 is white, 255 is black).

	unpurified (Figure 1b)	purified (Figure 2b)
background	27	23
lane 1 (Zn/10, Co/10)	204	101
lane 2 (Zn/10, Co)	186	78
lane 3 (Zn, Co/10)	146	60
lane 4 (Zn, Co)	120	48

Figure 3-1. [a.] PAGE for samples from cultures grown at different concentrations of zinc and cobalt assayed for CA activity using the color pH indicator bromcrescol purple, with low pH (CO_2 dissolution catalyzed by CA) indicated by light bands on the gel. [b.] Gel previously assayed with bromcresol purple for CA, dried and exposed to X-ray film. Samples are from cultures grown in media containing the following combinations of zinc and cobalt (relative to the standard Aquil concentrations): 1 = Zn/10, Co/10 2 = Zn/10, cobalt 3 = Zn, Co/10 4 = Zn, Co. The same amount of ^{57}Co was added to all cultures (18.5×10^4 Bq liter^{-1}). All four lanes are from the same gel. Dividing lines are provided to aid differentiation of lanes where the edges of bands overlap.

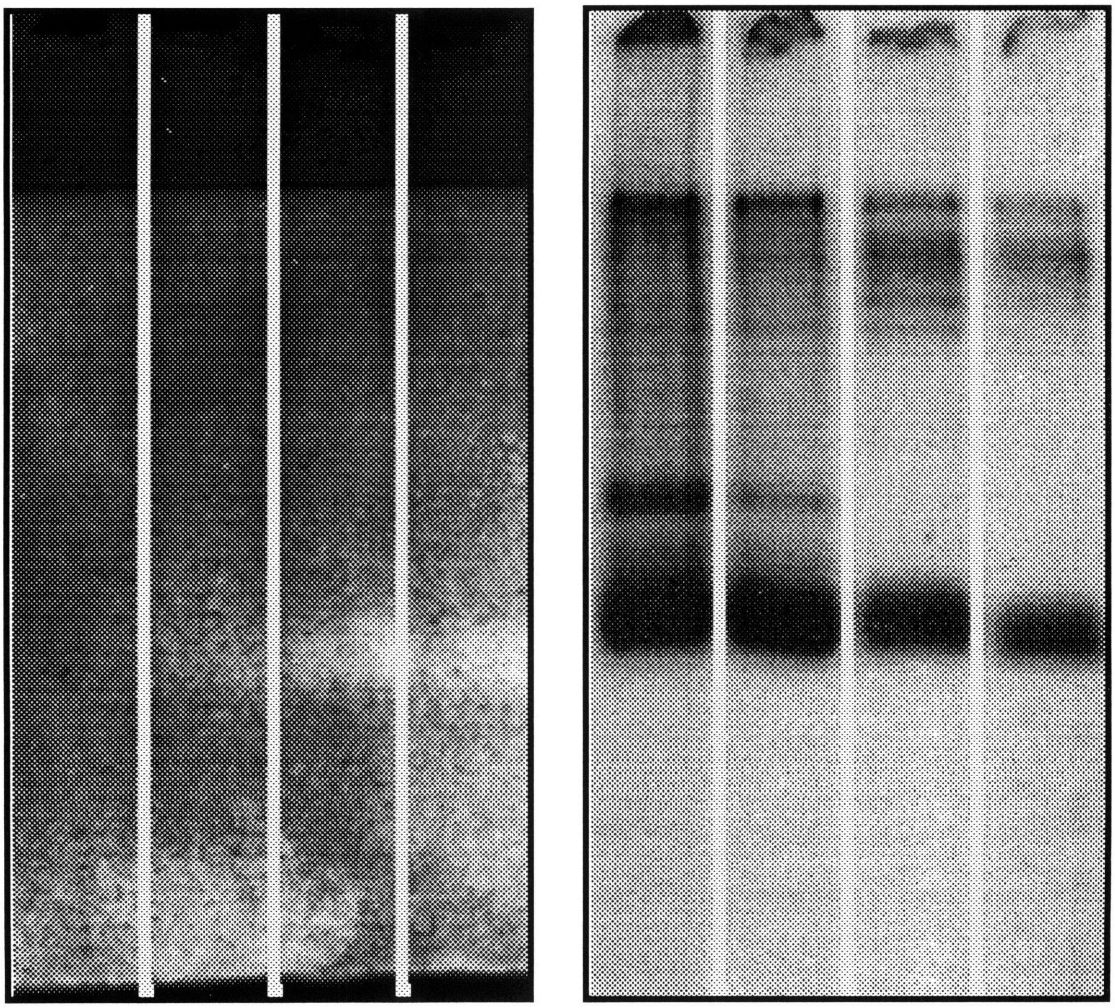


Figure 3-2. [a.] PAGE for samples from cultures grown at different concentrations of zinc and Co, purified and concentrated on a CA affinity column, then assayed for CA activity using the color pH indicator bromcrescol purple, with low pH (CO_2 dissolution catalyzed by CA) indicated by light bands on the gel. [b.] The same PAGE for samples purified on a CA affinity column. After a bromcresol purple assay for CA activity, the gel was dried and exposed to X-ray film. Samples are from cultures grown in media containing these combinations of zinc and cobalt (relative to the standard Aquil concentrations):1 = Zn/10, Co/10 2 = Zn/10, cobalt 3 = Zn, Co/10 4 = Zn, Co. The same amount of ^{57}Co was added to all cultures (18.5x 10^4 Bq liter^{-1}). All four lanes are from the same gel. Dividing lines are provided to aid differentiation of lanes where the edges of bands overlap.

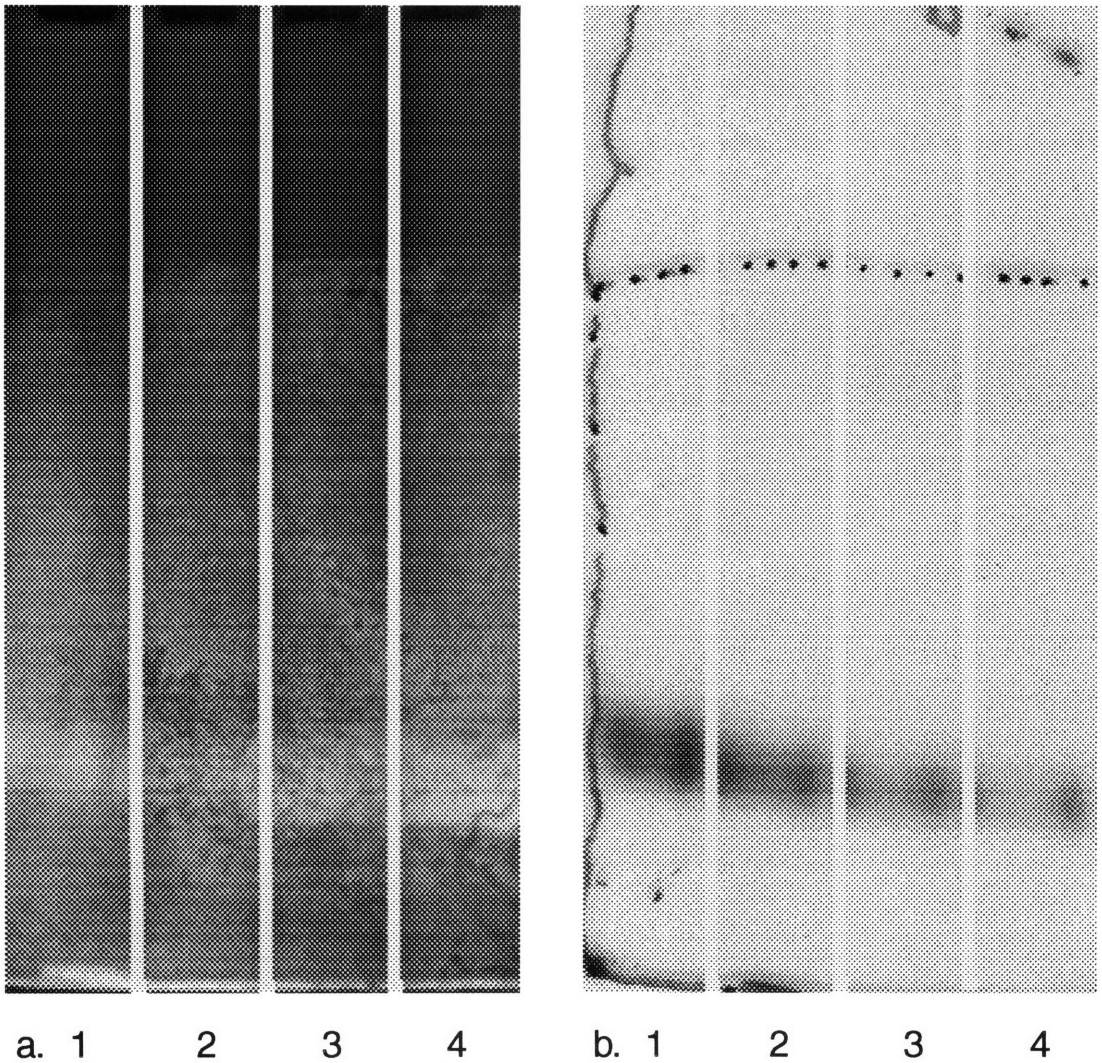


Figure 3-3a-c. Growth curves of *T. weissflogii* cultures containing different concentrations of zinc and cobalt (a = Zn/10, Co/10, b = Zn/10, Co, c = Zn, Co/10 relative to standard Aquil concentrations) and grown under different partial pressures of CO_2 (low = 100, med = 300, and high = 1,000 ppm).

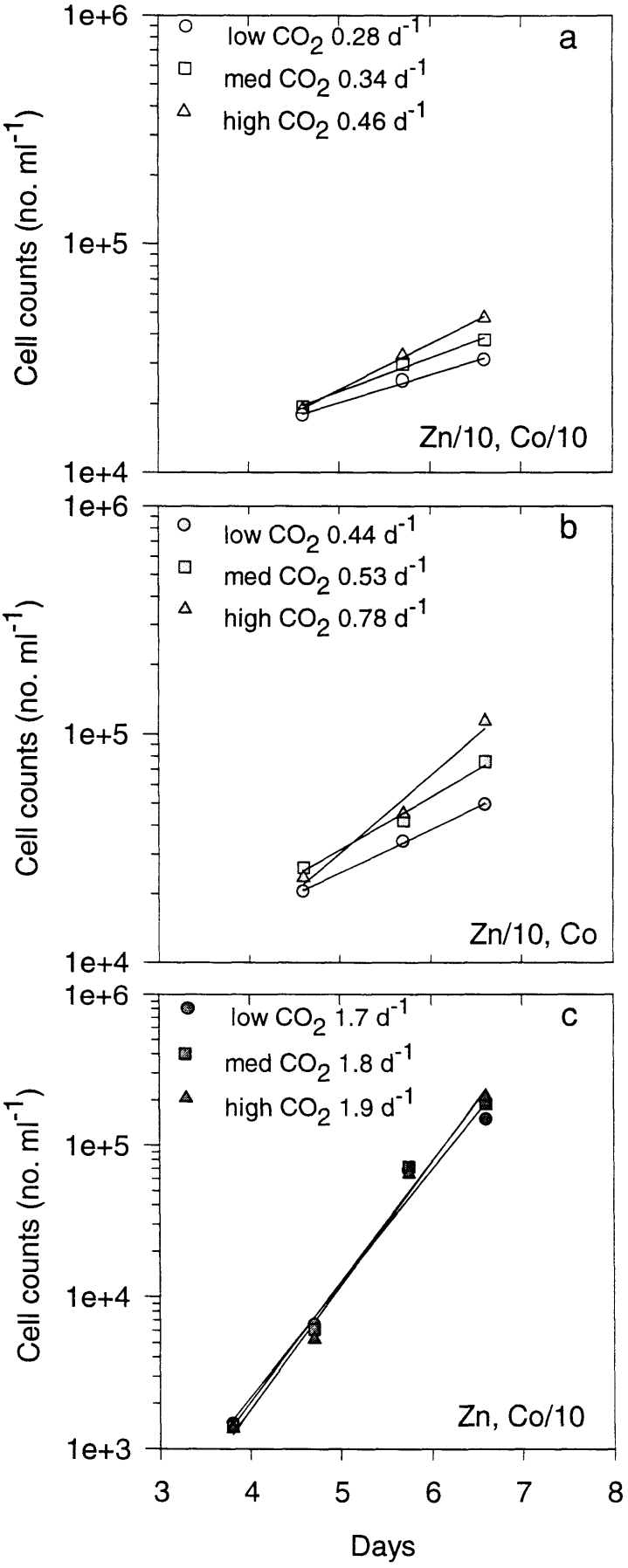

1e+6
1e+5
1e+4
Cell counts (no. ml-1)
low CO2 0.28 d-1
med CO2 0.34 d-1
high CO2 0.46 d-1
a
Zn/10, Co/10
1e+6
1e+5
1e+4
Cell counts (no. ml-1)
low CO2 0.44 d-1
med CO2 0.53 d-1
high CO2 0.78 d-1
b
Zn/10, Co
1e+6
1e+5
1e+4
1e+3
Cell counts (no. ml-1)
low CO2 1.7 d-1
med CO2 1.8 d-1
high CO2 1.9 d-1
c
Zn, Co/10
3
4
5
6
7
8
Days

Figure 3-3d. Growth rates for T. weissflogii bubbled under low, medium, and high partial pressures of CO_2 (100, 300, and 1000 ppm, respectively). Error bars indicate standard error on regression of growth slope.

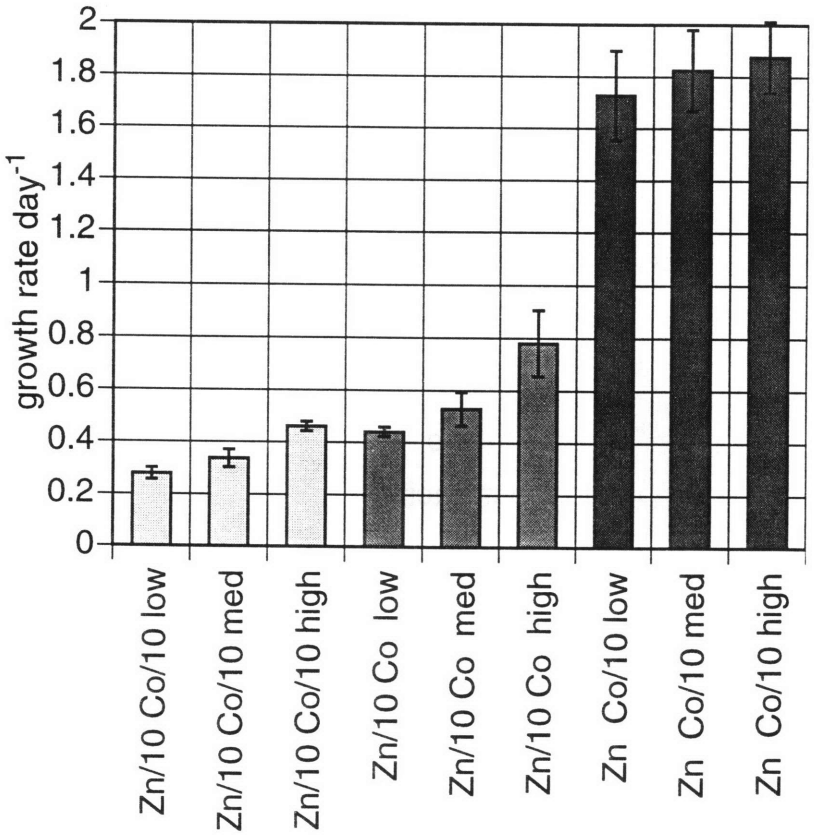
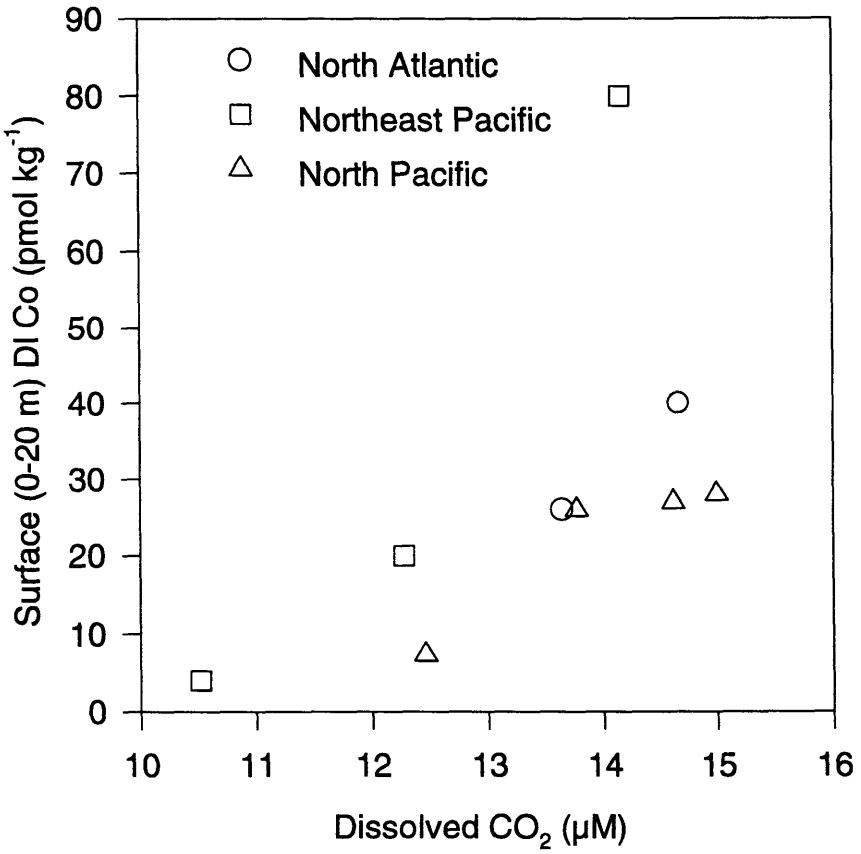


Figure 3-4. Comparison of surface dissolved inorganic cobalt concentrations and surface saturated CO_2 concentration from surface water temperature, using the equation of Edmond and Gieskes (Edmond and Gieskes 1970) for the Henry's Law constant. Surface cobalt and temperature measurements from Martin et al. (1990 1993) and Martin and Gordon (1988).



Chapter 4. Carbonic anhydrase in various marine phytoplankton

Introduction

The previous chapters establish two results: first, the ability to substitute cobalt for zinc is widespread, perhaps universal in phytoplankton and tends to be more effective in species from the open ocean, and second, one way in which cobalt can substitute for zinc in at least one phytoplankter is in the active center of the enzyme carbonic anhydrase (CA). Here we examine whether the high activity of CA we measure in *T. weissflogii* is also found in other species. If high CA activity is found in other species, it may explain the widespread ability of zinc-limited phytoplankton to increase their growth rates by substituting cobalt for zinc.

In addition to investigating the presence of CA activity in various phytoplankton species, we examine the roles of zinc or cobalt in the inorganic carbon acquisition and assimilation of a calcifying strain of the coastal coccolithophore *P. carterae*, and in the oceanic diatom *T. oceanica*. Cultures of these phytoplankton species were grown under conditions of metal sufficiency and deficiency at several partial pressures of CO_2 to uncover any such role.

Materials and Methods

Cultures of the oceanic diatoms *T. oceanica*, *T. partheneia*, and *T. subtilus*, and two coccolithophores, the oceanic *E. huxleyi* (CCMP 1516) and the coastal *P. carterae*, were grown in batch cultures of synthetic sea water media with the following modifications of the standard Aquil recipe (1): for the oceanic species, Zn and Co concentrations were decreased to one-fifth (3.2 and 4.4 pM inorganic metal, and 16 or 10 nM total metal, respectively) and one-fiftieth of the usual concentrations, and for *P. carterae*, the regular Zn and Co concentrations were used. Media for *E. huxleyi* cultures were further modified by decreasing the concentrations of the major nutrients to one-tenth of the usual Aquil levels (phosphate to 1 μM, nitrate to 30 μM). Cultures were maintained under continuous light flux of 900 μEinsteins m^{-2} sec^{-1} at a temperature of 22 °C.

Cells were counted using a Coulter counter (Multisizer II) and grown to late exponential phase (ca. 5-10x10^5 ml^{-1} for *T. oceanica*, *T. partheneia*, and *E. huxleyi*, and 5-10x10^4 ml^{-1} for *P. carterae* and *T. subtilus*.

Once cultures reached late exponential phase, the larger (*T. subtilus* and *P. carterae*) and smaller (*T. oceanica*, *T. partheneia*, and *E. huxleyi*) species were collected on 3 and 1 μm pore polycarbonate filters (Poretics) respectively, and stored in liquid nitrogen. Cells were later resuspended in deionized water with 20 μM leupeptin and 0.5 mM PMSF (Sigma) added as protease inhibitors. Resuspended cells were broken via sonication (Branson Sonifier 250) on ice at 50% power and a 30% duty cycle for two 45-s sessions with a 15-s pause for cooling. Unbroken cells and insoluble cell fragments were removed by centrifugation at 16,000 x g for 20 min. A volume of the supernatant from the cell homogenate was loaded onto each lane of a non-denaturing PAGE.

Electrophoresis was carried out at room temperature on a 10% gel buffered with 0.375 M TRIS-HCl at pH 8.8 and a stack of 5% polyacrylamide with 0.125 M TRIS-HCl at pH 6.8 . Bromphenol blue (0.1%) was added as a marker dye to the loading buffer of 50% v/v glycerol/water. The electrophoresis was carried out at a constant voltage of 100 V until the bromphenol blue front was approximately 1-2 cm from the edge of the gel.

After electrophoresis, the presence of carbonic anhydrase activity was assayed by a modification of the method of Patterson et al. (2) . The pH change from the catalyzed degassing of CO_2 was observed by blowing gaseous CO_2 on the surface of a gel stained in a 25 mM TRIS 250 mM glycine buffer (pH 7.6) containing 0.05% (wt/vol) of the color pH indicator bromthymol blue (Sigma) until saturation, then allowing the CO_2 to diffuse from the gel. The gel was photographed when a band of CA in a control lane (containing *T. weissflogii* homogenate) was detectable.

To examine the effects of dissolved CO_2, cultures of *P. carterae* and *T. oceanica* were grown, bubbling with artificial air containing 100, 300, and 500 ppm CO_2 , with either 100% or 10% of the standard Aquil concentrations of both Co and Zn for *P. carterae* , and 20% or 2% of

these metals for the *T. oceanica*. Cultures were maintained under continuous lighting of 900 μEinsteins m^{-2} sec^{-1} at a temperature of 22°C. The final pH varied from approximately 7.8 in the 500 ppm CO_2 cultures, to 8.5 in the 100 ppm cultures. Experiments with with *E. huxleyi* cultures under different P_{CO2} grew poorly and are not included here.

Subsamples of the cultures were taken periodically and measured by fluorometry (Turner 10-005R). Growth rates were determined by a linear least squares fit of the natural log of fluorescence versus time in days. Statistical significance of differences in growth rates was tested with a standard t-test with respect to the null hypothesis of no effect.

Results

Growth curves for all the *P. carterae*, *T. subtilus*, and *T. oceanica* are shown in Figure 4-1 (each culture was grown at only one metal level). Figure 4-2 shows growth curves for *T. partheneia* at low and high metals respectively; there is no significant difference between the growth rates at these metal concentrations ($0.73 \pm .03$ and $0.78 \pm .04$ day^{-1} respectively). The growth of *E. huxleyi* (CCMP 1516) at the two metal concentrations is shown in Figure 4-3. Here the effect of metal limitation is more pronounced, ranging from $0.57 \pm .06$ day^{-1} for the low metal condition to $0.81 \pm .06$ day^{-1} for higher zinc and cobalt.

The results of the electrophoresis gel assay for CA activity are shown in Figure 4-4. The lane containing the control, *T. weissflogii*, has distinct bands where the gel is darker, indicating CA activity . The only other samples with any detectable activity are the lanes for *T. partheneia*, which contain much less activity, but which each have at least one distinct band that migrates just behind the bromphenol blue front in the gel. A second faint band appears above the main band in the Zn/5 *T. partheneia* lane and is caused by the activity of a second band of CA.

Growth rates of *P. carterae* cultures bubbled in CO_2 are shown in Figures 4-5a and 4-5b. With low zinc and cobalt, growth rates for cultures at 100, 300, and 500 ppm CO_2 are $0.54 \pm .06$, $0.63 \pm .03$, and $0.68 \pm .05$ day^{-1} respectively. Under the same partial pressures of CO_2, growth

rates for cultures with higher zinc and cobalt are 0.96 ± .07, 0.87 ± .07, and .86 ± .05 day^{-1}, respectively.

Growth rates for *T. oceanica* cultures under different partial pressures of CO_2 are shown in Figure 4-6a and 4-6b. Growth rates for cultures with low metals bubbled with 100, 300, and 500 ppm CO_2 are 0.73 ± .08, 0.87 ± .06, and 0.80 ± .03 day^{-1} respectively. Under the same partial pressures of CO_2, growth rates for cultures with higher zinc and cobalt are 0.96 ± .10, 1.28 ± .02, and 1.19 ± .03 day^{-1}, respectively.

Discussion

We found no evidence of carbonic anhydrase activity in most of the phytoplankton species we studied. Only one of the three diatoms and neither of the coccolithophores examined here have measurable quantities of the enzyme. The lack of detectable CA in most of these other phytoplankters may be caused by several factors.

