

Zinc, cadmium, and cobalt interreplacement and relative use efficiencies in the coccolithophore *Emiliana huxleyi*

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Abstract

We investigate the interreplacement of Zn, Cd, and Co in the cosmopolitan coccolithophore *Emiliana huxleyi* by examining its growth rate, cellular metal quotas, and cellular metal uptake rates under different combinations of Zn, Cd, and Co concentrations in the medium. Co and Zn can fully replace each other, except perhaps for small individual requirements that are met by minute metal concentrations supplied as contaminants. Zn is used at 75% efficiency by *E. huxleyi* compared with Co. In contrast, Cd can only partially replace Zn or Co and is used at 66% efficiency compared with Co. Up to 50% of the cellular Zn or Co can be replaced by Cd, once the minimum cellular Zn or Co requirement is fulfilled. The limitation by Zn, Cd, and Co and the interreplacement of the three metals occurs over a range of unchelated metal concentrations, 0.2–5 pmol L⁻¹, that is relevant to surface oceanic waters. Based on our laboratory results and the published metal concentrations in surface oceanic waters, we calculate that interreplacement of Zn, Cd, and Co should occur in regions where Zn is highly depleted.

Zinc serves as a cofactor for hundreds of metalloproteins involved in all aspects of metabolism and is known to be an essential micronutrient for marine phytoplankton. Surface depletion and the presence of subpicomolar concentrations of unchelated Zn in oceanic water suggest that Zn can potentially be a limiting factor in the ocean (Bruland 1989; Lohan et al. 2002; Ellwood 2004). Indeed, growth data from laboratory cultures show that model species are limited by unchelated Zn concentrations that have been reported for surface seawater (Anderson et al. 1978; Brand et al. 1983; Sunda and Huntsman 1992). To date, however, only few field enrichment experiments have shown any effect of Zn on phytoplankton productivity in the open ocean, and the effect that is seen is far less substantial than that of Fe, a known limiting micronutrient in the oceans (Coale 1991; Crawford et al. 2003; Lohan et al. 2005). Elucidating the potential role of Zn in the productivity of the oceans clearly requires a better understanding of the Zn physiology of marine phytoplankton.

Laboratory experiments have shown that oceanic species such as *Thalassiosira oceanica* and *Emiliana huxleyi* have much lower Zn requirements for growth than coastal

species (Brand et al. 1983; Sunda and Huntsman 1992). A part of this difference may result from the efficiency with which oceanic species replace Zn with other metals for biological functions. Except in marine cyanobacteria, which have an absolute Co requirement, Co and Zn are interreplaceable in many marine phytoplankton (Price and Morel 1990; Sunda and Huntsman 1995; Saito et al. 2002). In the model diatom *Thalassiosira weissflogii*, a primary role of Zn and Co is to serve as metal cofactors in carbonic anhydrase (CA), which is involved in inorganic carbon acquisition for photosynthesis (Yee and Morel 1996; Lane and Morel 2000b). Cd has also been found to serve as an alternative metal center in CA in diatoms; this is the only documented biochemical function of Cd (Lane and Morel 2000a; Lane et al. 2005). The growth of other phytoplankton, such as the chlorophyte *Tetraselmis maculata* and the prymnesiophyte *Pleurochrysis carterae*, has also been shown to be promoted by Cd addition (Lee and Morel 1995), but the primary biochemical role of Cd has not yet been identified in these species.

Coccolithophores are dominant eukaryotic phytoplankton in the open ocean, and *E. huxleyi* is numerically the most abundant coccolithophore, found in all except the polar oceans (Brown and Yoder 1994; Winter et al. 1994). Several lines of evidence suggest that, as in diatoms, Cd and Co replacement for Zn is a strategy adopted by *E. huxleyi* to reduce its Zn requirement: (1) *E. huxleyi* has a relatively high Cd quota compared with diatoms when grown in the presence of Cd under Zn-replete conditions (Sunda and Huntsman 2000; Ho et al. 2003); (2) it has an increased Cd uptake rate under Zn limitation (Sunda and Huntsman 2000); (3) it is able to grow in the absence of added Zn when Co is included in the medium (Sunda and Huntsman 1995; Timmermans et al. 2001) and has been found to have a higher maximal growth rate when grown with Co alone

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than it does with Zn alone (Sunda and Huntsman 1995). However, the available evidence for a beneficial effect of Cd on growth in *E. huxleyi* is ambiguous (Lee and Morel 1995). Further, we do not know the primary role of Zn, Cd, or Co in this organism where CA activity is either absent or very low (Nimer et al. 1994; Elzenga et al. 2000).

In this study, we examine the effect of Cd on *E. huxleyi* growth and the requirements of this organism for Zn, Cd, and Co. We also estimate the relative use efficiencies for these three metals by analyzing the growth data quantitatively with the empirical Droop equation. This allows us to extrapolate the laboratory culture results to oceanic conditions.