Not all phytoplankton may possess carbonic anhydrase or require it for growth under most conditions. In the cultures of phytoplankton tested here, the highest growth rates were at most slightly more than half those of *T. weissflogii*, which can be as high as 1.8 day^{-1}. Additionally, the fastest growing species, *T. oceanica* and *E. huxleyi*, are both small, with cell diameters of approximately 3 and 5 μm respectively, compared to 10 μm for *T. weissflogii*. The combination of a factor of 2 smaller daily carbon requirement per unit cell volume (because of slower growth) and 2 to 3 times greater diffusive CO_2 flux per unit cell volume (because of smaller size) results in a net carbon dioxide demand to supply ratio that is effectively 16 to 25 percent of that for *T. weissflogii*. Given that CO_2 uptake can account for 10 percent of the maximum inorganic carbon requirement in *T. weissflogii* (3) , it would appear that even without bicarbonate utilization, these phytoplankters should not be severely limited by carbon. Similarly, bubbling of *T. weissflogii* cultures at partial pressures of CO_2 only three times the atmospheric concentration decreases CA production to nearly undetectable levels (4) . Therefore, any carbonic anhydrase present in these

other phytoplankters might be at much lower (and therefore undetectable) concentrations than in *T. weissflogii.*

The insufficient sensitivity of the gel assay for carbonic anhydrase may be another reason for no detected activity in most of the phytoplankton. Typically, the control lane with bovine CA can contain a minimum of one μg of protein and still be easily detected. Activity for bovine erythrocyte carbonic anhydrase typically ranges from 2000 to 3000 units mg^{-1} protein in commercial preparations (Sigma) . Literature values of measured CA activity for other phytoplankton have ranged from 7 to 20 units mg^{-1} protein in crude fractions (5, 6). The maximum protein that we can load into one lane of a gel is approximately 50 μg, and thus the amount of CA activity for many of the species, if less than 1 unit, may be below the detection limit of the assay .

Aside from the sensitivity of the assay, a lack of activity measured by the method described here also does not conclusively prove the absence of CA in these phytoplankton; CA has been found in different locations within the cell for various phytoplankton ranging from inside the chloroplast in *Chlorella vulgaris* (7) , to the periplasmic space in *Dunaliella tertiolecta* (8) , to outside the cell, in *Chlamydomonas reinhardtii* (9, 10) . The CA found in the supernatant fraction of *T. weissflogii* may have been a fragmented CA released from a membrane. The presence of CA in the soluble fraction of *T. partheneia* but not the other diatoms might represent differences in the location of CA and in its tendency to become solubilized in the supernatant during our treatment.

Nimer and co-workers report the measurement of carbonic anhydrase activity in a fraction separated via gradient centrifugation of homogenates from a calcifying *E. huxleyi* (6) . This activity, which coincides with the fraction of homogenate with the maximum chlorophyll concentrations (assumed to be the chloroplasts), is present in both stationary phase and log phase cultures but is inhibited by combining this fraction with other homogenate fractions from log phase cultures. Separation via non-denaturing PAGE should similarly separate CA from most other cellular proteins and components, so inhibition by such a cellular components is unlikely in our

experiments.

Non-denaturing PAGE cannot be used on insoluble proteins, and thus a membrane-bound CA would not be detected in this assay. In light of the membrane-bound forms of CA found previously in other species and strains (6, 7, 9, 10), there is a possibility that such insoluble forms of CA exist in these species and are not detected. Liquid phase assays with suspensions of insoluble material can circumvent this limitation, but cannot separate the carbonic anhydrase from other proteins bound to the same membranes.

Since the results of the CA activity assay are inconclusive, we also examined the possible role of zinc and cobalt in carbon acquisition (and perhaps in CA function) directly by observing the combined effects of P_{CO2} and metal concentrations on phytoplankton growth (as was done in Morel et al. (4)). From the results of growth experiments for cultures bubbled with air under different partial pressures of CO_2, it appears that CO_2 may partially compensate for zinc or cobalt limitation, i.e. alleviate a zinc-cobalt-carbon co-limitation, at least in *P. carterae* .

The results for *P. carterae* demonstrate that zinc and cobalt additions may compensate for the effects of low P_{CO2}. At low metals, growth rate increases with increased carbon dioxide ($p < 0.2$) , but at high metals, CO_2 has either no impact or a negative effect on growth. This is similar to the results for *T. weissflogii* in Morel et al. (4); in high zinc neither high nor low CO_2 affects growth. In our experiments with *P. carterae* however, there seems to be a negative effect of high P_{CO2} in cultures with sufficient metals. A detrimental effect of high CO_2 has been previously found in other coccolithophores under similar CO_2 aeration (11). We have seen this effect in other cultures of other species, and it may be the result of low pH in the medium. As noted previously in Chapter 3, although bubbling with high or low CO_2 changes pH in the medium, inorganic metal concentrations only increase 3 % at pH 7.5 (high CO_2). Therefore neither the negative effect of high P_{CO2} on metal-sufficient cultures nor the positive effect on metal-limited cultures is likely to be caused by the influence of pH on the availability of metals (either toxic or beneficial).

The results of experiments changing P_{CO2} are less convincing for *T. oceanica* . In conditions of either low or high metals, low P_{CO2} may slightly decrease growth of *T. oceanica* ($p < 0.3$). Again, a P_{CO2} of 500 ppm appears to have a slightly deterimental effect on growth as compared to normal air values of around 300 ppm. Nevertheless, *T. oceanica* may exhibit interactions between P_{CO2} and Zn' and Co' similar to those observed in *T. weissflogii* and *P. carterae* ; higher P_{CO2} results in higher growth in metal-limited cultures. It is therefore possible that zinc and cobalt also play a role in carbon acquisition in this open ocean diatom.

The interaction between P_{CO2} and Zn-Co observed in our cultures implies that the increase in the concentration of one of these improves either the uptake or the assimilation of the other. It is doubtful that P_{CO2} has any effect on metal uptake or assimilation since it has little effect on trace metal speciation in the medium. It thus seems probable that there is a role for zinc and/or cobalt in carbon uptake or metabolism, in light of our previous results with *T. weissflogii*. The effect of CO_2 at low Zn-Co in the absence of measurable soluble CA activity indicates that the role of these metals may be in a membrane-bound form of CA in some species.

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Figure 4-1. Growth curves for batch cultures of :

■ *T. subtilus,* Zn' = 3.2 nM Co' = 4.4 nM

● *T. oceanica,* Zn' = .32 nM Co' = .44 nM

▲ *P.carterae* Zn' = 3.2 nM Co' = 4.4 nM

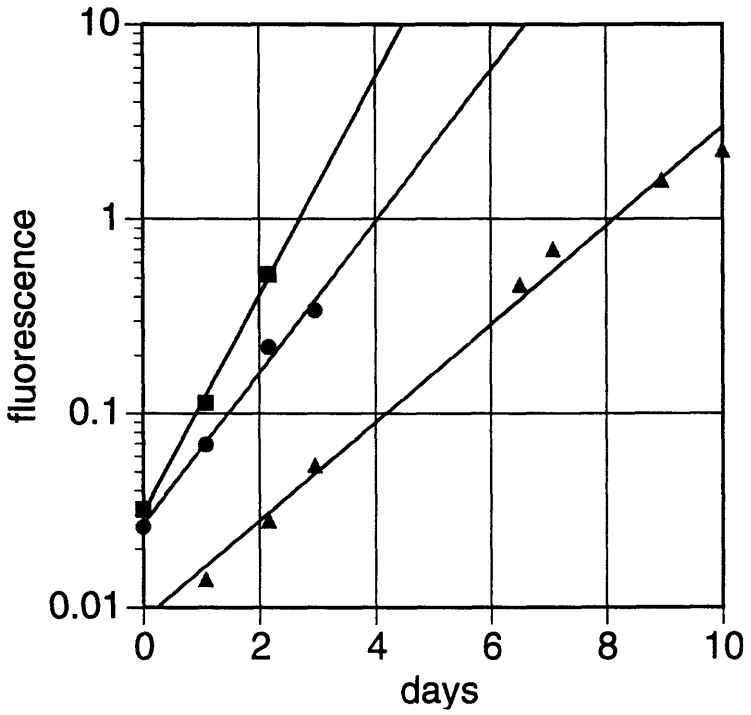


Figure 4-2. Growth curves for *T. partheneia* batch cultures grown in Aquil

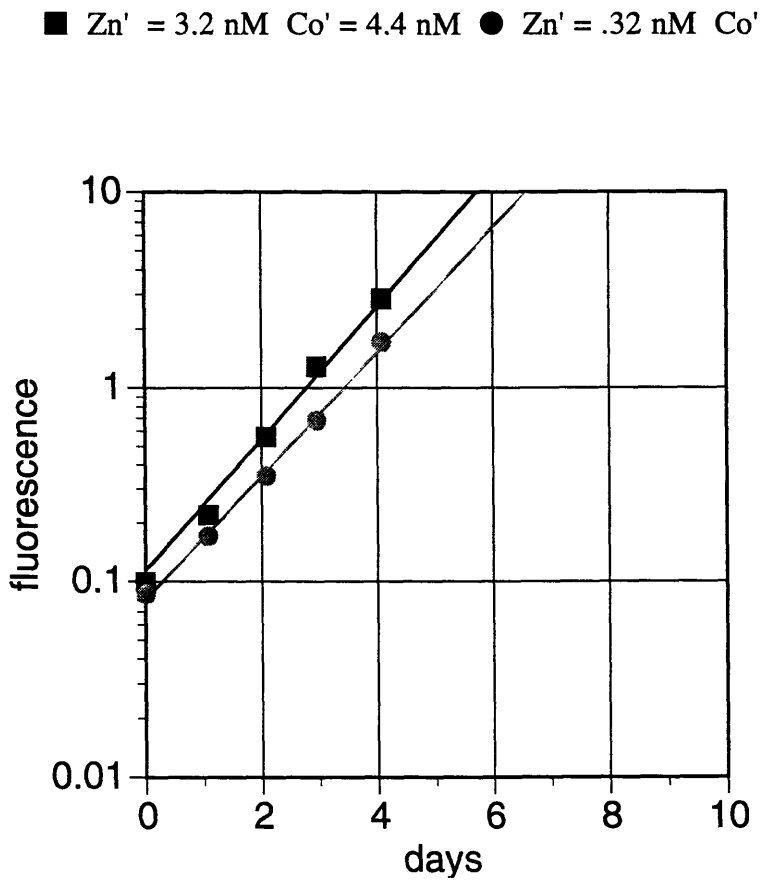


Figure 4-3. Growth curves for *E. huxleyi* batch cultures

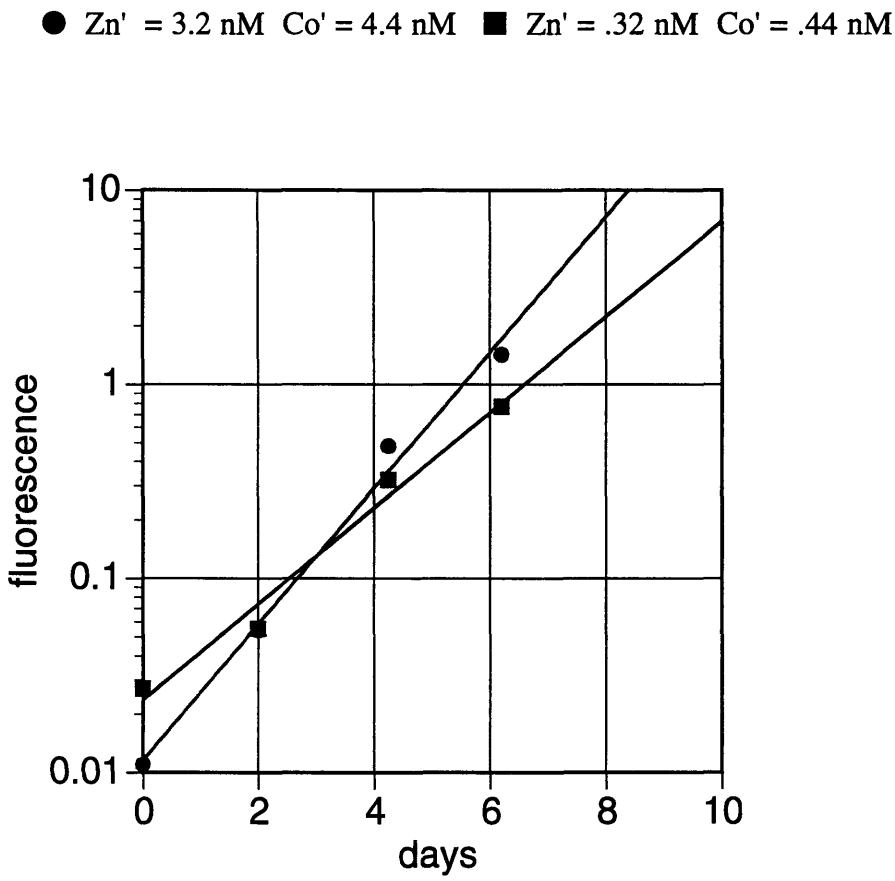


Figure 4-4. PAGE gel with assay for CA activity in six phytoplankton species

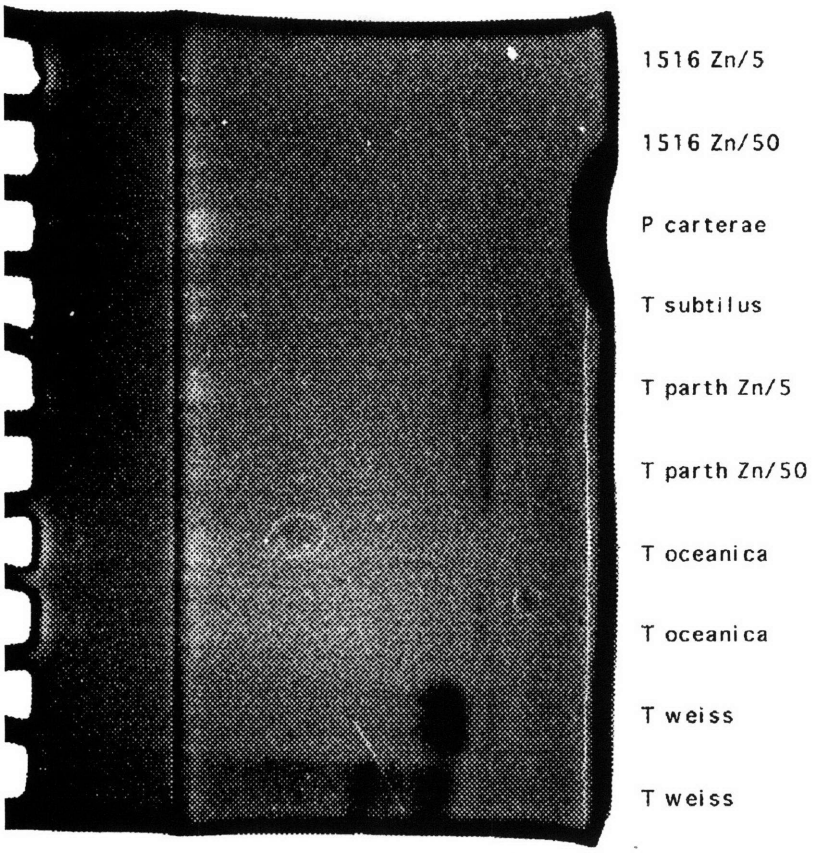


Figure 4-5. Growth curves for *P. carterae* under, ■ 100 ● 300 ▲ 500 ppm CO_2

In Aquil medium with Zn'/10 = 1.6 nM Co'/10 = 2.2nM

In Aquil medium with Zn' = 16 nM Co' = 22nM

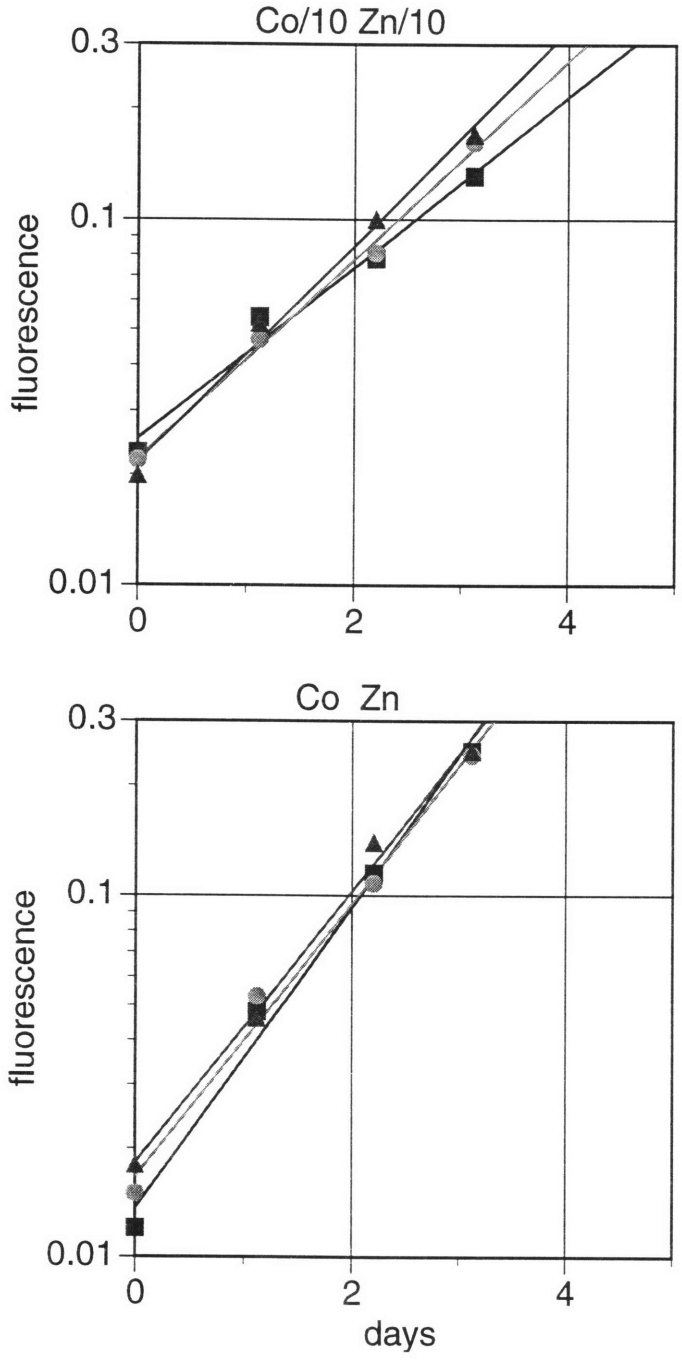


Figure 4-5b. Growth rates for *P. carterae* under 100 (low), 300 (med), 500 ppm (high) CO_2 error bars indicate standard error of growth rate calculation.

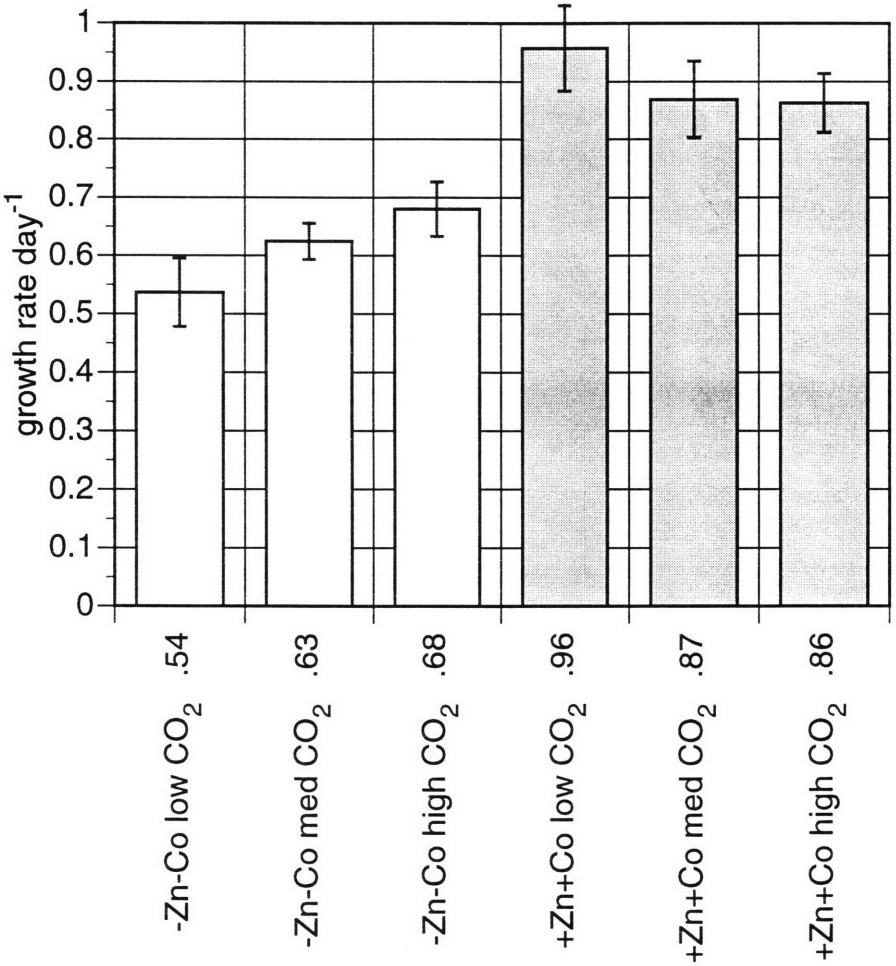


Figure 4-6. Growth curves for *T. oceanica* under, ■ 100 ● 300 ▲ 500 ppm CO_2
In Aquil medium with Zn'/10 = 1.6 nM Co'/10 = 2.2nM
In Aquil medium with Zn' = 16 nM Co' = 22nM

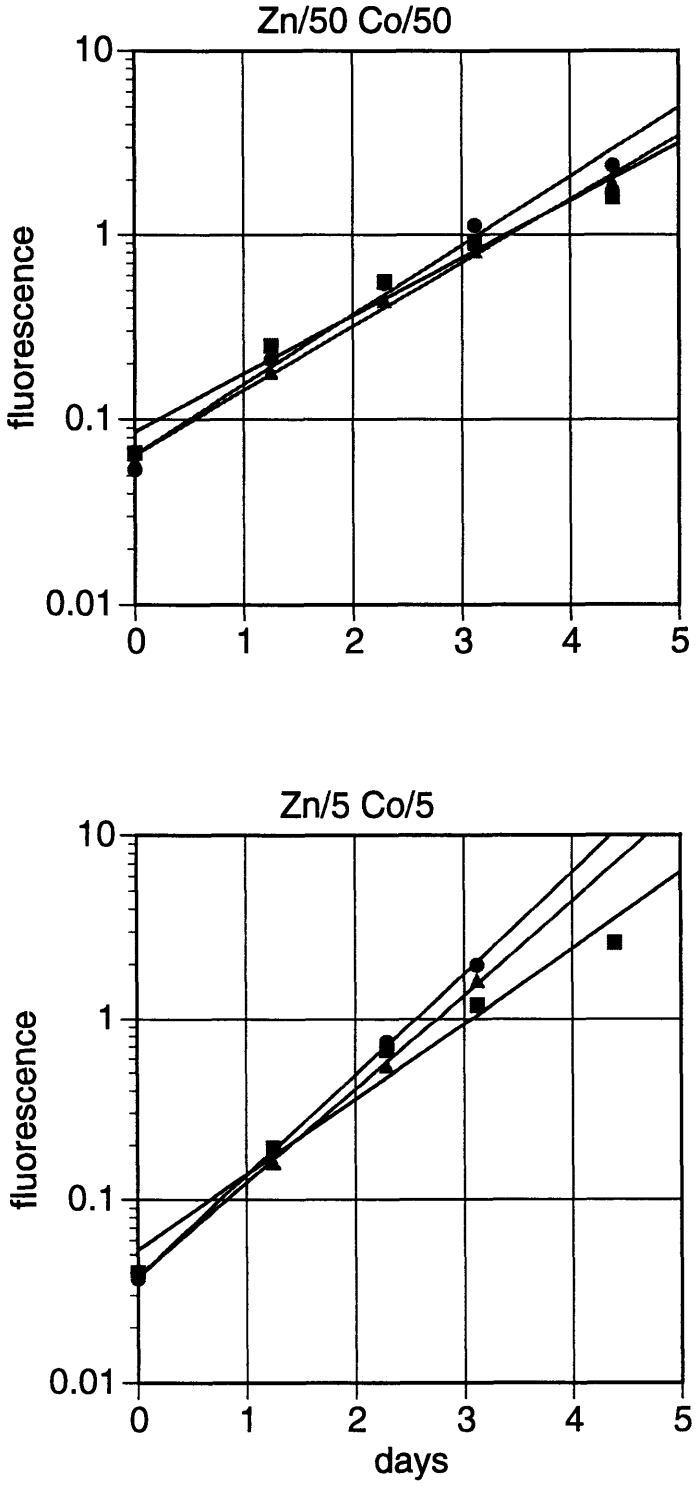
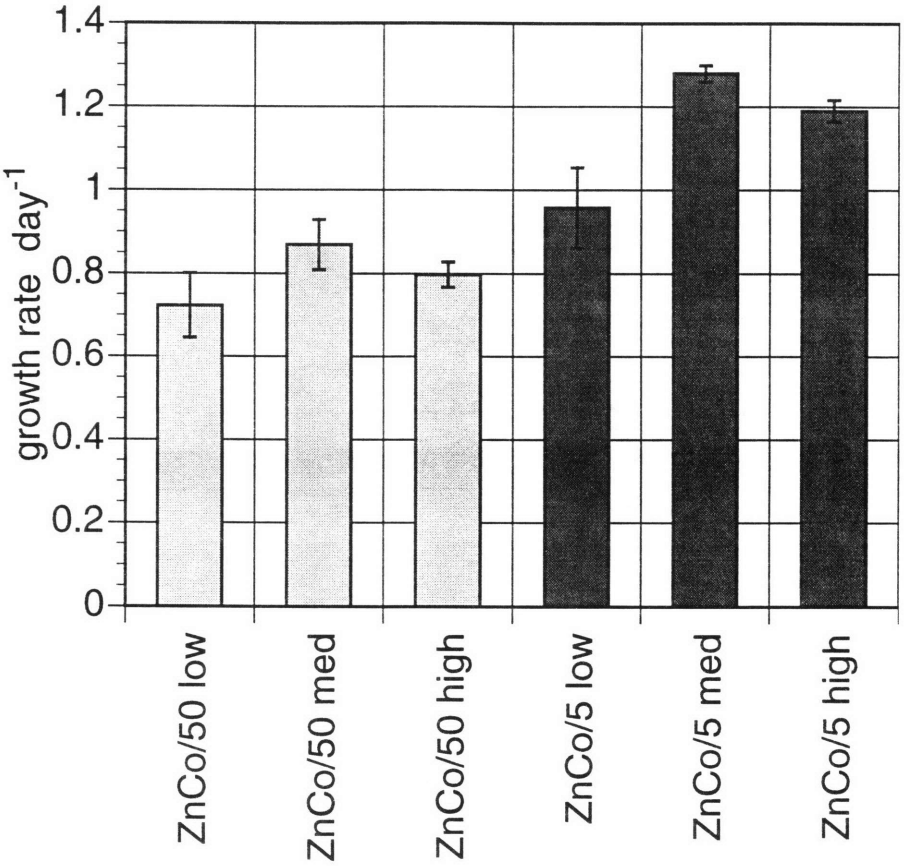


Figure 4-6b. Growth rates for *T. oceanica* under 100 (low), 300 (med), 500 ppm (high) CO_2 error bars indicate standard error of growth rate calculation.



Chapter 5. Zinc and cobalt in other metabolic functions

Introduction

In the previous chapter we examined a number of species for the presence of a soluble form of the zinc enzyme carbonic anhydrase (CA) to determine if it is a common function in which cobalt might substitute for zinc. The results were negative; we could measure CA activity in only one species, suggesting that the high activity of a soluble form of CA in *T. weissflogii* is not characteristic of many phytoplankton species. Thus either the major function of zinc that can be replaced by cobalt in these organisms is in membrane-bound CA or in enzymes other than CA. Because carbonic anhydrase has been found in insoluble fractions of the cell in other phytoplankton (1, 2, 3, 4, 5, 6), ranging from outer membranes to chloroplasts, in this chapter we investigate the distribution of zinc and cobalt between the soluble and insoluble fractions of the cell. We also examine the presence of zinc and cobalt in nucleic acid associated proteins, and in superoxide dismutase. Finally we compare the distribution of zinc and cobalt among soluble cellular proteins separated by electrophoresis.

Some possible zinc functions become apparent from examination a list of known zinc enzymes (7) (See Table 1-1 in Chapter 1). Most of the enzymes listed are from bacteria, mammals and yeast, but a number are from plants as well. One group of enzymes on which some work has been done in all the kingdoms is the nucleic acid polymerases, which are required by all known organisms. The work done by Vallee and co-workers (8, 9, 10) on the protist *Euglena gracilis* (grown in the dark) paved a trail in the study of zinc-limitation responses of eukaryotic organisms. These authors noted severe depression of RNA and protein synthesis, doubling in the cellular volume and DNA content, and accumulation of Ca, Mn and Fe. This response suggested a role for zinc in the regulation or production of nucleic acid polymers, which was soon revealed to be the DNA and RNA polymerases in work by others in bacterial systems (11, 12). Likewise in *E. gracilis*, Vallee and co-workers found that in high-zinc cells, there are three RNA polymerases, for

generating ribosomal, transfer, and messenger RNA, but in low-zinc cells, there was only one, which appears to make all three forms of RNA. This indicates one possible means by which cellular response can compensate for zinc deficiency.

Another means of satisfying zinc requirements in low zinc environments is by substitution with another metal, such as cobalt. In *Escherichia coli* grown in medium with low zinc and high cobalt, synthesis of a cobalt containing RNA polymerase occurs, and this enzyme possesses nearly the same activity and accuracy as the zinc enzyme (13). Metal limitation of bacterial growth did not occur in zinc-depleted (~10 nM total zinc) medium treated with Chelex in that work, but addition of excess cobalt (5 μM total cobalt) resulted in production of the cobalt-containing RNA polymerase. Because our work and that of others have shown (14, 15, 16, 17) that marine algae are limitable by zinc deficiency (in contrast to *E. coli* in that study), addition of cobalt at lower concentrations might also cause a similar substitution in eukaryotic phytoplankton, and we investigate substitution in polymerases and other RNA and DNA-binding compounds as one mechanism by which cobalt substitution for zinc occurs.

Superoxide dismutase is another function for zinc that exists for a range of organisms across kingdoms. Mechanisms for combating oxidizing radicals are likely to be important in photosynthetic organisms such as algae which grow in oxic environments with high light fluxes. All the species of green algae examined in one study were revealed to possess enzymes with superoxide dismutase (SOD) activity (18). There are three forms of SOD, containing iron, or manganese, or copper and zinc (Cu-Zn SOD), and some of the algae included in the study have been shown to have this Cu-Zn form of SOD. In vitro studies on metal substitution in bovine Cu-Zn SOD indicate that the copper ion is the redox active metal in this enzyme, and substitution of zinc by a number of different metals resulted in nearly full activity (19). The non-specificity of the metal in this site suggests that it is one function in which we might find cobalt substituting for zinc, and this role is examined here.

Materials and Methods

Algal culturing- Cultures of phytoplankton species were grown in Aquil medium with the following modifications to the standard recipe (20); EDTA was decreased to 10 μM to maintain a high specific activity of the radioactive metal added. Trace metal nutrient concentrations were lowered proportionately to compensate. Zinc and cobalt were added to the cultures at the following concentrations; for *E. huxleyi* 1516, *P. carterae*, and *T. weissflogii*, zinc was added to achieve a final inorganic species concentration of 16 or 0.64 pM (8 or 0.32 nM total) in the high and low zinc cultures respectively, and cobalt was added to reach a final inorganic species of 22 or 0.88 pM (5 or 0.2 nM total). The medium for *T. oceanica* contained zinc added at 1.6 or 0.16 pM (0.8 or 0.08 nM total).

Synthetic seawater was prepared in acid-washed 1 liter polycarbonate bottles (Nalgene), to which Chelexed major nutrients were added, and microwaved for 15 minutes on high power, swirling every 3 minutes. After the bottles cooled, 1000x concentrated trace metal stock mix (minus zinc, cobalt, and iron but including EDTA) that had been previously filter-sterilized (Gelman Acrodisc, 0.22 μm pore) was added to the medium, along with a concentrated mix of vitamins from the Aquil recipe. A constant 20 μCi of radioactive ^{57}Co or ^{65}Zn (Amersham) was added to each liter of medium. The medium was left to equilibrate overnight (at least 12 hours) after all additions were made.

Inocula from stock cultures maintained in Aquil with full trace metals (with one-tenth the major nutrients phosphate, nitrate and silicate for *E. huxleyi* stocks) were transferred into clean tubes with no added trace metals and allowed to grow for at least two doublings in density as measured by fluorescence (Turner 10/005R) before inoculation into polycarbonate bottles. Cultures were maintained under continuous light at 900 μEinsteins m^{-2} sec^{-1} at 22°C. Cells were counted with a hemacytometer and allowed to grow until late exponential phase. Cells were then collected on (Poretics) polycarbonate filters (3 μm pore size for the larger cells, and 1 μm pore size

for smaller cells) using a gentle vacuum of -0.3 atmospheres or less. 20 ml of 1 mM DTPA was passed through the cells at a rate of 2 ml min^{-1} while still collected on the filter to remove non-specifically bound surface metals, followed by wash of 10 ml synthetic seawater (Aquil without any nutrients or EDTA added). After the first wash the filter was removed from the filter apparatus and cells were resuspended in 8 ml of SOW as a second wash. Cells were centrifuged into a tight pellet in a swinging-bucket rotor at ~2000 x g for 5 minutes. The supernatant was decanted, and collected cells were stored frozen at -20 °C.

Cells were resuspended in a 50 mM NaCl 10 mM phosphate buffer at pH 7.0, with 1 mM phenylmethylsulfonyl fluoride (PMSF) added as a protease inhibitor, and diluted to a concentration of 5 x 10^5 cells ml^{-1} for the smaller species, and to 1 x 10^5 cells ml^{-1} for the larger ones. Cells were disrupted by three passes through an N_2 pressure bomb (Yeda press) cooled to 0 °C. The one exception to this method of disruption was the coccolithophore *E. huxleyi*, for which collected cells were disrupted using a sonicator (Branson Sonifier) on 50 % power, 30% cycle, for 1 minute with the sample was on ice.

Crude partitioning- Disrupted cell homogenate was transferred to 1.5 ml microcentrifuge tubes, and centrifuged for 1 hour at 25000 x g in a refrigerated centrifuge. The supernatant was carefully removed by pipetting, transferred to another tube, and the centrifugation continued for another 30 minutes. The latter step was repeated several more times (generally twice was sufficient) until the supernatant was clear, or no visible change in the size of the pellet appeared after a centrifugation step.

Nucleic acid precipitation- Nucleic material was precipitated by a modification of the method of Treisman (21) for purification of plasmid DNA from bacteria. The step for LiCl precipitation of RNA was omitted, and RNAase was not added. To 1 volume of cell homogenate in a microcentrifuge tube, 0.5 volume of 30 % (w/v) polyethylene glycol (PEG 8000, Sigma) was added and mixed well. The mixture was cooled at 0 °C for 15 minutes, followed by centrifugation at 20,000 g for 20 minutes. A higher final PEG concentration (10 % w/v) was chosen because the

original concentration as published (6.5 % w/v) did not recover much of the DNA (as determined by ethydium bromide staining of agarose gels) in trial runs with algal cell homogenate preparations.

Supernatant was pipetted from the tube, carefully avoiding the pellet, which generally appeared as a translucent speck at the bottom of the tube. Where the pellet of nucleic acids was not visible, fluid was removed with the pipette tip away from the side of the tube which was oriented outward during centrifugation. The supernatant was transferred to another tube.

All fractions (pellet, precipitated nucleic acid, remaining supernatant) were counted for ^{57}Co or ^{65}Zn activity using a gamma counter (Wallac 1480).

PAGE- Non-denaturing electrophoresis was performed according to the Laemmli method (21) at room temperature on a 10% polyacrylamide gel. After electrophoresis, the gel was assayed for carbonic anhydrase by a modification of the method of Patterson et al. (22). The pH change from the catalyzed evasion of CO_2 was observed by blowing gaseous CO_2 on the surface of a gel stained in a 25 mM TRIS, 250 mM glycine buffer (pH 7.6) containing 0.05 % (w/v) of the color pH indicator bromthymol blue (Sigma) until the entire gel was CO2 saturated, then allowing the gel to sit until bands of CA activity appeared. The gel was photographed using color positive film (Fuji, ASA 100), and the image scanned and digitized (Leaf 45) in grayscale. Brightness and contrast were adjusted so that the highlights and shadows spanned the entire range of grayscale (256 levels).

For detection of SOD activity, electrophoresis was performed as described above. After electrophoresis, the gel was assayed for SOD activity using the method of Beauchamp and Fridovich as described by Flohe and Otting (23). A small volume of solution of 0.25 mg ml^{-1} of 4-Nitro blue tetrazolium chloride (NBT, Sigma) and 0.10 mg ml^{-1} riboflavin was prepared in Milli-Q water. The gel was soaked in this solution by pipetting ~ 1 ml per 100 cm^2 gel surface in the dark. Excess solution is drained. After 20 minutes, a solution of 0.01 mg ml-1 N,N,N',N'-Tetramethylethylenediamine (TEMED, Sigma) was also pipetted onto the gel and allowed to soak

for 20 minutes, and similarly drained. The gel was then exposed to light, until a bluish-purple color developed in the gel, and the gel was then washed in distilled water to prevent further development.. SOD activity appeared as clear regions in the blue background. Gels assayed for SOD activity photographed using color positive film (Fuji, ASA 100), and the image scanned and digitized (Leaf 45) in grayscale. Brightness and contrast were adjusted so that the highlights and shadows spanned the entire range of grayscale (256 levels).

Results

Growth curves

The same general trends in growth rate observed in Chapter 2 are also observed here: cultures with higher zinc and cobalt tend to grow faster; the oceanic species have a narrower range between their highest and lowest growth rates; and the oceanic species have smaller differences between growth rates in high zinc (with low cobalt) and growth rates in high cobalt (with low zinc). Table 5-1 summarizes the growth rates of cultures grown on radioactive zinc and cobalt. As can be seen, growth rates are less systematic than the results in Chapter 2: the low EDTA medium (necessary to maintain high specific activity in radiolabeled metal experiments) requires smaller additions of the nutrient metals, and the combination of pipetting errors for small volumes and the larger contribution of background contamination may explain the greater variations.

Cellular zinc and cobalt

Zinc an cobalt quotas versus medium concentrations- For all the species grown, cellular zinc quotas (Figures 5-1a through 5-1d) are roughly proportional to the Zn' of the medium, and are also proportional to cell size. Cobalt quotas are also roughly proportional to the concentration in the medium and to cell size in all the phytoplankton studied (Figures 5-2a through 5-2d) but are about an order of magnitude smaller than the quotas of zinc at similar concentrations.

In *T. weissflogii,* the largest species studied, zinc quotas vary from 45 to 53 amol cell^{-1} zinc when cells are grown with high zinc concentrations (Zn' 16 pM) in the medium. A 25-fold

decrease in the Zn' of the medium (to 0.64 pM) results in an approximately proportional decrease in the zinc quota, to between 2.4 and 3.9 amol cell^{-1}. In *P. carterae*, the next largest phytoplankter, zinc quotas range from 14 to 19 amol cell^{-1} when grown in medium with high zinc and decrease to between 0.94 and 0.82 amol cell^{-1} when grown in medium with low zinc. In *E. huxleyi* , about one-tenth the volume of *T. weissflogii*, the cellular zinc ranges from 5 to 4 amol cell^{-1} when grown in high zinc medium, almost exactly one-tenth that of *T. weissflogii*. The cellular zinc concentrations of *E. huxleyi* grown at low Zn' vary somewhat more, ranging from 0.3 amol cell^{-1} when Co' in the medium was also low, to 0.08 amol cell^{-1} when grown at high Co'. *T. oceanica*, also about one-tenth as large as *T. weissflogii*, contains much less zinc, about 0.56 amol cell^{-1} , but once adjusted for the lower zinc concentration (1.6 pM Zn') in the "high" zinc medium, the quotas are still proportional to the medium concentration, and quotas at "low" zinc (0.16 pM Zn'), are also proportional, at about 0.06 amol cell^{-1}.

Cobalt quotas of each of the species are also proportional to the concentration of the medium, within a factor of three, but almost an order of magnitude lower than zinc quotas for comparable concentrations in the medium. The cobalt quota of *T. weissflogii* at the higher Co' of 22 pM (5 nM total Co) ranges from 8 to 4.4 amol cell^{-1} in cultures grown in low and high zinc, respectively. When Co' is decreased to 0.88 pM (0.2 nM total) in the medium, quotas for *T. weissflogii* decrease to 0.35 to 0.12 amol cell^{-1}, approximately 25 times less. *P. carterae* has the next highest cobalt quota, 2.6 and 2.4 amol cell^{-1} for cultures in low and high zinc, respectively, which decreases to 0.04 and 0.07 amol cell^{-1} when grown in low cobalt. *E. huxleyi* follows the same trend when grown at high cobalt, with quotas of 0.98 and 0.88 amol cell^{-1} in cultures in low and high-zinc medium, respectively, approximately one-tenth the quotas for *T. weissflogii* at the same metal concentrations. Cobalt quotas for both the oceanic species deviate from direct proportionality. *E. huxleyi* grown at the lower Co' deviates the most from proportionality, decreasing only 10-fold to 0.09 amol cell^{-1} in cultures grown at low zinc, but decreasing proportionally 30-fold (to 0.03 amol cell^{-1}) in cultures grown with high zinc. Similarly, *T.*

oceanica cobalt quotas are not directly proportional to quotas in *T. weissflogii* scaled for size. *T. oceanica* grown at the higher cobalt concentration (Co' of 2.2 pM) takes up between 0.28 and 0.18 amol $cell^{-1}$, about three times higher than the quotas expected for a cell 10 times smaller in medium with 10 times less cobalt (the expected quotas would be between 0.08 and 0.04 amol $cell^{-1}$ if the proportionality to size and medium concentration were strict).

Effect of Co on Zn quotas and of Zn on Co quotas - Cobalt concentrations have little effect on the zinc quotas of phytoplankton except for *E. huxleyi*, in which high cobalt decreases cellular zinc at low zinc concentrations in the medium. For most of the phytoplankton at all cobalt concentrations, increased zinc in the medium generally decreases cellular cobalt.

As the data of Figures 5-1a through 5-1d show, inhibition (or enhancement) of accumulation of one metal by the other metal varies greatly among the species. For inhibition of zinc accumulation by cobalt, *T. oceanica* is the simplest case: neither low nor high Co' has any effect on zinc quota at either high or low zinc concentrations. The effects in *T. weissflogii* and *P. carterae* are of the same order of magnitude, but are virtually mirror images of each other. When Zn' is low, zinc quotas of *P. carterae* decrease 10 % when cobalt is increased, whereas *T. weissflogii* in low zinc medium takes up nearly twice as much zinc per cell when cobalt is increased. When Zn' is high , *T. weissflogii* grown in high cobalt have 15 % lower quotas than cells at the same Zn' but at a low cobalt concentration. Conversely, *P. carterae* grown in high zinc takes up almost 40 % more zinc at high Co' than it takes up at low cobalt. Unlike the other species, in *E. huxleyi* zinc accumulation is always inhibited by higher cobalt: at high zinc, the effect of high cobalt is slight, causing a 20 % decrease in zinc quota, but at low zinc, the effect of high cobalt is very pronounced, causing a 70 % decrease in zinc quota.

The effect of zinc concentration on cobalt quotas is more pronounced in all the species we studied. In *T. weissflogii*, *T. oceanica*, and *E. huxleyi*, increasing zinc decreases cobalt quotas, whether at low or high cobalt. The magnitude of this inhibition varies, and is greatest in *E. huxleyi* and *T. weissflogii* at low cobalt concentrations. At high cobalt concentrations, the effect

of zinc on *P. carterae* is small, decreasing cobalt quotas slightly at high zinc. However, at low cobalt, addition of zinc increases cobalt quotas in this phytoplankter.

Table 5-2 shows the Zn':Co' ratio in the medium, and the ratio of cellular Zn:Co to the medium Zn':Co'. This relative enrichment factor (REf), is equivalent to the ratio of the enrichment factors (often expressed as the ratio of cellular metal concentrations to ambient metal concentrations) for zinc relative to cobalt in these phytoplankton; if quotas of both metals in a cell are in a ratio equal to their proportions in the medium, this REf is 1. One thing apparent from the table is that the oceanic phytoplankton have a smaller ratio of zinc to cobalt under most circumstances. Zinc is very enriched compared to cobalt in all species under all conditions. The table is also subdivided to indicate these REfs for different crude fractionations of the cells, which will be described below.

Cellular fractionation- The soluble fraction of cellular zinc (excluding the portion associated with nucleic acids) varies among species, from as high as 45 % in *T. weissflogii* to as low as 4 % in *E. huxleyi*, and varies within species depending upon the zinc and cobalt concentration of the medium in which the phytoplankton are grown. Data on partitioning of zinc in cell fractions are given in amol $cell^{-1}$ in Tables 5-3a through 5-3d for the four species, and shown as percentages of total cell quotas in Figures 5-3a through 5-3d. In general, addition of cobalt or zinc to the medium increases the fraction of zinc which appears in the soluble portion. A sizable amount of zinc is associated with nucleic acids, which are also soluble, but can be separated from the rest of the soluble material by precipitation. The amount of zinc associated with the nucleic acid (NA fraction) increases with increasing cobalt or zinc in the medium. In contrast, the fraction of cobalt in the soluble fractions is always at or greater than 45 % of the total cell quota, and these percentages do not change with changing concentrations of zinc and cobalt in the medium (Figures 5-4a through 5-4d). Likewise, percentages of total cobalt associated with the NA fraction also remains constant within each species, at 7% or less of the total zinc for all the phytoplankton we studied.

In Figures 5-3a through 5-3d, pie charts are sized so that the areas of the whole pies are proportional to the zinc content per cell for each species. Each pie is sliced to indicate the distribution between the pellet, consisting of insoluble material in cell homogenates, the nucleic acids in the supernatant, which includes any DNA, RNA, and proteins associated with nucleic acids which precipitate in this fraction (which is referred to as NA), and the non-NA fraction of the supernatant. Figures 5-4a through 5-4d show the distribution of cobalt in each fraction for these phytoplankters in a similar manner.

The distribution of cobalt is similar among three of the phytoplankters: for *T. weissflogii*, *T. oceanica*, and *E. huxleyi*, the fractionation of cobalt among pellet, NA, and the remaining supernatant fractions remains constant within each species for different external medium conditions. However, the distributions in these fractions differ among the species. In *T. weissflogii* and *T. oceanica*, approximately 55 to 60 percent of the cobalt is in the supernatant, between 35 to 40 percent is in the pellet, and the remaining 5 percent is associated with precipitated NA. The distribution in *E. huxleyi* is similar: between 45 and 50 percent is in the supernatant fraction, roughly the same amount is in the pellet, and the remaining 5 percent is associated with precipitated NA. Curiously, the distribution of cobalt varies more in *P. carterae*. At low zinc and cobalt, only 25 % of the cobalt is associated with the pellet. This fraction increases to about 34 % of the total with addition of either zinc or cobalt, but decreases to 16 % when high concentrations of both zinc and cobalt are added. The fraction of zinc in nucleic material remains low and fairly constant at about 2 % for cultures of *P. carterae* under all conditions.

In contrast, fractionation of zinc varies more among the species. With the exception of *E. huxleyi*, increasing zinc in the medium increases the fraction of zinc in the supernatant. For *T. weissflogii*, the change is largest on an absolute scale, changing from 12 % in cells grown in medium with low zinc and low cobalt, to about 45 % in cells grown at high zinc (whether grown at low or high cobalt). On a relative scale however, the change in *P. carterae* is the greatest, ranging from 2 % of the total cellular zinc when grown at low zinc and cobalt, up to 20 % of the total. The

relative change in supernatant zinc concentration is also large in *T. oceanica*, increasing from 6 % in of cellular zinc in cells from low zinc and cobalt medium, to 20 % in cells grown in high zinc and cobalt. In all cases (again, excepting *E. huxleyi*), an increase in cobalt in the medium increases the percentage of zinc in the supernatant fraction, albeit to a lesser degree than an increase in zinc in the medium does. The distribution of zinc in the supernatant fraction of *E. huxleyi* remains constant, at between 3 and 4 % under all conditions tested.

Similar to the effects of zinc and cobalt on the zinc content of the supernatant, increasing zinc and cobalt in the medium increases the fraction of zinc that appears in the nucleic acid fraction. In *T. weissflogii*, the effect is small, changing from about 6 % of cellular zinc in cells grown at low zinc and cobalt, to 9 % with additions of either cobalt or zinc or both metals. For *T. oceanica* and *P. carterae*, the change the fraction of zinc associated with DNA and RNA is more dramatic, increasing from less than 1 % of total cellular zinc in cells grown in medium with low zinc and cobalt, to a maximum of approximately 10% of the total zinc quota in cells from high-zinc medium. Like the distribution of zinc in the supernatant of *E. huxleyi* , zinc in the nucleic material of this phytoplankter remains constant, at between 3 and 4 % of the total zinc quota.

Protein gel electrophoresis

Carbonic anhydrase- Consistent with the results of Chapters 3 and 4 we found a large fraction of soluble zinc and cobalt in large bands with CA activity in PAGE for proteins from *T. weissflogii*. We were unable to detect any bands with CA activity in gels from other cultures of phytoplankton, in spite of a change from sonication to Yeda press (N_2 pressure bomb) in the method used to break the cells. Sonication may cause localized heat stress in the breaking solution, which sometimes results in inactivation of enzymes. Furthermore, concentrations of cells in the breaking buffer were increased to increase the concentration of proteins in the sample, but neither of these changes resulted in any increase in detectable activity for the other species.

An assay for CA activity of a PAGE gel for *T. weissflogii* shows activity in most of the lanes, with the most activity per lane in lanes for cultures with high zinc, and the next highest

amount of activity in the lanes with low zinc but high cobalt (Figure 5-5a). In some of the lanes, a faint second band of CA activity appears immediately above the main CA band. The autoradiograph of the same gel for ^{65}Zn (left four lanes of Figure 5-5b) shows a large fraction of the soluble cellular zinc appears in the main band with CA activity, and a smaller amount of zinc also appears in the second CA band. Because the same amount of radioactive ^{65}Zn was added to each of the lanes, the specific activity of zinc is approximately 25 times higher in the left two lanes (with less added cold zinc) as compared to the next two lanes. For samples in the leftmost lane (with low zinc and cobalt), *T. weissflogii* contains a very small amount of CA, and thus very little zinc is associated with the CA band. The addition of cobalt increases CA activity, and the fraction of total zinc with the CA band also increases. Addition of zinc increases the CA activity even more, and the soluble zinc and CA in the cells increase greatly. Although the ^{65}Zn bands in the high zinc lanes appear smaller, the specific activity is 25 times higher than in the low zinc lanes. Once this difference is noted, it is apparent that the quantities of zinc in the CA bands for these lanes are distinctly higher than in the others.