Methods

Cell culture—*E. huxleyi* strains CCMP374 and CCMP1949 were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton in Maine, strains DWN53/74/9 and CCMP1516 from the Plymouth Culture Collection of Marine Algae in the UK. Unless a specific strain was indicated, strain CCMP374 was used for the experiments. All the culture experiments were conducted in trace metal ion buffer system in acid-cleaned polycarbonate bottles using 0.2- μm filtered Gulf Stream seawater (Sunda et al. 2005). The culture media were enriched with chelexed and filter-sterilized 150 $\mu\text{mol L}^{-1}$ NaNO_3 , 10 $\mu\text{mol L}^{-1}$ PO_4^{3-} , or 1 $\mu\text{mol L}^{-1}$ filter-sterilized α -glycerophosphate (the source of P had not effect on the growth of *E. huxleyi*) and 20 $\mu\text{g L}^{-1}$ thiamine. Free trace metal ion concentrations were controlled by 100 $\mu\text{mol L}^{-1}$ ethylenediaminetetraacetic acid (EDTA), with the total concentrations of 87 nmol L^{-1} Fe, 19.6 nmol L^{-1} Cu, 100 or 50 nmol L^{-1} Mn, 10 nmol L^{-1} Se, and varied concentrations of Zn, Cd, and Co. Background Zn, Cd, and Co concentrations were estimated to be 0.2, 0.03, and 0.03 nmol L^{-1} from the seawater, respectively (Sunda and Huntsman 1998). The concentrations of Zn, Cd, and Co from the nutrient stocks were measured by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) as 1.0, 0.03, and 0.02 nmol L^{-1} , respectively, resulting in total background of 1.2, 0.06, and 0.05 nmol L^{-1} of Zn, Cd, and Co, respectively. The unchelated metal concentrations in the medium were computed from the total metal concentrations and available conditional stability constants (Sunda et al. 2005). Note that if our estimated background concentrations were off by 50% (because of uncontrolled contaminations, for example), the calculated unchelated concentrations of Zn and Cd would shift by 0.1 pmol L^{-1} and that of Co by 0.01 pmol L^{-1} . The computed ratios for unchelated metals to free metal ion concentrations are 1.51 for Zn, 34 for Cd, and 1.49 for Co (Sunda et al. 2005). The cultures were grown at 20°C under continuous light (80–100 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$). Cells were acclimated in the medium without Zn, Cd, or Co for a week before transferring into experimental media. Cell numbers were counted daily using a Multisizer II Coulter Counter, and the specific growth rates were then computed during exponential growth. Although sterile techniques were used throughout the experiments, it is possible that

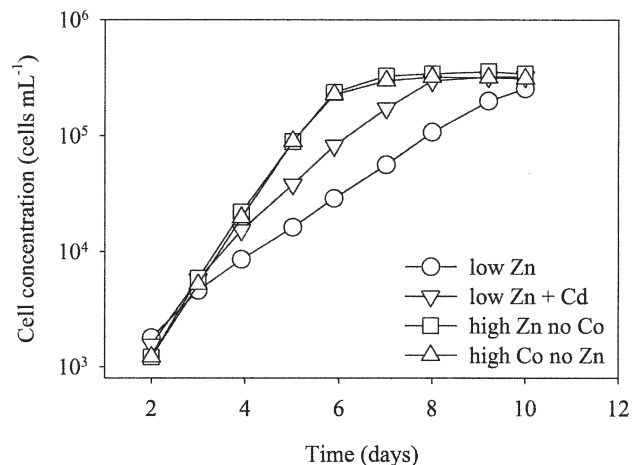


Fig. 1. Growth curves for *E. huxleyi* cells grown with different metal additions. Low Zn: $\text{Zn}' = 0.7 \text{ pmol L}^{-1}$ and no Co or Cd added; low Zn + Cd: $\text{Zn}' = 0.7 \text{ pmol L}^{-1}$, $\text{Cd}' = 20 \text{ pmol L}^{-1}$, and no Co added; high Zn no Co: $\text{Zn}' = 15 \text{ pmol L}^{-1}$ and no Co or Cd added; high Co no Zn: $\text{Co}' = 15 \text{ pmol L}^{-1}$ and no Zn or Cd added.

the experiments had low levels of bacterial contamination.

Metal quota measurement—Cellular metal quotas were measured as described by Tang and Morel (2006). Cells were harvested when the cell numbers reached 200,000 cells mL^{-1} . About 100 mL of cell culture was filtered onto 1- μm acid-cleaned polycarbonate membranes under low vacuum (~6.7 kPa) and rinsed five times with chelexed NaCl solution. No complexing agents were used to wash cells since most of the metals are intracellular (Tang and Morel 2006). Filtered samples were then digested in Teflon tubes with 50% HNO_3 (Optima grade, Fisher) at 100°C for 4 h. After digestion, Milli-Q water was added into the tube to give the final concentration of 5% HNO_3 . Cellular concentrations of P, Ca, Zn, Cd, and Co were determined by ICP-MS (*Element 2*, ThermoFinnigan) and were reported as a ratio, mmol metal : mol P.