Adding only cobalt to low-zinc cultures increases CA activity, and the CA band also contains more ^{57}Co, even though the specific activity is much lower. However, addition of high zinc to the medium decreases the cobalt in CA greatly. Unlike zinc, cobalt does not appear in the second CA band in any of the lanes. No gels for any of the other species show any CA activity .

Superoxide dismutase- In all four species of phytoplankton we examined, we find at least one band with superoxide dismutase activity. For two of the species, bands of ^{65}Zn also appear to be associated with proteins showing this activity, and for one of these species, ^{57}Co also appears with SOD activity.

An assay for SOD activity in *T. weissflogii* reveals high activity in all the lanes in one broad major band, and a narrower, less intense minor band slightly above it (Figure 5-6a). An autoradiograph of the same gel (Figure 5-6b) shows that a band of zinc appears at the top of the major band of SOD activity, and another ^{65}Zn band co-elutes with the minor SOD band. A very

small amount of ^{57}Co seems to migrate in two bands parallel to the ^{65}Zn where the SOD bands appear, but this quantity of cobalt is much less than found in the CA band. All other cobalt bands do not appear to migrate parallel to any zinc bands, save for at the interface between the stack and main gel, where larger proteins cannot migrate beyond.

Similar to the result in *T. weissflogii*, the assay for SOD in *T. oceanica* indicates activity in all the lanes (Figure 5-7a). There are few differences in SOD activity among the lanes, except that all the lanes grown with radioactive zinc showed a second band of SOD activity. Comparison to an autoradiograph of the same gel (Figure 5-7b) shows that ^{65}Zn activity appears in a band which migrates with this second band of SOD activity. No bands of ^{57}Co appear parallel to any of the zinc bands. One other notable feature is a very large amount of cobalt activity which migrates with the solvent front in the gel (the large dark band across the entire gel in Figure 5-7a, which appears as a large cloud of radioactive cobalt in the autoradiograph, Figure 5-7b), which is also found in other gels and will be described below.

The assay for SOD activity in *P. carterae* also shows some activity in all the lanes; this is not clear from Figure 5-8a because the gel overdeveloped in the light, and the oxidation rate eventually exceeded the capacity of the *P. carterae* SOD to prevent reaction with the dye in most of the lanes. SOD activity still appears clearly in only two lanes, the fourth and eighth, which perhaps not coincidentally contain the highest concentrations of zinc and cobalt. However, an autoradiograph of the same gel does not reveal any zinc or cobalt with this band (Figure 5-8b). Other than the very large cloud of cobalt activity at the solvent front (as seen in the autoradiograph for *T. oceanica*), only one cobalt band appears distinctly in the ^{57}Co lanes. Likewise, only two bands appear in the lanes with ^{65}Zn, one in the second through fourth lanes, and a second much fainter band in the fourth lane.

E. huxleyi also shows SOD activity in cultures from all concentrations of zinc and cobalt in a single band that appears in all the lanes (Figure 5-9a). An autoradiograph of that gel shows ^{65}Zn in bands that migrate with the SOD activity (Figure 5-9b). Very faint bands appear in the

^{57}Co lanes that also migrate with the SOD band. Like the gels for the other species (other than *T. weissflogii*), a very large band of ^{57}Co activity migrates with the solvent front.

Other protein bands- Several other protein bands appear in each of the gels, but there is little in common among the gels for the different species except for the very large band with cobalt activity which appears in the solvent front This is a highly mobile (small and/or highly charged) species, perhaps a complex of cobalt with EDTA or DTPA from the culture medium or the wash solution. However, no such band appears in any of the zinc lanes (other than some bleed from the leftmost ^{57}Co lanes into the rightmost lanes for ^{65}Zn), indicating that this compound most likely originates from inside the cell. Although the band appears to be broad, it is likely to have been originally much narrower, because assays for both SOD and CA activity require a soaking step to allow the coloring reagents to diffuse into the gel. During this step, small compounds, such as those that migrate at the front of the gel, may also migrate omnidirectionally within the gel as well as out of the gel and into the soaking buffer.

Outside of the bands associated with CA and SOD, there does not seem to be any common pattern between the major bands of ^{65}Zn and ^{57}Co for each of the species. For example, in the *T. weissflogii* autoradiograph (Figure 5-5b) the zinc band near the top of the gel has no parallel counterpart in any of the cobalt lanes. Likewise, the double cobalt band in the lanes on the right have no zinc counterpart. This lack of parallel bands (other than in SOD or CA) is also seen in gels for the other species.

Discussion

The work shown here confirms and expands upon some results of the previous chapters. Cellular zinc quotas in all the phytoplankters, including *E. huxleyi*, are higher than their cellular cobalt quotas when the metal concentrations are the same in the medium. These quotas are comparable to quotas previously given in the literature for these and other phytoplankton (15, 16, 17, 24). Zinc quotas are higher in the coastal than in the oceanic species, a trend which seems

coherent with the relatively high Zn' at which the coastal species become limited in their growth (Chapter 2) . In our study, zinc quotas also appear to be roughly proportional to cell volume, but this may be a consequence of having chosen large coastal and small oceanic species, as the results of Chapter 2 and of previous authors (14, 17) have shown low zinc requirements for large oceanic species and high zinc requirements for small coastal species.

The lower zinc requirement of the oceanic phytoplankton also correlates with a higher extent of cobalt substitution, which was shown in Chapter 2 in the effects on growth rates of these metals. This effect is manifested again here, in the relative quotas of zinc to cobalt. In medium with high cobalt and low zinc, the absolute cobalt quotas of all species are higher than their zinc quotas, but all of the phytoplankton accumulate zinc preferentially to cobalt compared to the relative inorganic concentrations of these metals in the medium. This preference for zinc over cobalt is shown in Table 5-2 as REfs (i.e. the cellular Zn:Co ratios over the Zn:Co ratios in the medium) of greater than 1. For all the phytoplankton, this ratio is greater than 1, but in the neritic phytoplankton, this ratio is virtually always greater than 10, whereas the same ratio for the oceanic species is almost always less than 10 for the same conditions in the medium. The selectivity for zinc is thus greatest in the coastal species, which are more similar to each other than they are to oceanic species from their own class.

Although cellular zinc is found predominantly in the insoluble fraction of most cultures, this fraction and the fraction in precipitated nucleic acids change greatly as a function of metal concentrations in the medium for some species. At low metal concentrations in the medium, less zinc is in the soluble or nucleic acid fractions of the cell. When either zinc or cobalt was increased in the medium, the quantity and proportion of cellular zinc in the soluble and nucleic acid fractions of the cell increase dramatically. In contrast, cobalt remains distributed among the cell fractions at fairly constant proportions of the total cobalt quota for cells under all conditions. One species, *E. huxleyi*, has an idiosyncratic response to changing metal conditions: a large (> 90 %) and constant fraction of its changing zinc quota always remains in the insoluble fraction. This is in stark

contrast to *T. weissflogii*, for which well over half of its zinc quota is contained in the soluble fraction (of which 75 % or more may be in CA) and in the nucleic acid fraction.

T. weissflogii might not be typical however, because little or no soluble CA is found in most other species we examine. However, a role of zinc in CA (and thus also of cobalt) cannot be ruled out. Research on other species has found CA associated with membranes and organelles, and the results of experiments with high and low P_{CO2} show an effect of zinc and/or cobalt on carbon acquisition in *P. carterae* and perhaps also in *T. oceanica*. The large amount of zinc contained in the insoluble fraction of species other than *T. weissflogii* may indicate that this is the fraction in which CA might be found.

Our data also indicate that the primary mechanism of cobalt substitution is not replacement of zinc in the polymerases and other proteins associated with the NA fraction. Although the percentage of total zinc in the nucleic acid fraction varies for each species (except in *E. huxleyi*) with changing cobalt and zinc conditions, the percentage of cobalt remains invariant. Adding cobalt to zinc-limited cells increases the amount of zinc in the NA fraction. This preferential inclusion of zinc is what we expect if cobalt does not substitute for zinc extensively in proteins in this fraction. Nonetheless, it is still possible that cobalt might substitute for zinc in polymerases and other proteins bound to nucleic acids. Although the percentage of the total cellular cobalt in the NA fraction remains constant with increased Co' in the medium, the total amount of cobalt in the NA fraction increases. Additional data, such as PAGE gels, are needed to determine with certainty whether this cobalt is specifically bound to proteins or non-specifically precipitated with the NA fraction.

It appears that SOD may be one soluble zinc enzyme in which cobalt substitutes for zinc directly, in at least some phytoplankton species. In the PAGE gels of two of the phytoplankters we studied, SOD was one of the most visible protein bands; in three species zinc co-elutes with SOD activity, and in two cases cobalt co-elutes with this band. Several other bands of zinc and cobalt appear in gels, but they generally do not migrate together. The apparent lack of similar

elution characteristics in PAGE for the major cobalt and zinc bands however does not necessarily indicate that they have different functions; previous work on cadmium substitution in *T. weissflogii* showed that cadmium appears in a form of CA that does not co-elute with the major zinc CA band (17). Other than in *T. weissflogii* however, the quantity of zinc in the soluble fraction is much smaller than in the insoluble fraction, and therefore the role of zinc and cobalt in SOD may be secondary to a role in membranes.

Although a much larger portion of the cellular cobalt quota is in the soluble fraction, the cobalt in protein bands associated with SOD activity is dwarfed by cobalt associated with another compound in the soluble phase. The large quantity of this low molecular weight cobalt compound is quite apparent in the autoradiographs of the SOD assay gels. The identity of this compound is unknown, but one intriguing possibility is that it might be cobalt complexed with phytochelatin. Although cobalt does not induce phytochelatin production in *T. weissflogii*, even at very high cobalt concentrations (about 100 times the highest Co' we tested), the constitutive quantity of phytochelatin produced is between 5 and 10 amol $cell^{-1}$(25). which is greater than the cobalt quota under most conditions. Another result in *T. weissflogii* may provide more clues: cadmium, like cobalt, can behave like a nutrient in zinc-limited cells (26). However, even when cadmium is provided at concentrations that improve the growth of cells, the production of phytochelatin is higher than in zinc-sufficient, low-cadmium cultures. Thus, even when using a metal such as cadmium to replace zinc and improve growth, a phytoplankter may need to sequester that metal to control its distribution and metabolism within the cell. The same may be true of cobalt, and the low molecular weight cobalt compound might therefore be a phytochelatin complex.

B_{12} is another small cobalt-containing compound which is likely to be found in phytoplankton. Although eukaryotic phytoplankton are not known to produce B_{12} , we add it to all our culture media at concentrations of 0.4 nM. If exchange of radioactive cobalt occurs with B_{12} in solution or within the cell, a radioactive form of this small cobalt compound would be found in our autoradiographs. In spite of steps to sterilize the medium, cultures may become

contaminated with bacteria (or are not available axenic, such as *E. huxleyi* CCMP 1516). These bacteria in cultures might produce a radioactive B_{12} from the inorganic cobalt in the medium, which would then be taken up by the algae. PAGE gels tailored for separation of low molecular weight compounds or column chromatography (such as HPLC) might distinguish between radioactive B_{12} and phytochelatin-bound cobalt.

The results of this chapter, in concert with the results from the other chapters, illustrate the large differences in the metabolism of cobalt versus zinc for coastal and oceanic species of phytoplankton. These differences reflect differences in the relative distribution of these metals in different environments, and the evolutionary adaptations that have occurred in phytoplankton to allow optimum survival in their respective niches. These biological adaptations in phytoplankton may in turn cause the differences in cobalt distribution that we see in different regions of the oceans.

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Table 5-1. Growth rates of phytoplankton cultures for radioactive metal quota experiments in low EDTA medium.

	-Zn-Co	-Zn+Co	+Zn-Co	+Zn+Co
T. weissflogii ^{65}Zn	0.38	0.59	0.82	1.3
T. weissflogii ^{57}Co	0.47	0.68	0.85	0.79
T. oceanica ^{65}Zn	0.7	0.7	0.71	0.66
T. oceanica ^{57}Co	0.63	0.66	0.65	0.71
P. carterae ^{65}Zn	0.49	0.54	0.71	0.75
P. carterae ^{57}Co	0.26	0.19	0.77	0.47
E. huxleyi ^{65}Zn	0.63	1.37	1.09	0.95
E. huxleyi ^{57}Co	0.63	0.52	0.69	1.26

Table 5-2. Relative enrichment of the Zn:Co ratio,

i.e. $REf = (Zn:Co)_{cell} / (Zn':Co')_{medium}$

in whole cells, and in different cell fractions for various phytoplankton species.

	whole cell	pellet	NA	supernatant
	REf	REf	REf	REf
P. carterae				
-Zn-Co	32.05	135.44	6.40	1.03
-Zn+Co	10.88	28.58	15.72	0.98
+Zn-Co	10.92	28.25	25.32	1.69
+Zn+Co	10.54	47.52	42.42	2.57
E. huxleyi				
-Zn-Co	4.46	8.92	3.18	0.40
-Zn+Co	3.04	6.24	2.82	0.23
+Zn-Co	10.15	18.74	9.80	0.74
+Zn+Co	6.45	12.39	4.13	0.43
T. oceanica				
-Zn-Co	3.45	8.72	0.24	0.35
-Zn+Co	2.85	6.59	0.56	0.60
+Zn-Co	4.33	8.12	7.07	1.36
+Zn+Co	4.34	8.11	6.12	1.67
T. weissflogii				
-Zn-Co	9.19	18.76	10.90	2.07
-Zn+Co	16.53	22.03	36.73	10.85
+Zn-Co	25.78	33.58	64.90	18.75
+Zn+Co	14.24	16.56	30.20	11.56

Table 5-3a. Partitioning of zinc and cobalt in *P. carterae*, values given in amol cell^{-1}

Zinc	-Zn-Co	-Zn+Co	+Zn-Co	+Zn+Co
pellet	0.91	0.75	12.09	13.09
NA	0.00	0.02	0.51	1.88
supernatant	0.02	0.05	1.41	3.73

Cobalt	-Zn-Co	-Zn+Co	+Zn-Co	+Zn+Co
pellet	0.01	0.91	0.02	0.38
NA	0.00	0.05	0.00	0.06
supernatant	0.03	1.64	0.05	2.00

Table 5-3b. Partitioning of zinc and cobalt in *E. huxleyi* 1516, values given in amol cell^{-1}

Zinc	-Zn-Co	-Zn+Co	+Zn-Co	+Zn+Co
pellet	0.27	0.08	4.62	3.87
NA	0.01	0.00	0.19	0.11
supernatant	0.01	0.00	0.17	0.13

Cobalt	-Zn-Co	-Zn+Co	+Zn-Co	+Zn+Co
pellet	0.04	0.45	0.01	0.43
NA	0.00	0.03	0.00	0.04
supernatant	0.04	0.51	0.01	0.41

Table 5-3c. Partitioning of zinc and cobalt in *T. oceanica*, values given in amol cell^{-1}

Zinc	-Zn-Co	-Zn+Co	+Zn-Co	+Zn+Co
pellet	0.05	0.05	0.41	0.39
NA	0.00	0.00	0.06	0.05
supernatant	0.00	0.01	0.10	0.12

Cobalt	-Zn-Co	-Zn+Co	+Zn-Co	+Zn+Co
pellet	0.01	0.11	0.01	0.07
NA	0.00	0.02	0.00	0.01
supernatant	0.01	0.15	0.01	0.10

Table 5-3d. Partitioning of zinc and cobalt in *T. weissflogii* , values given in amol cell^{-1}

Zinc	-Zn-Co	-Zn+Co	+Zn-Co	+Zn+Co
pellet	1.94	2.14	24.93	20.76
NA	0.14	0.35	4.86	3.56
supernatant	0.29	1.39	23.25	20.73

Cobalt	-Zn-Co	-Zn+Co	+Zn-Co	+Zn+Co
pellet	0.14	3.34	0.04	1.72
NA	0.02	0.33	0.00	0.16
supernatant	0.20	4.40	0.07	2.47

Figure 5-1a. Zinc quotas for *P. carterae*

+Zn = 16 pM Zn', +Co = 22 pM Co', and -Zn = 0.64 pM Zn', -Co = 0.88 pM Co'

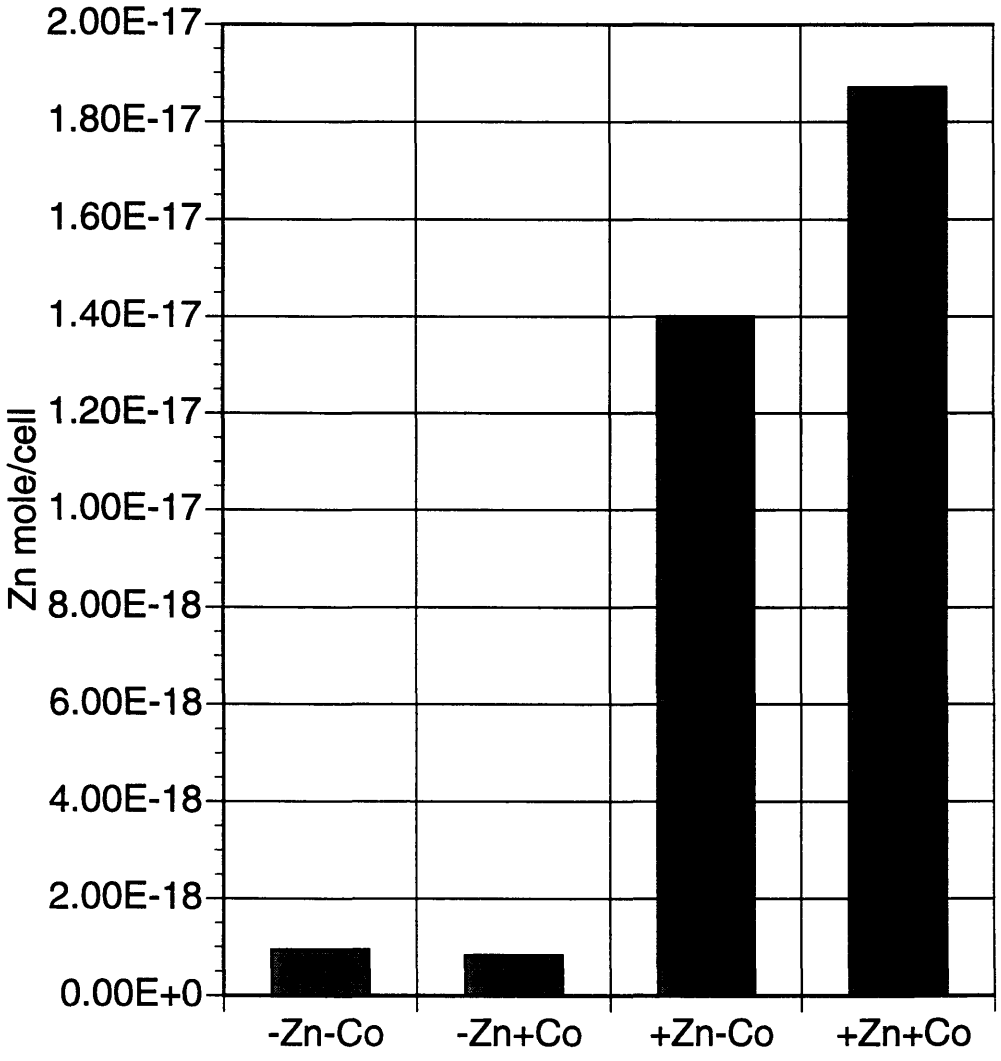


Figure 5-1b. Zinc quotas for *E. huxleyi*

+Zn = 16 pM Zn', +Co = 22 pM Co', and -Zn = 0.64 pM Zn', -Co = 0.88 pM Co'

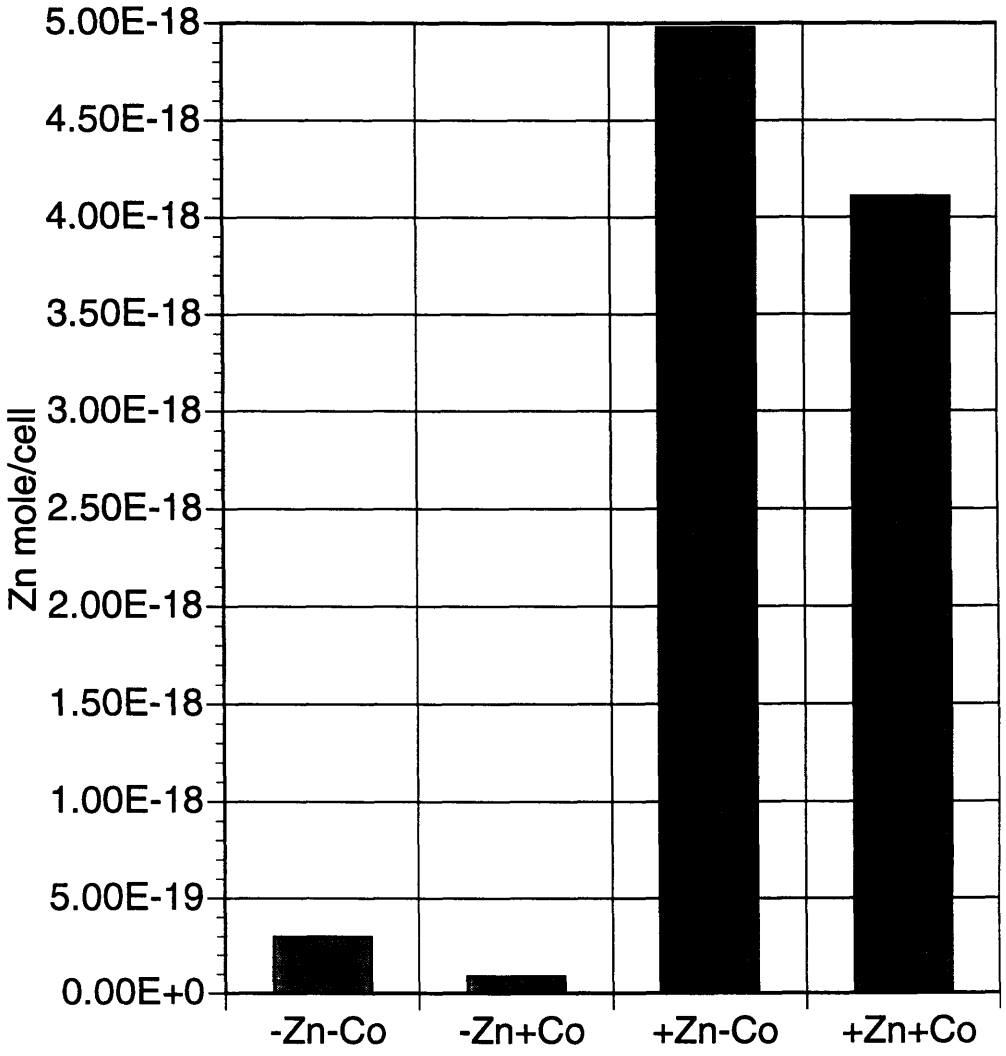


Figure 5-1c. Zinc quotas for *T. oceanica*

+Zn = 1.6 pM Zn', +Co = 2.2 pM Co', and -Zn = 0.16 pM Zn', -Co = 0.22 pM Co'

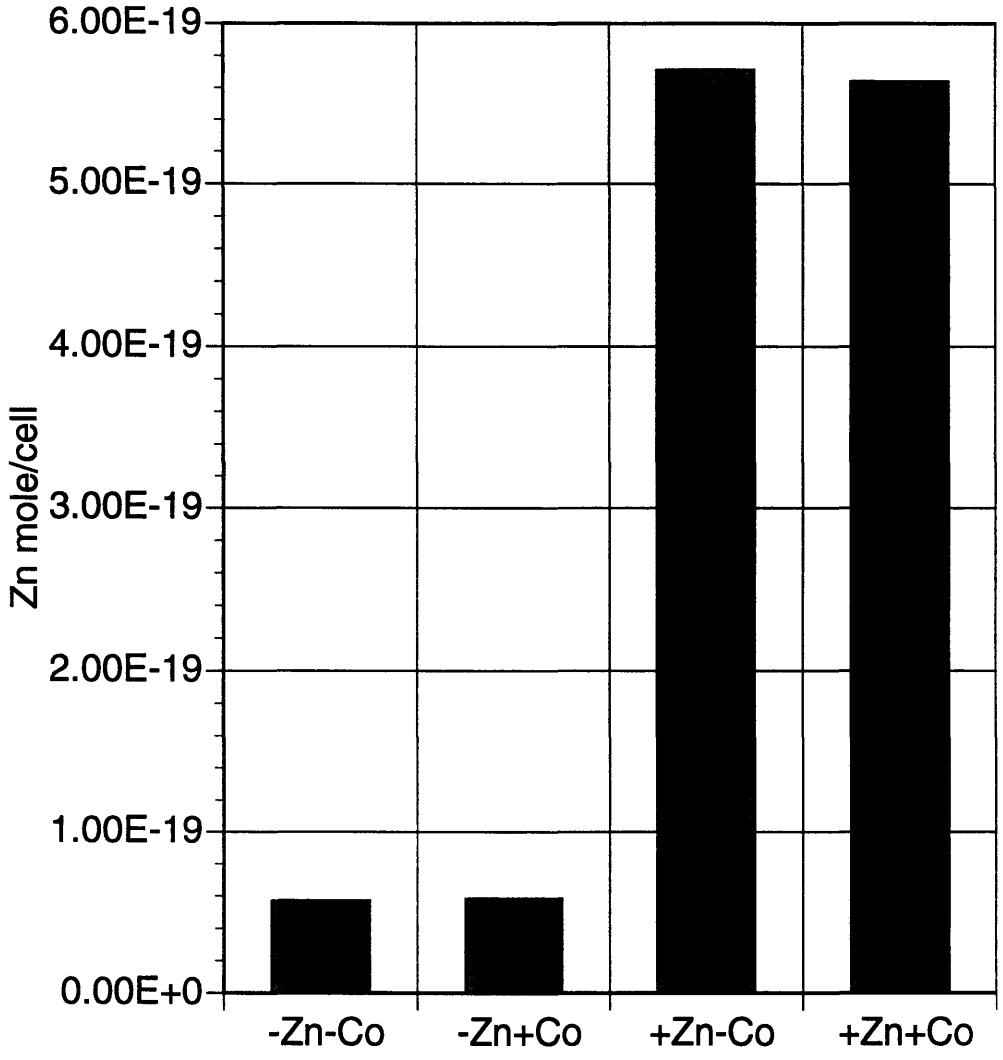


Figure 5-1d. Zinc quotas for*T. weissflogii*

+Zn = 16 pM Zn', +Co = 22 pM Co', and -Zn = 0.64 pM Zn', -Co = 0.88 pM Co'

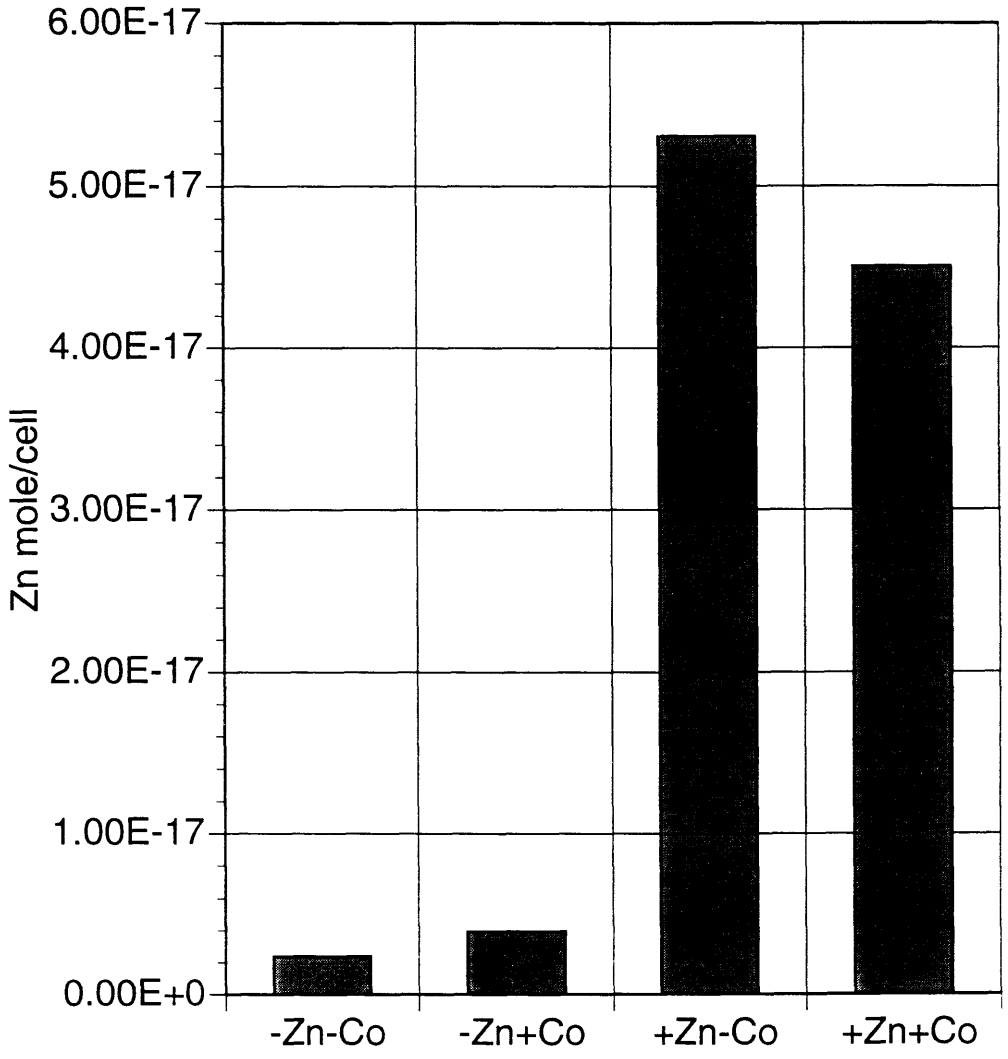


Figure 5-2a. Cobalt quotas for *P. carterae*

+Zn = 16 pM Zn', +Co = 22 pM Co', and -Zn = 0.64 pM Zn', -Co = 0.88 pM Co'

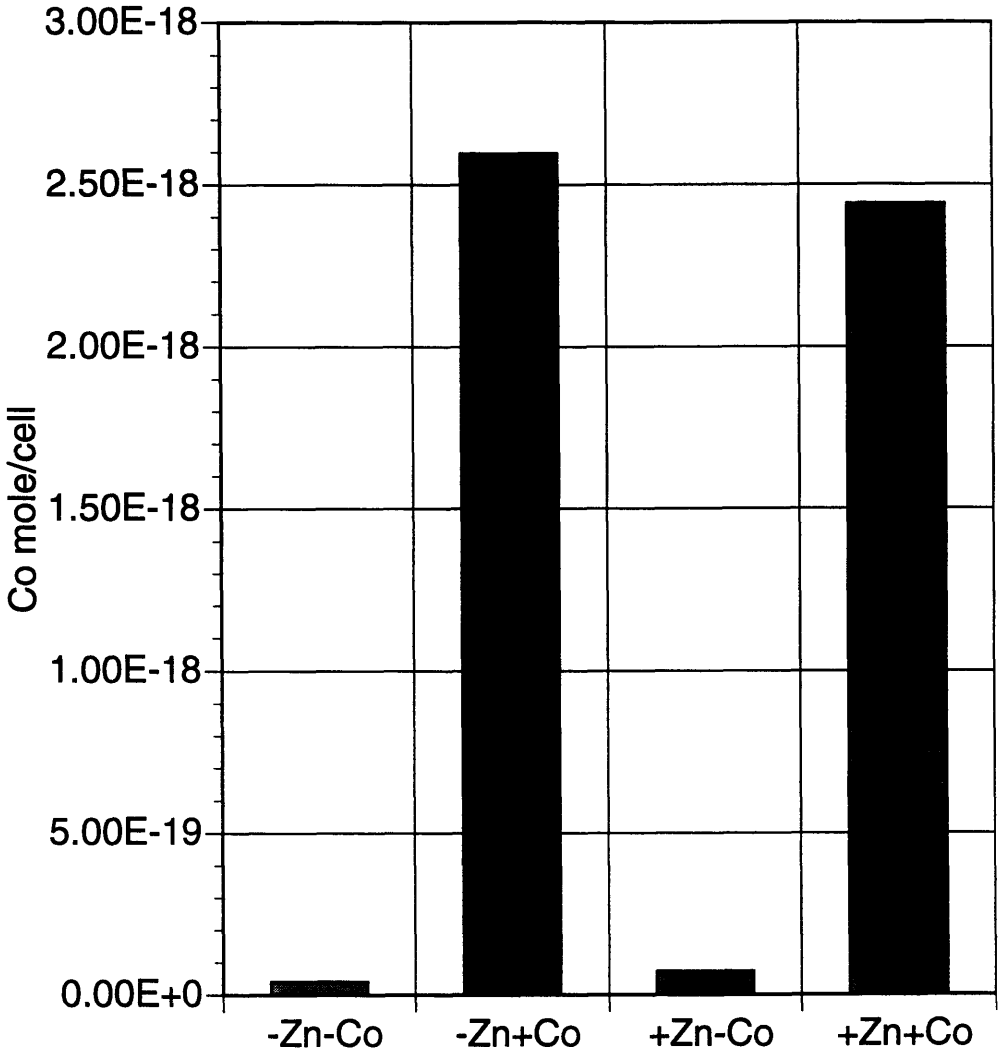


Figure 5-2b. Cobalt quotas for *E. huxleyi*

+Zn = 16 pM Zn', +Co = 22 pM Co', and -Zn = 0.64 pM Zn', -Co = 0.88 pM Co'

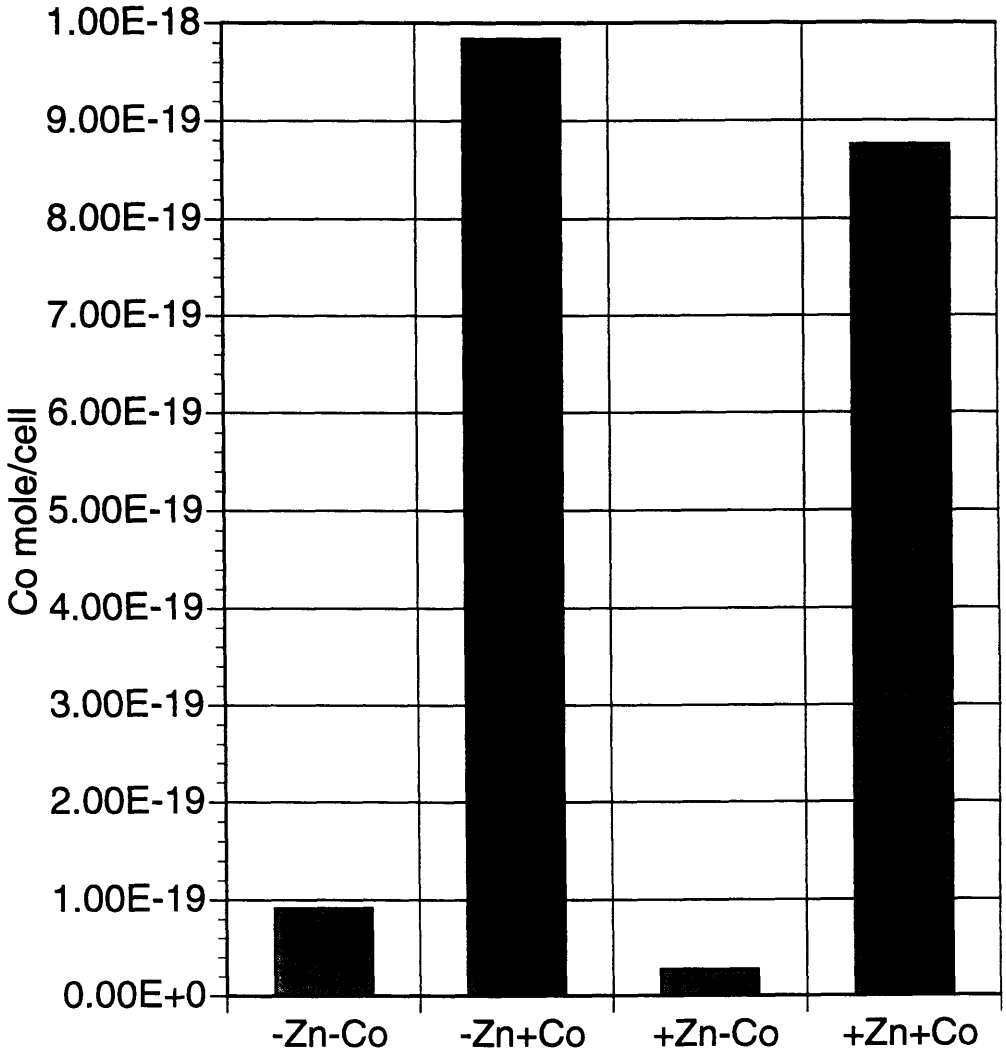


Figure 5-2c. Cobalt quotas for *T. oceanica*

+Zn = 1.6 pM Zn', +Co = 2.2 pM Co', and -Zn = 0.16 pM Zn', -Co = 0.22 pM Co'

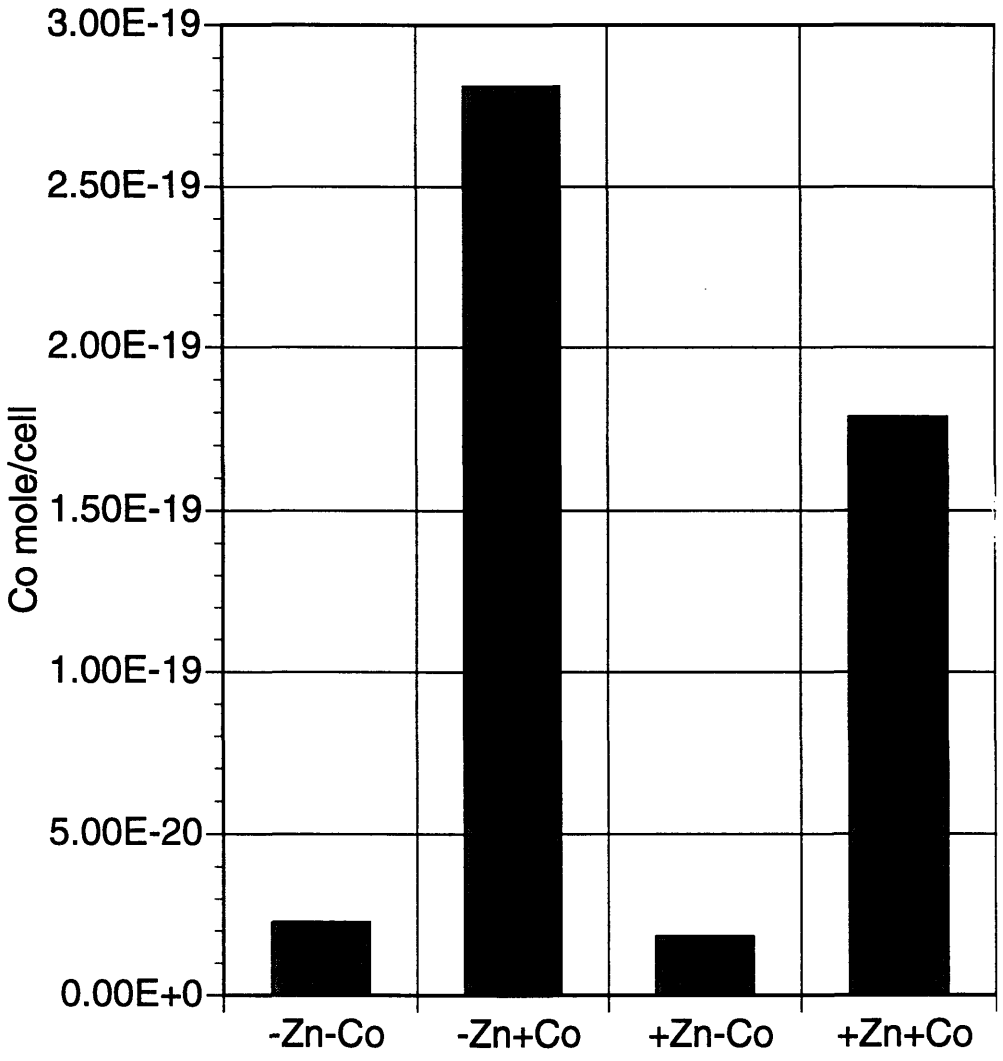


Figure 5-2d. Cobalt quotas for *T. weissflogii*

+Zn = 16 pM Zn', +Co = 22 pM Co', and -Zn = 0.64 pM Zn', -Co = 0.88 pM Co'

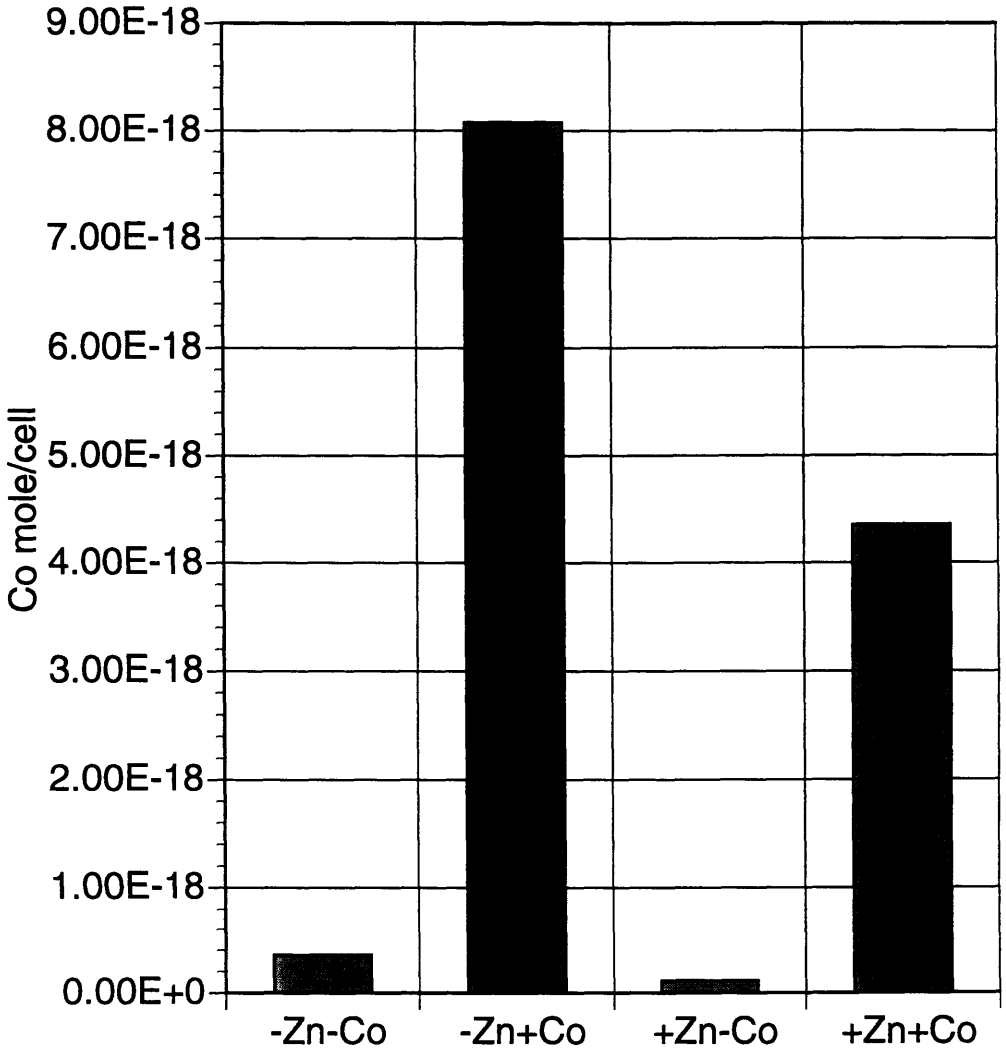


Figure 5-3a. Zinc fractionation in*P. carterae*

+Zn = 16 pM Zn', +Co = 22 pM Co', and -Zn = 0.64 pM Zn', -Co = 0.88 pM Co'

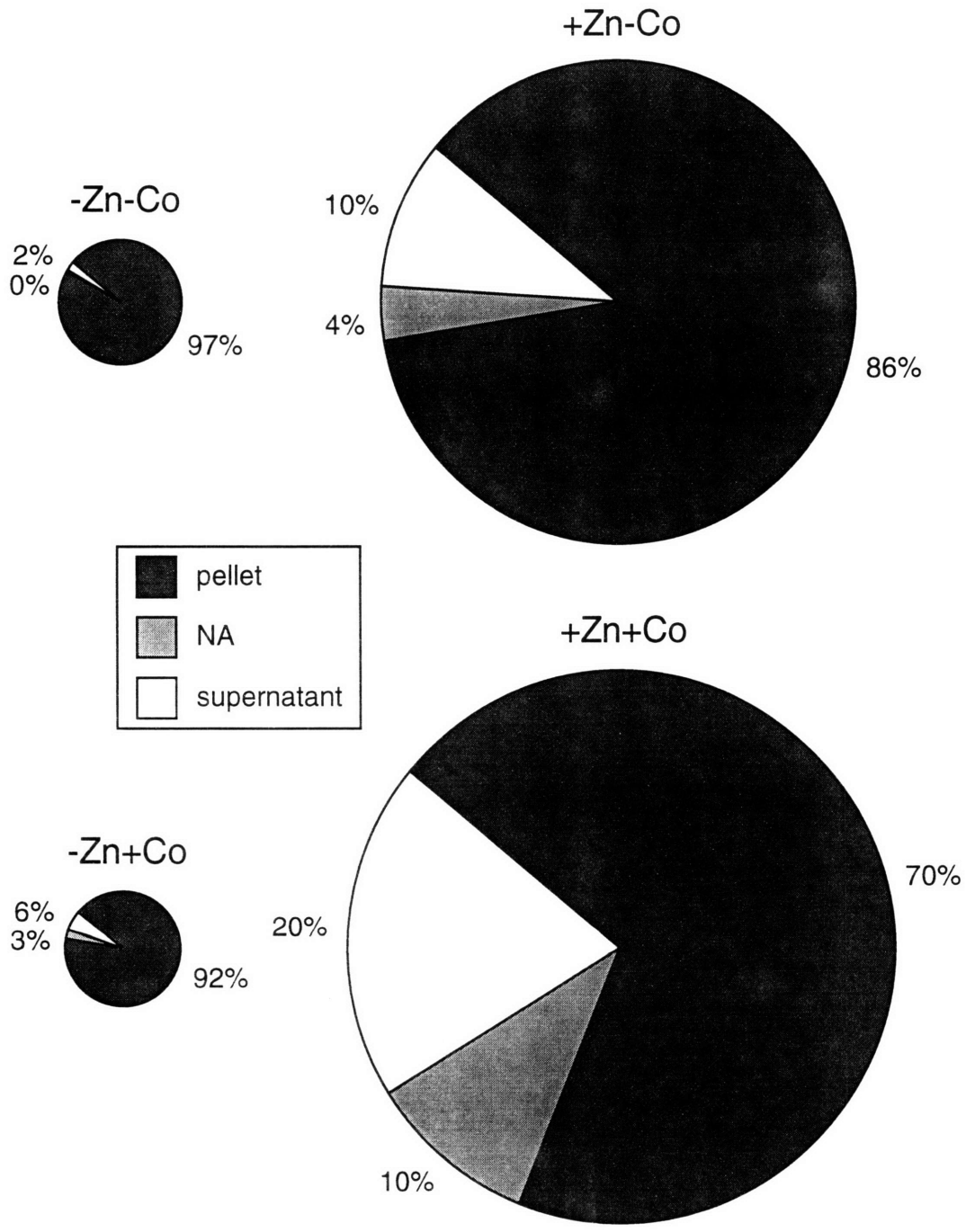


Figure 5-3b. Zinc fractionation in *E. huxleyi*

+Zn = 16 pM Zn', +Co = 22 pM Co', and -Zn = 0.64 pM Zn', -Co = 0.88 pM Co'

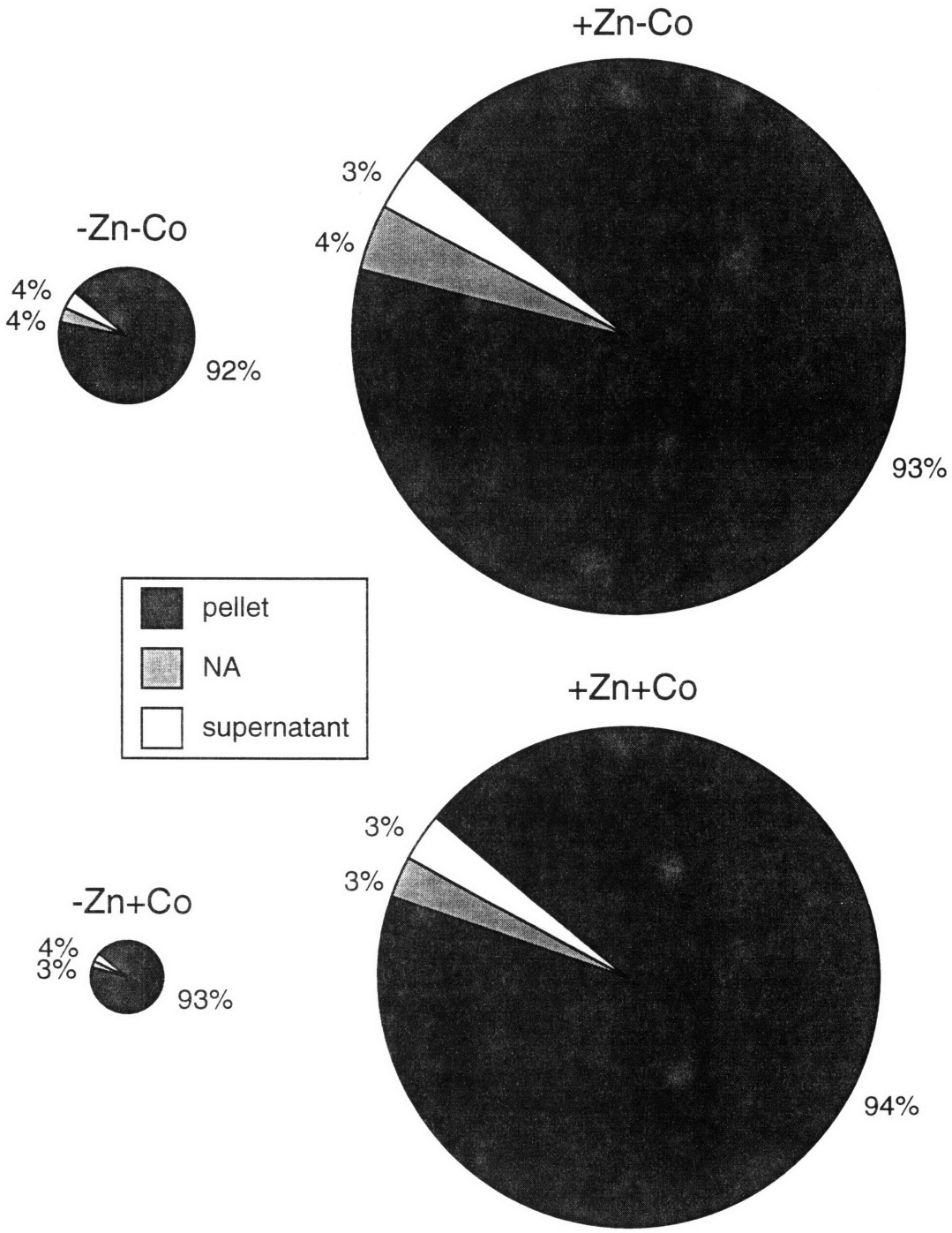


Figure 5-3c. Zinc fractionation in*T. oceanica*

+Zn = 1.6 pM Zn', +Co = 2.2 pM Co', and -Zn = 0.16 pM Zn', -Co = 0.22 pM Co'

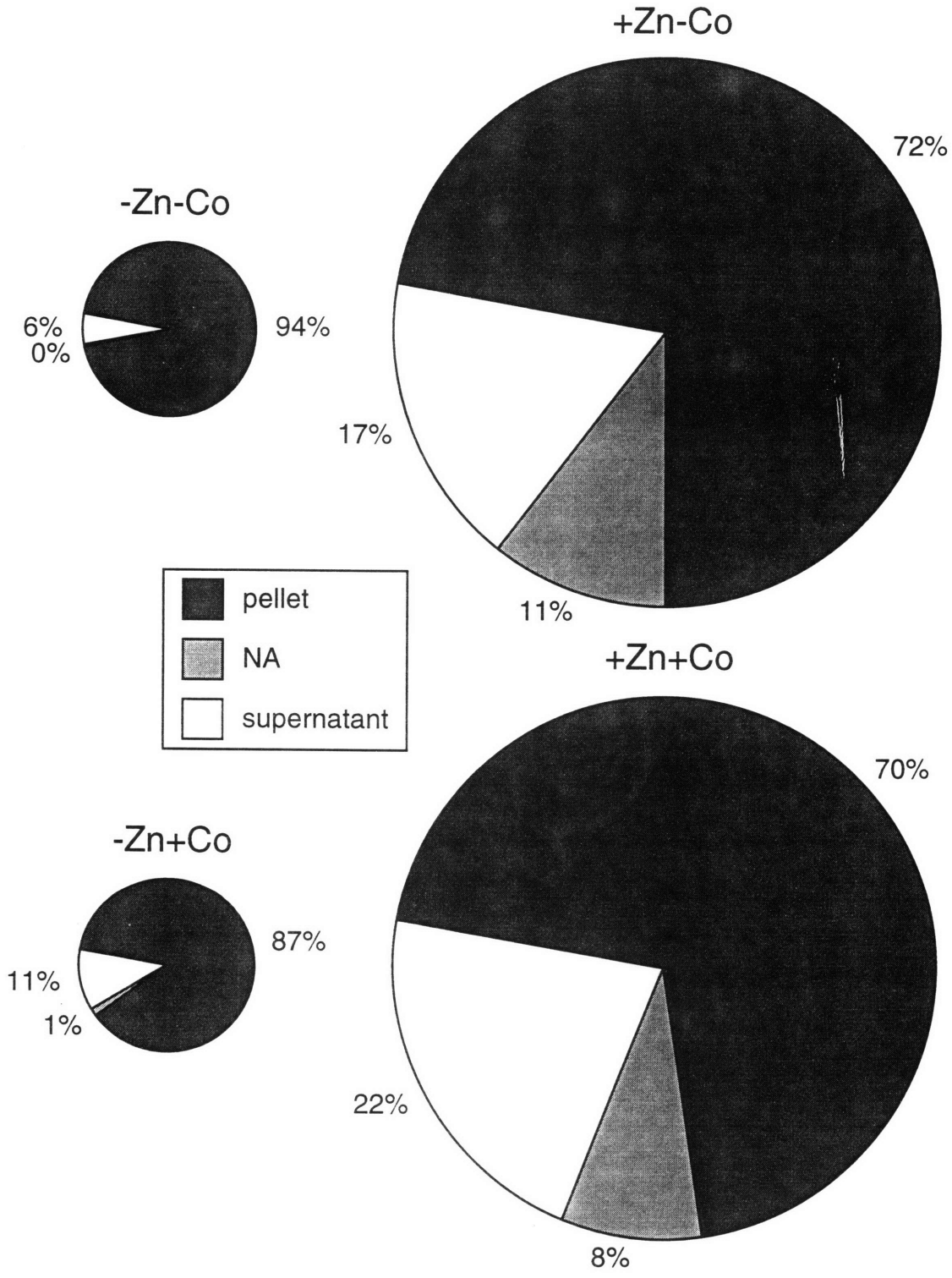


Figure 5-3d. Zinc fractionation *T. weissflogii*

+Zn = 16 pM Zn', +Co = 22 pM Co', and -Zn = 0.64 pM Zn', -Co = 0.88 pM Co'

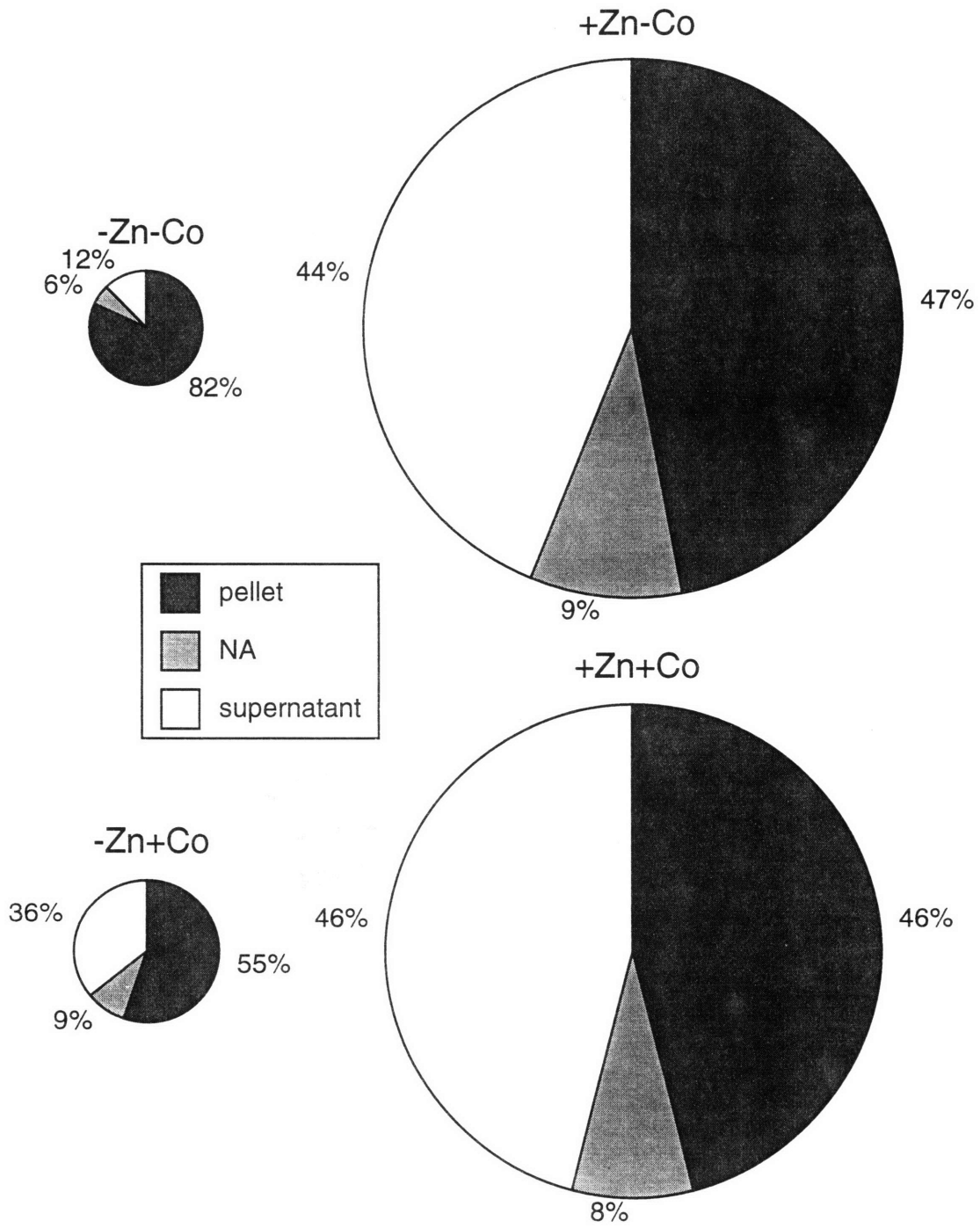


Figure 5-4a. Cobalt fractionation in *P. carterae*

+Zn = 16 pM Zn', +Co = 22 pM Co', and -Zn = 0.64 pM Zn', -Co = 0.88 pM

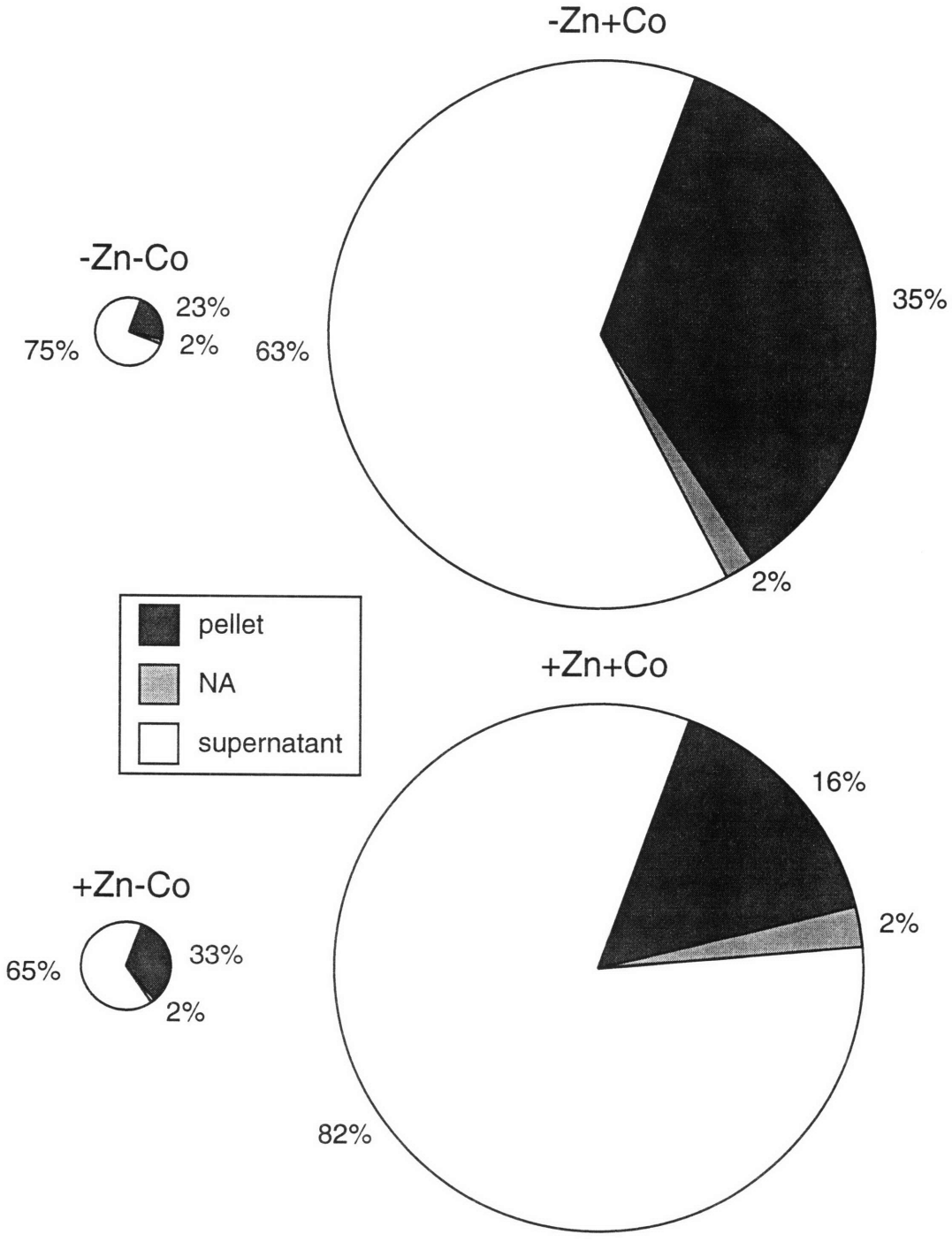


Figure 5-4b. Cobalt fractionation in *E. huxleyi*

+Zn = 16 pM Zn', +Co = 22 pM Co', and -Zn = 0.64 pM Zn', -Co = 0.88 pM Co'

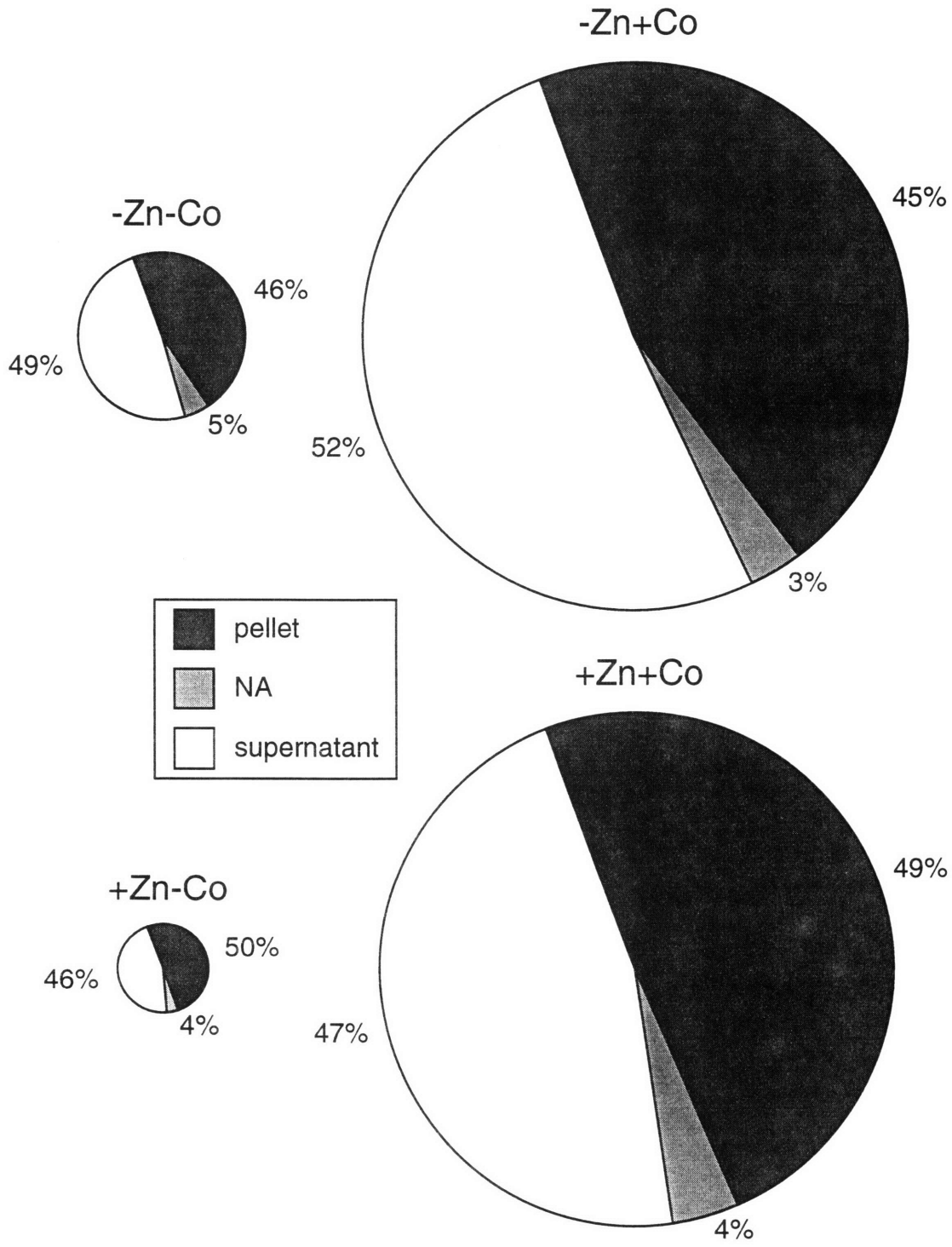


Figure 5-4c. Cobalt fractionation in *T. oceanica*

+Zn = 1.6 pM Zn', +Co = 2.2 pM Co', and -Zn = 0.16 pM Zn', -Co = 0.22 pM Co'

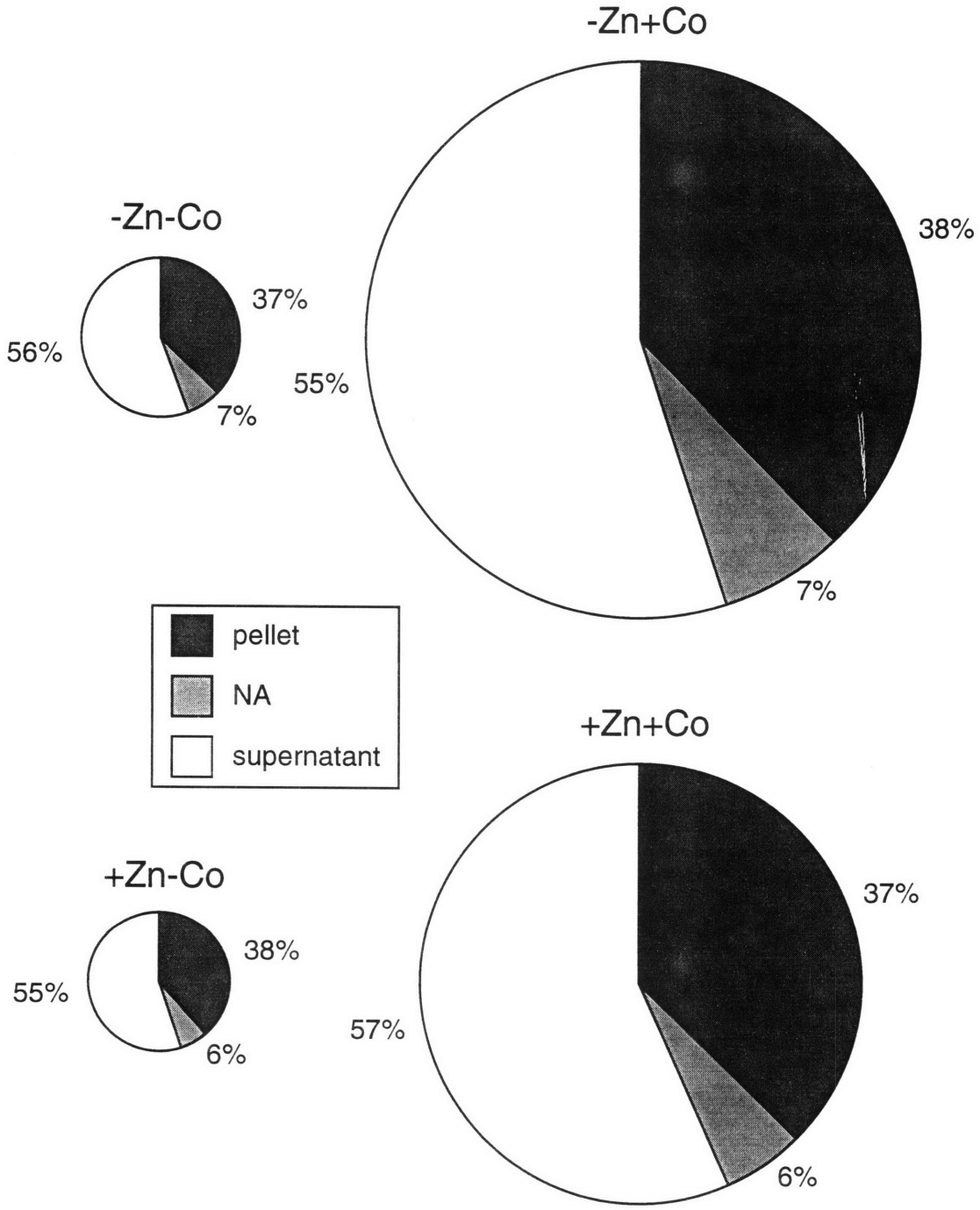


Figure 5-4d. Cobalt fractionation in *T. weissflogii*

+Zn = 16 pM Zn', +Co = 22 pM Co', and -Zn = 0.64 pM Zn', -Co = 0.88 pM Co'

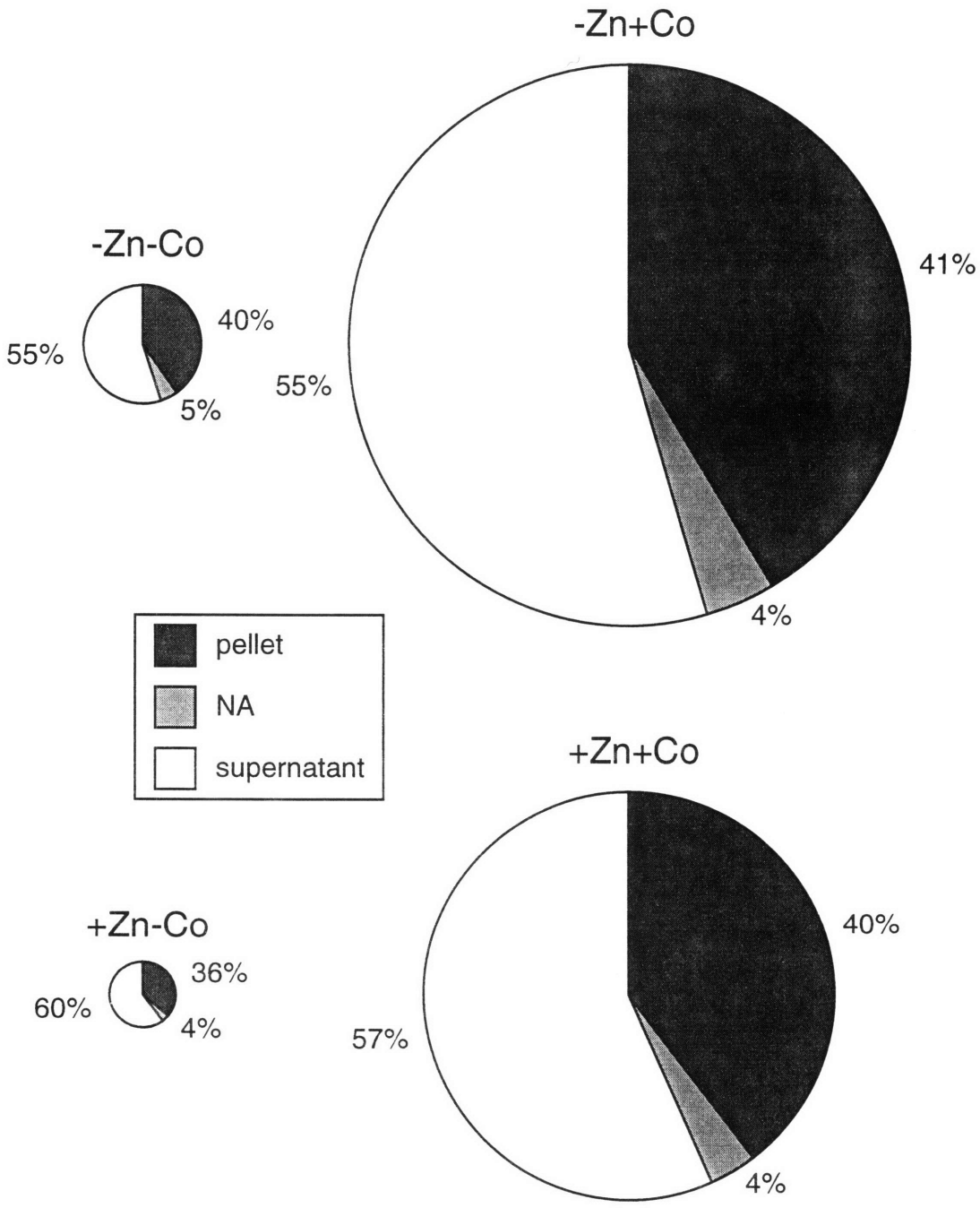
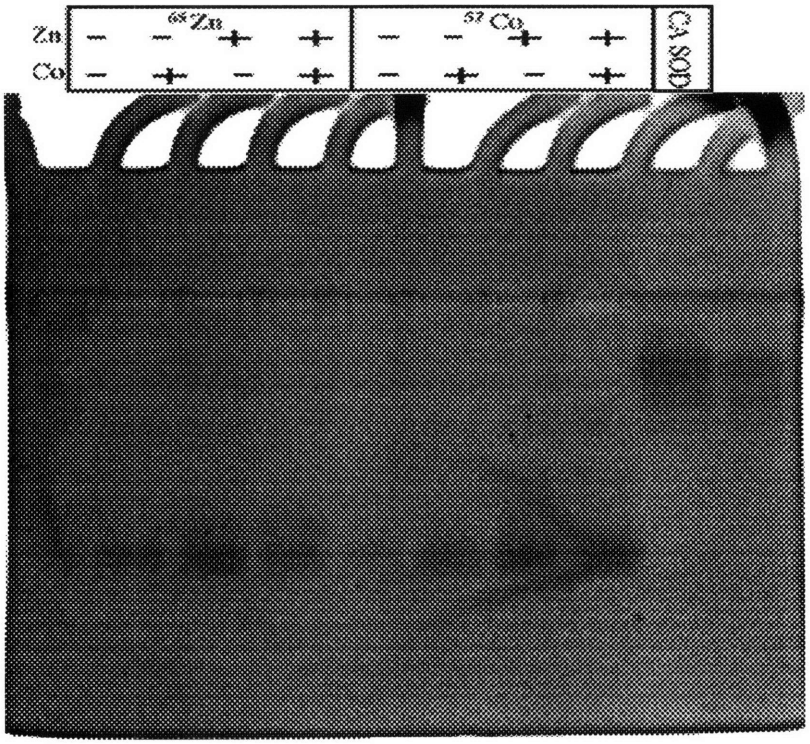
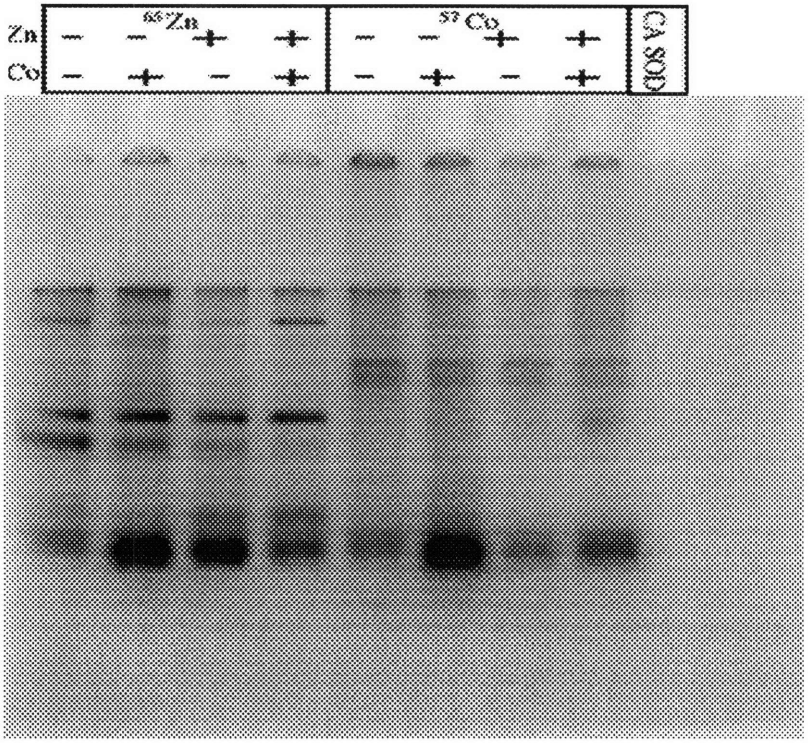


Figure 5-5a. CA activity in *T. weissflogii*



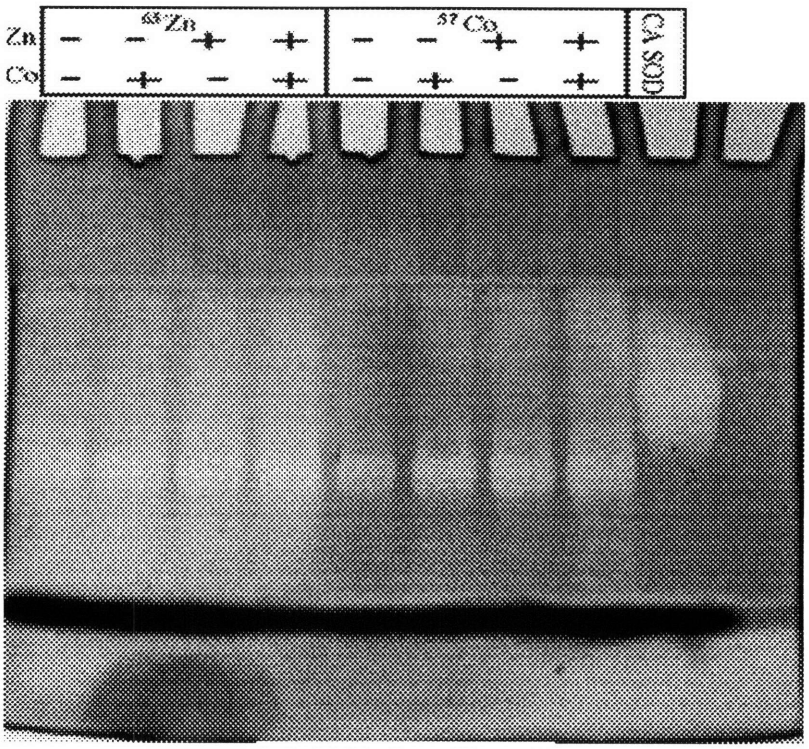
T. Weissflogii

Figure 5-5b. ^{65}Zn and ^{57}Co activity in *T. weissflogii*, CA gel



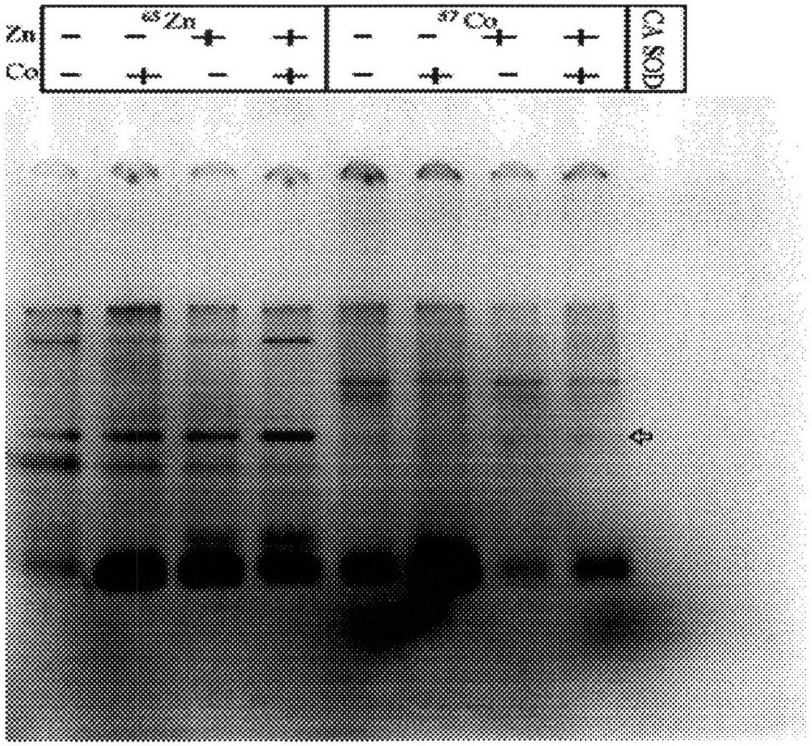
T. Weissflogii

Figure 5-6a. SOD activity in *T. weissflogii*



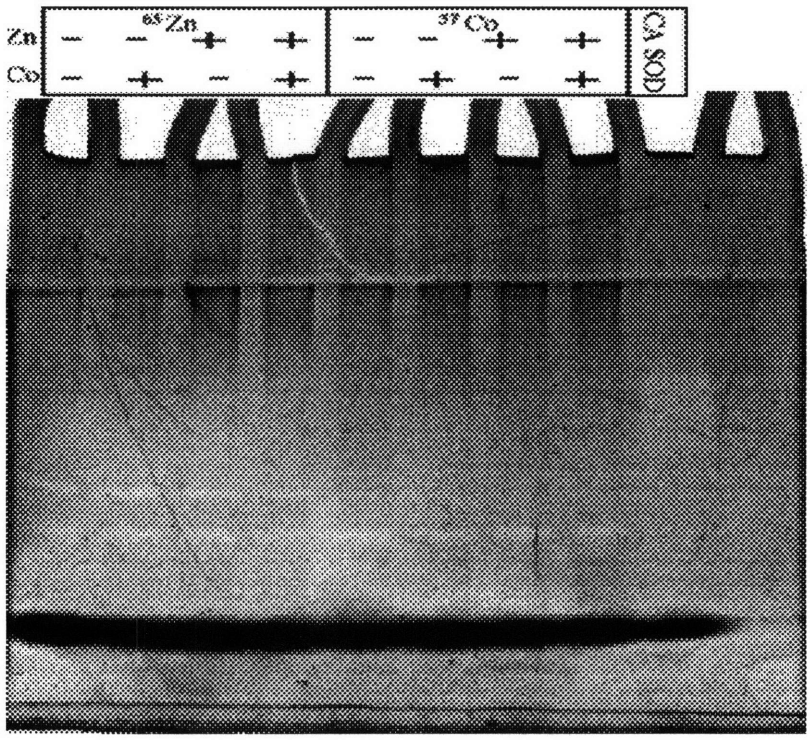
T. Weissflogii

Figure 5-6b. ^{65}Zn and ^{57}Co activity in *T. weissflogii*, SOD gel arrow indicates location of SOD activity



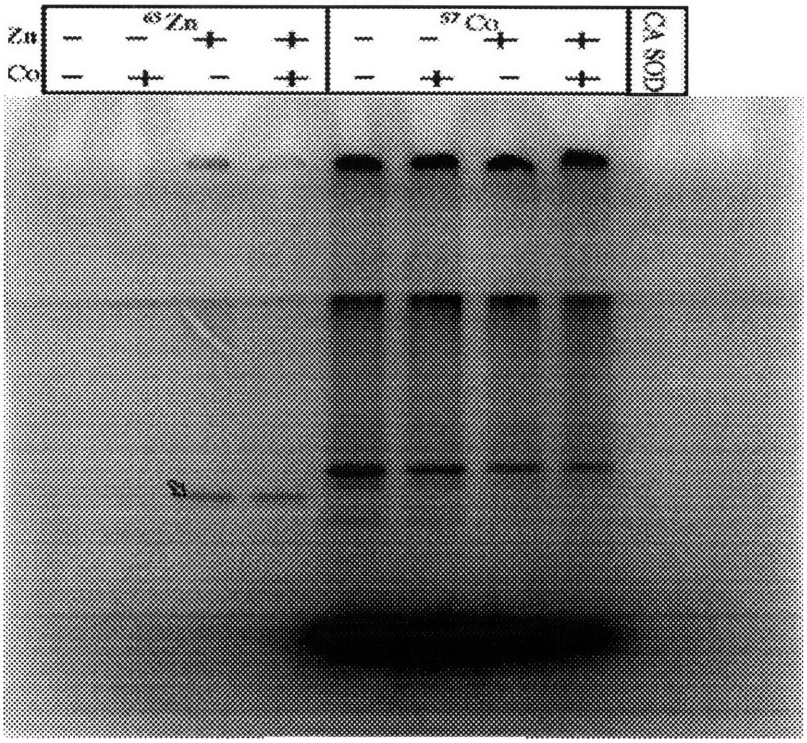
T. Weissflogii

Figure 5-7a. SOD activity in *T. oceanica*



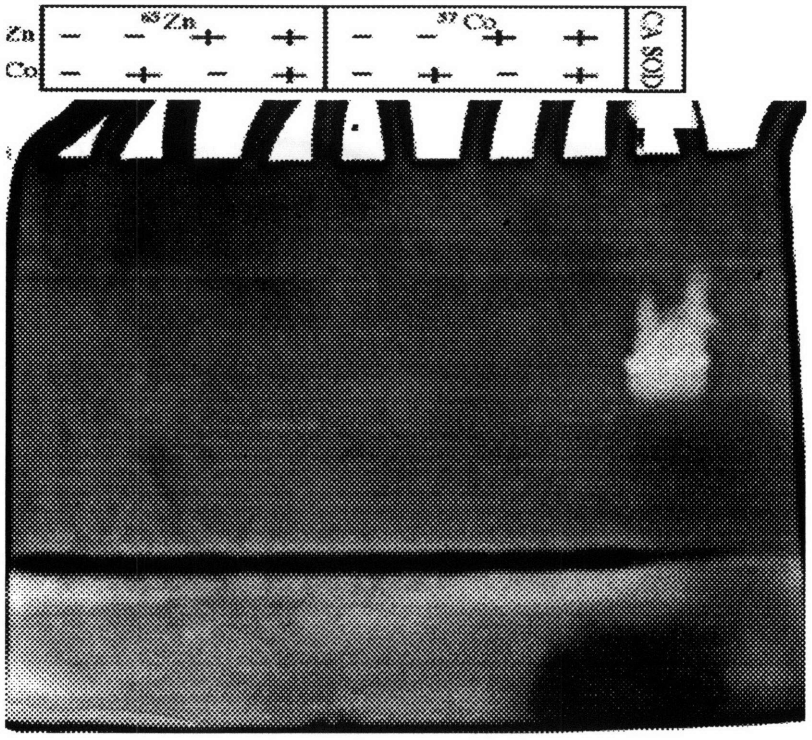
T. Oceanica

Figure 5-7b. ^{65}Zn and ^{57}Co activity in *T. oceanica*, SOD gel arrow indicates location of SOD activity



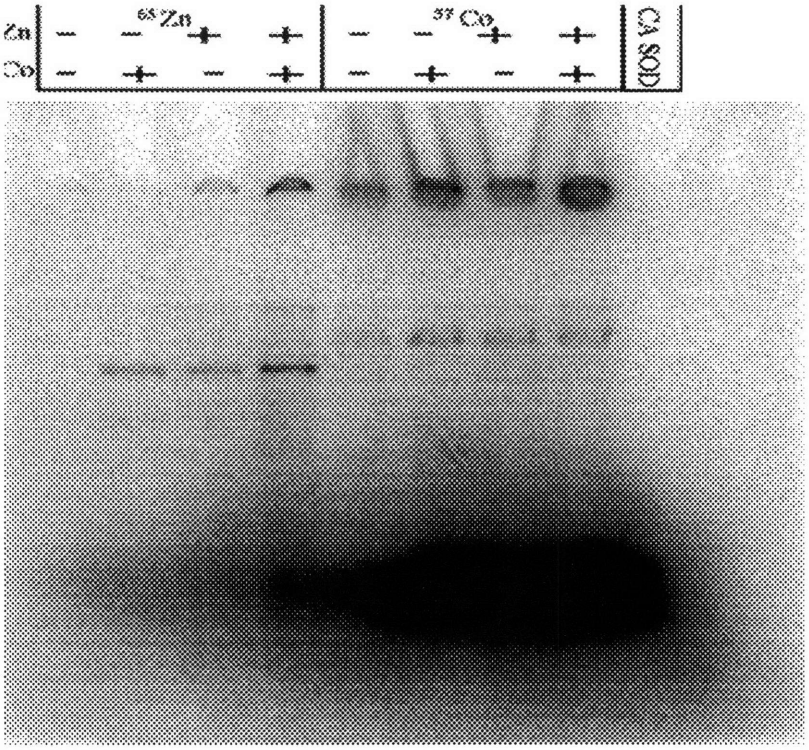
T. Oceanica

Figure 5-8a. SOD activity in *P. carterae*



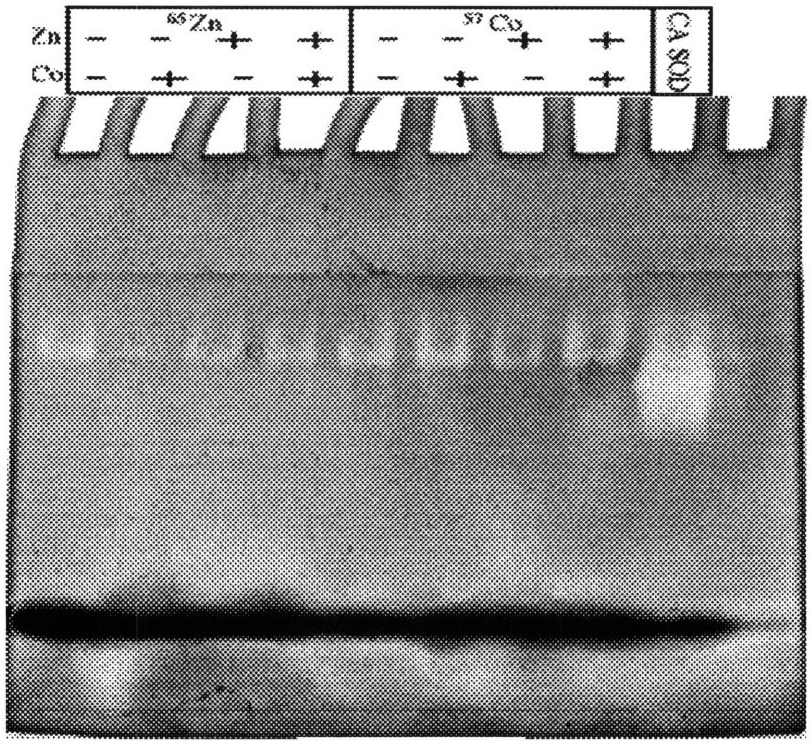
P. Carterae

Figure 5-8b. ^{65}Zn and ^{57}Co activity in *P. carterae*, SOD gel



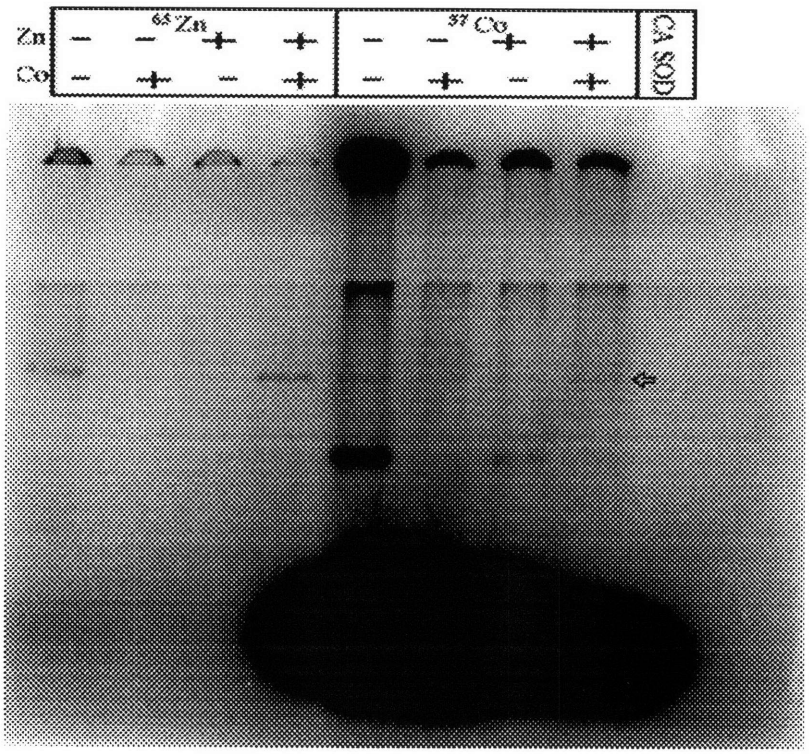
P. Carterae

Figure 5-9a. SOD activity in *E. huxleyi*



E. Huxleyi

Figure 5-9b. ^{65}Zn and ^{57}Co activity in *E. huxleyi*, SOD gel arrow indicates location of SOD activity



E. Huxleyi

Chapter 6. Conclusions and Future Work

In agreement with previous work (1, 2), we find substitution of cobalt for zinc in a range of marine phytoplankton species when available zinc is low. Despite some differences in results, concentrations of zinc that are found to limit growth of phytoplankton are quite similar for different laboratories, even using different media and growth conditions. The ranges of zinc concentrations that cause growth limitation of coastal and oceanic phytoplankton in laboratory cultures (less than 6 and 2 pM Zn', respectively) also coincide with ranges of coastal and oceanic measurements for inorganic zinc (3, 4). With few exceptions, oceanic species in cultures can survive with much less zinc in their growth medium, much as they do in their natural environment.

Evolutionary adaptations to environmental conditions are also reflected in the abilities of coastal and oceanic phytoplankton to substitute cobalt for zinc. Neritic species, from areas where zinc is seldom limiting, grow poorly in cultures with only cobalt added to the medium. In contrast, oceanic species grow at nearly their maximum rates in low-zinc medium with only cobalt added, conditions that parallel the relative enrichment of cobalt in some oceanic waters. These macroscopic effects on growth are also apparent in accumulation of zinc and cobalt by different species; the coastal species have a strong preference for zinc over cobalt and accumulate more zinc, whereas the oceanic species are less selective, especially when zinc concentrations are very low. These preferences do not appear to follow phylogenetic patterns.

When an essential metal is present at low concentrations, other metals are often accumulated by phytoplankton, even when these metals have a negative effect on growth (5, 6, 7, 8, 9, 10). This is not the case here; without fail, increased accumulation of cobalt in zinc-limited phytoplankton increases growth. Improved growth with increased cobalt indicates a metabolic role for cobalt, and this role is mainly in substitution for zinc, because cobalt improves growth primarily when zinc is low.

Cobalt has been shown to substitute for zinc in vivo and in vitro in enzymes of organisms such as mammals and bacteria (11, 12, 13), but the mechanisms of cobalt substitution in phytoplankton were not known. Because addition of cobalt to zinc-limited cultures increases CA expression in *T. weissflogii* (14), substitution of cobalt for zinc in this enzyme was suspected. This mechanism is shown here to be direct substitution in the native zinc-containing CA. Cellular zinc distributions in other species reveal that cobalt substitution in a soluble CA (such as is found in *T. weissflogii*) is perhaps atypical for marine phytoplankton; most other species have more than half their total zinc in insoluble cellular material, i.e. membranes.

One of these zinc functions in membranes may still be CA however, because CA activity has been found in organelles and cell membranes in some phytoplankton (15, 16, 17, 18). Our results point to such a role; even in species of phytoplankton with no soluble CA, zinc-limited cultures appear to benefit from increasing CO_2. Adding cobalt to the medium of zinc-limited phytoplankton cultures displaces zinc and increases the amount of cobalt in the insoluble fraction of cells, and a membrane-bound CA might be one protein in which this replacement occurs.

A role for cobalt as a substitute for zinc in CA is also supported by the field data. Figures 1-2 and 3-4 show that in low zinc regions, low surface cobalt concentrations (normalized to salinity) are correlated to high temperature, and thus to low (calculated) equilibrium dissolved CO_2 concentrations in the ocean. Carbonic anhydrase production is induced in laboratory cultures over this range of CO_2 concentrations, and increased demand for CA increases requirements for zinc. Under conditions of low zinc, part of this CA demand might be satisfied by cobalt. Lower surface cobalt concentrations in areas where both low zinc and high temperature exist together thus fortify the argument for zinc-cobalt-carbon co-limitation of phytoplankton (14). Although high temperature also causes stratification, which decreases mixing of surface waters with deeper waters (with higher cobalt concentrations), normalizing cobalt concentrations to salinity accounts for this effect.

Our evidence for the existence of CA in phytoplankton other than *T. weissflogii* is indirect. Confirming this hypothesis requires that the presence of CA in other marine phytoplankton in cultures be established directly. By applying methods used by others to find CA in other cellular components (15, 16, 17, 18) we might find insoluble forms of CA in species which do not have a soluble CA. The location of CA in membranes and organelles of other phytoplankton would explain the partitioning of zinc that we find in many species in culture, and help solidify the hypothesis that this enzyme constitutes a major portion of the zinc requirement in many algae. Future work extracting these forms of CA from the membrane is necessary to demonstrate that these are zinc enzymes and that cobalt substitutes for the zinc in these enzymes in low zinc cultures. The same methods used on laboratory cultures might be used to find CA in phytoplankton in the field.

Another biochemical question remaining that needs to be addressed is the identity of the low molecular weight cobalt-containing compound(s) found in most of the species. This compound can be separated from other soluble cellular components (e.g. via HPLC or PAGE designed to separate smaller compounds). The mobility of this compound using these methods can be compared to B_{12} and phytochelatin, and more specific methods such as derivatization and HPLC separation (used in measuring phytochelatin) might also be applied.

The preferential incorporation of zinc over cobalt into the nucleic acid (NA) fraction also needs to be confirmed. Methods to separate polymerases and other bound proteins from precipitated nucleic acids, followed by separations of these proteins by PAGE or other methods, might be used to determine whether zinc and cobalt are found in the same NA-associated proteins. Amino acid sequencing and immunoassays (Western blots) might then be applied to find if there are homologies in these proteins to polymerases and zinc fingers or other known nucleic acid-binding proteins.

The hypothesis that there is zinc-cobalt-carbon co-limitation of marine phytoplankton needs to be tested in the field. Enrichment experiments with zinc have been attempted previously, but

showed little effect. In one experiment (19), the phytoplankton were perhaps not zinc-limited because of a moderately high initial zinc concentration of 0.3 nM total Zn (6 pM Zn' assuming the same degree of complexation as in Bruland (3)). In another experiment (20), the high initial fugacity of CO_2 (500 µatm in the water) meant that phytoplankton would perhaps not require CA for maximum growth. In warm open ocean surface waters away from upwelling regions, there should exist a combination of low dissolved zinc and low dissolved CO_2 conditions, where zinc-limited phytoplankton will utilize cobalt. Improved growth of natural phytoplankton assemblages from these regions with additions of either zinc, cobalt, or carbon dioxide individually would demonstrate that this putative limitation really occurs. Co-limitation would be shown if adding any one of these nutrients decreases the requirement for the others (e.g. addition of zinc or cobalt causes a decreased effect of CO_2 on growth).

In situ oceanic additions of metals are difficult at best, and directly changing CO_2 in situ is practically impossible, so such experiments would need to be on-board incubations of natural phytoplankton assemblages. Metals can be added to bottle incubations, and high or low CO_2 conditions can be created by aeration with tank gases. At the 59 °N (20 °W) and T-7 (50 °N 145 °W) stations in the studies of Martin et al. in the North Pacific and North Atlantic for example, surface concentrations of phosphate and nitrate were not totally depleted (0.5 and 0.7 µM phosphate, and 6.1 and 7.0 µM nitrate respectively), but zinc was low (0.25 and 0.07 nM dissolved Zn) and cobalt only slightly depleted (~50% of its concentration at depth, 43 and 28 pM, respectively). These waters are at low surface temperatures (10.8 and 10.1 °C respectively) and thus might not be limited in carbon. Incubations of such waters at low P_{CO2} would demonstrate whether zinc would become depleted by additional demand for CA, and whether cobalt would also become depleted as a result. Assays for CA activity compared to controls (incubations with atmospheric CO_2 levels) could be used to compare the expression of CA in phytoplankton under these different conditions.

Even if CA is not a primary function for zinc and cobalt in most phytoplankters, bottle experiments can provide direct evidence of cobalt substituting for zinc in natural phytoplankton assemblages in the field. On-board cobalt enrichments of water samples from regions with low surface zinc concentrations might illustrate this substitution occurring in natural populations. Comparisons to growth in controls (with no metals added) and zinc-enriched incubations would show whether the high degree of cobalt substitution seen in laboratory cultures of oceanic species holds true in the field. Furthermore, changes in species distributions with different treatments in bottle experiments might also provide evidence of whether there are significant phylogenetic differences in cobalt substitution and zinc limitation of natural phytoplankton populations.

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Appendix

Figure A2-1. Growth curves for *T. weissflogii*, grown in Aquil media with co-varying amounts of zinc and cobalt added to each. Within each graph for a given Co, the symbols represent different Zn additions as follows:

(■) Zn (●) Zn/5 (▲) Zn/25 (◆) no Zn

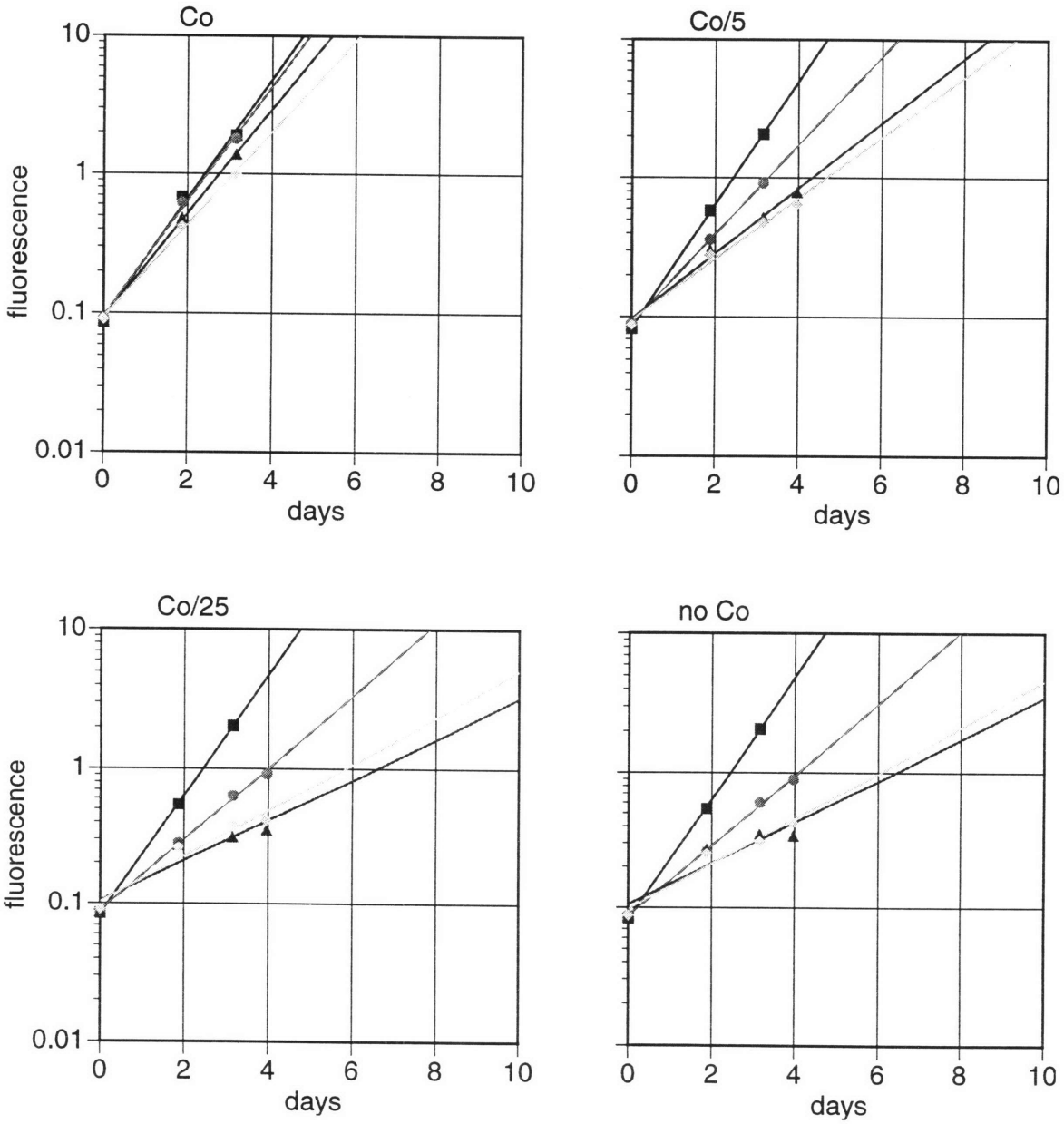


Figure A2-2. Growth curves for *P. carterae*, grown in Aquil media with co-varying amounts of zinc and cobalt added to each. Within each graph for a given Co, the symbols represent different Zn additions as follows:

(■) Zn (●) Zn/5 (▲) Zn/25 (◆) no Zn

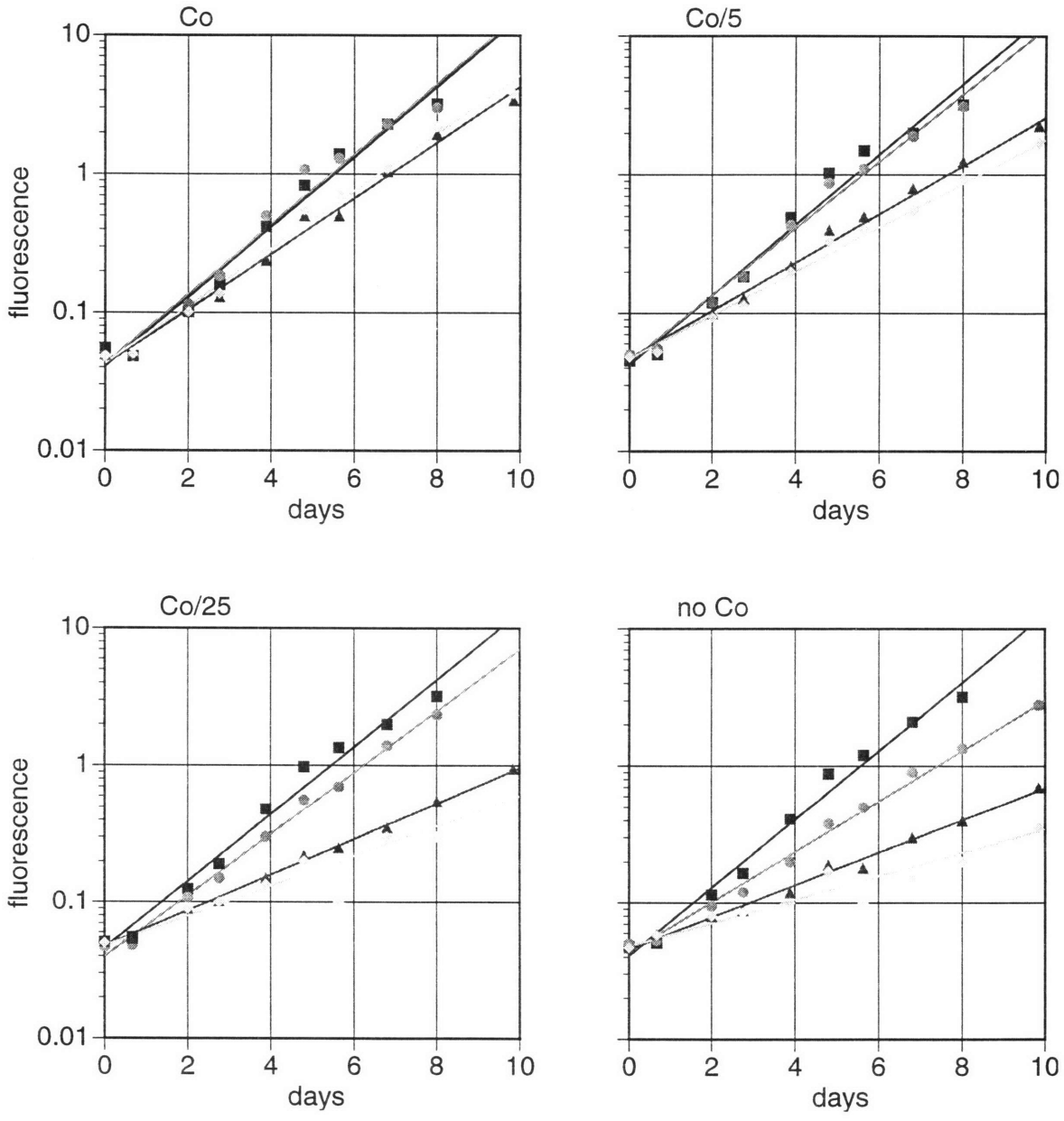


Figure A2-3. Growth curves for T. oceanica, grown in Aquil media with co-varying amounts of zinc and cobalt added to each. Within each graph for a given Co, the symbols represent different Zn additions as follows:

(■) Zn (●) Zn/5 (▲) Zn/25 (◆) no Zn

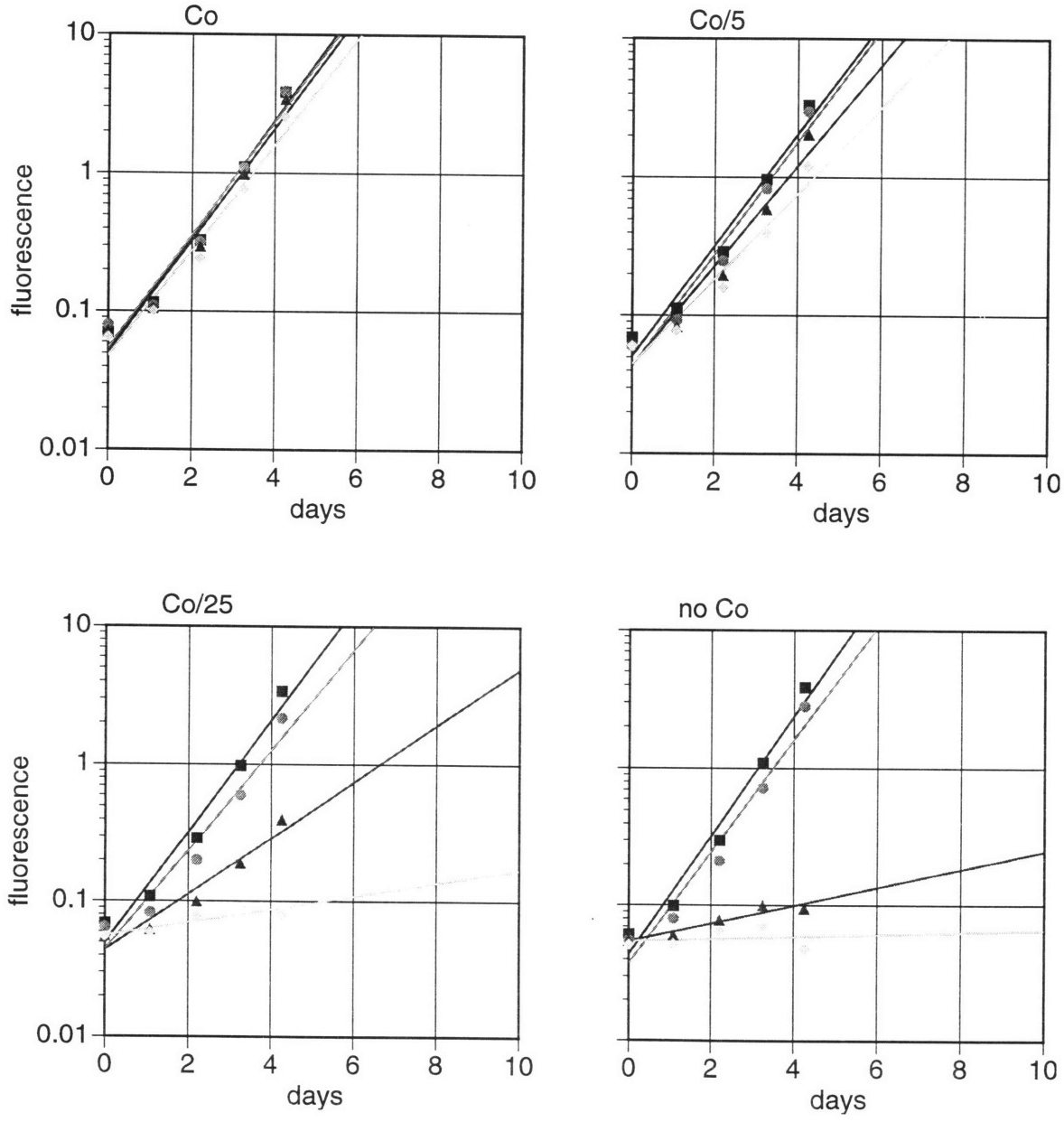


Figure A2-4. Growth curves for E. huxleyi BT6, grown in Aquil media with co-varying amounts of zinc and cobalt added to each. Within each graph for a given Co, the symbols represent different Zn additions as follows:

(■) Zn (●) Zn/5 (▲) Zn/25 (◆) no Zn

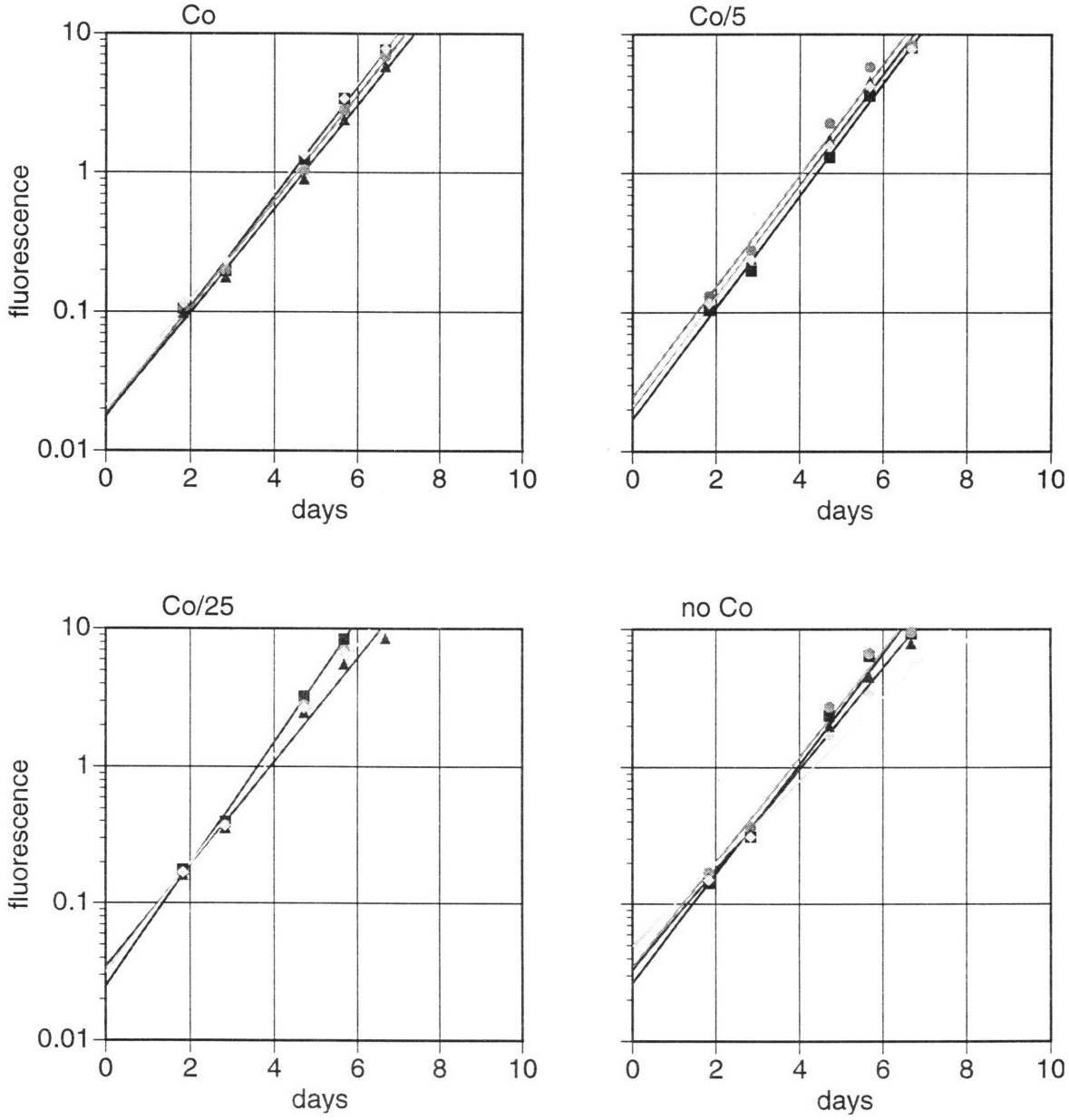


Figure A2-5. Growth curves for E. huxleyi CCMP1516, grown in Aquil media with co-varying amounts of zinc and cobalt added to each. Within each graph for a given Co, the symbols represent different Zn additions as follows:

(■) Zn (●) Zn/5 (▲) Zn/25 (◆) no Zn

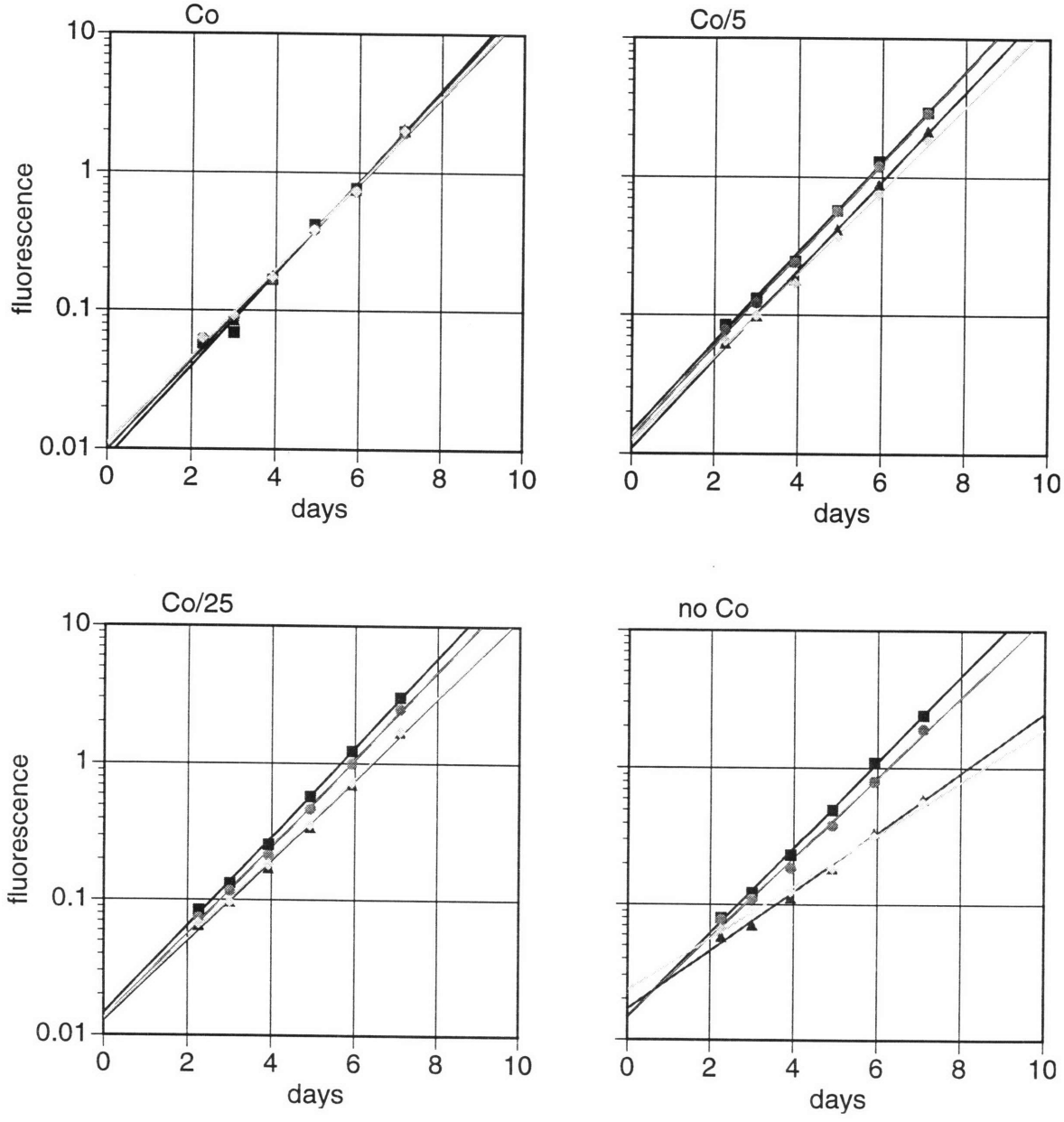
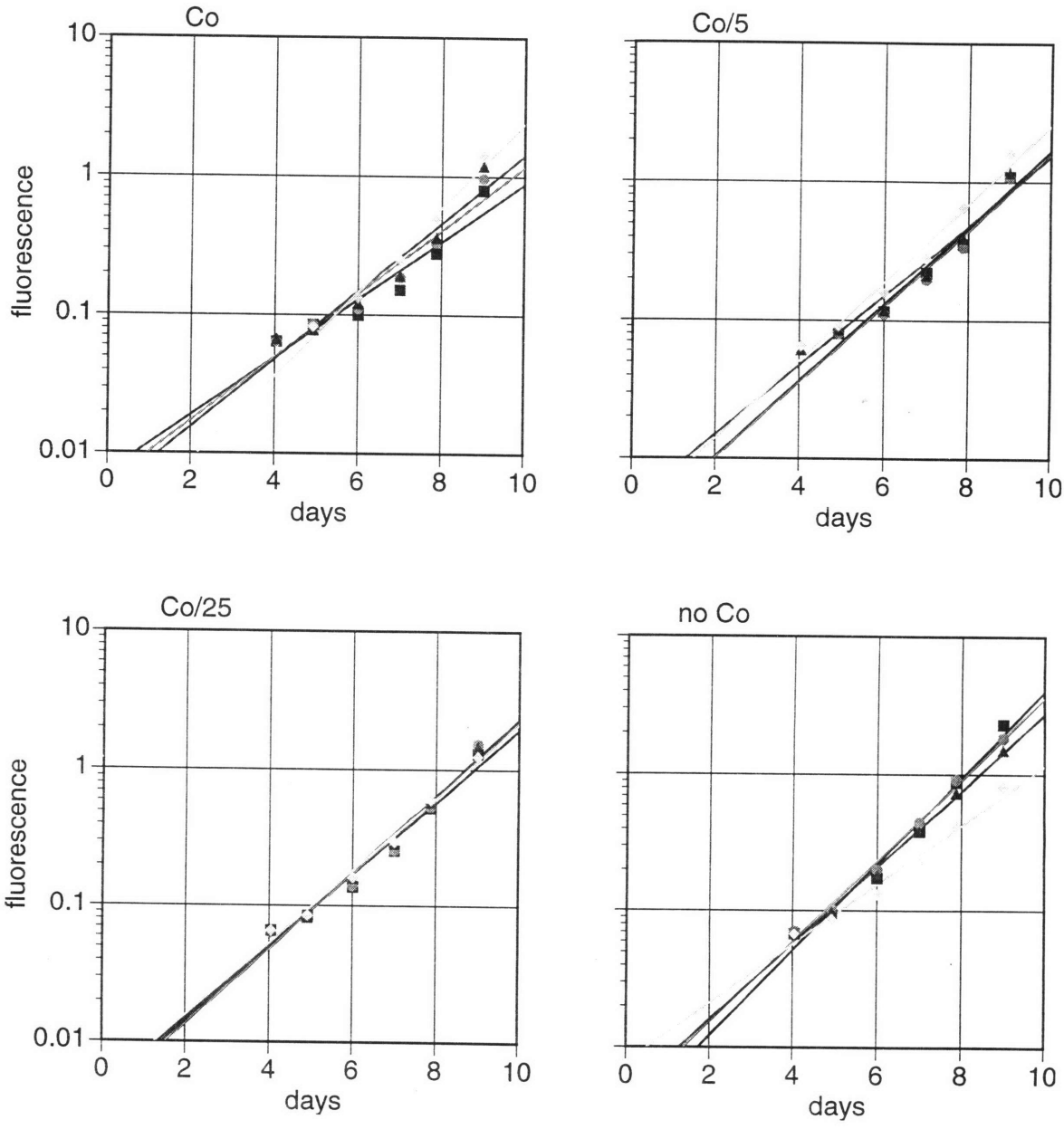


Figure A2-6. Growth curves for T. subtilus 50AiT, grown in Aquil media with co-varying amounts of zinc and cobalt added to each. Within each graph for a given Co, the symbols represent different Zn additions as follows:

(■) Zn (●) Zn/5 (▲) Zn/25 (◆) no Zn



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