Results

Zn, Cd, and Co interreplacement for growth—We examined the effect of Zn, Cd, and Co on the growth of *E. huxleyi* strain CCMP374 (Fig. 1 and Table 1). The growth rate at a Zn' of 0.7 pmol L^{-1} in the absence of Cd and Co was reduced to 50% of the maximum rate achieved at $\text{Zn}' = 15 \text{ pmol L}^{-1}$ (0.65 vs. 1.33 d^{-1}). In the presence of 20 pmol L^{-1} Cd' at 0.7 pmol L^{-1} Zn' , the growth rate was enhanced to 70% of the maximum rate (0.92 vs. 0.65 d^{-1} , *t*-test, $p < 0.05$) demonstrating clearly that Cd is beneficial to the growth of Zn-limited *E. huxleyi*. In the absence of added Zn, the growth rate reached its maximum at a Co' of 15 pmol L^{-1} (1.36 d^{-1}) and additional Zn didn't affect the growth rate, indicating that Co can fully replace Zn for growth. The difference in the effects of Cd and Co on growth suggests that Cd is less efficient than Co as a replacement for Zn (*see below*).

Table 1. Effect of Zn, Co, and Cd on growth rates and cellular Zn quotas in different *E. huxleyi* strains. Growth rates are reported as the average from two independent cultures.

Strain	Location of isolation	Ca:P (mol mol P ⁻¹)	Growth rates (d ⁻¹) and cellular Zn quotas (in parentheses, mmol mol ⁻¹ P)			
			0.7 pmol L ⁻¹ Zn'	0.7 pmol L ⁻¹ Zn' 20 pmol L ⁻¹ Cd'	15 pmol L ⁻¹ Zn'	15 pmol L ⁻¹ Co'
CCMP374	Gulf of Maine 42°N 69°W	0.04±0.01	0.65±0.01	0.92±0.06	1.33±0.02	1.36±0.01
CCMP374 (naked cells)	Gulf of Maine 42°N 69°W	0.11±0.01	0.41±0.01 (0.57±0.04)	0.65±0.01 (0.46±0.07)	1.09±0.05 (5.11±0.51)	1.23±0.02
A1387	NA*	0.09±0.07	0.21±0.02† (1.11±0.03)	0.93±0.02 (0.41±0.06)	1.09±0.00 (3.56±0.15)	1.18±0.03
DWN53/74/6	24°N 20°W	92.21±9.03	0.58±0.01 (0.30±0.01)	0.90±0.01 (0.23±0.02)	1.17±0.01 (2.19±0.20)	1.23±0.01
CCMP1516	South Pacific 2°S 82.7°W	1.36±0.01	0.47±0.02 (0.41±0.03)	0.69±0.01 (0.35±0.03)	1.03±0.02 (5.20±0.16)	1.03±0.01
CCMP1949	Gulf of Maine 42°N 69°W	16.88±11.04	0.37±0.01 (0.44±0.11)	0.73±0.00 (0.33±0.1)	0.84±0.00 (6.75±1.14)	0.86±0.04

* Not available.

† Biphasic exponential growth was observed. Growth rate was 0.51 ± 0.02 d⁻¹ in the first phase and 0.21 ± 0.02 d⁻¹ in the second phase as reported in the table, and cells were collected during the second phase for cellular metal quota measurement.

We further examined the effect of Cd on growth rate over a range of Zn', from the background level of 0.2 pmol L⁻¹ (background Zn_T ~ 1.2 nmol L⁻¹, corresponding to Zn' ~ 0.2 pmol L⁻¹) to 1.5 pmol L⁻¹ (Fig. 2), representing values found in the open ocean (see Table 4). With no Zn added to the medium, Cd addition had no effect on the growth rate, indicating that *E. huxleyi* has a minimum Zn requirement that cannot be replaced by Cd (Fig. 2). Cd was beneficial to the growth of Zn-limited cells within the range of Zn addition tested (0.39 to 1.54 pmol L⁻¹ of Zn', Fig. 2) with a growth rate enhancement from 0.2 to 0.4 d⁻¹. The presence of Cd had no significant effect on growth when enough Zn was present to support the maximum growth rate.

Since the previous experiments had high Cd concentrations, we conducted another set of experiments with constant Zn' (0.7 pmol L⁻¹) but various Cd' in the medium to evaluate the Cd requirement for growth

(Fig. 3). The growth rates and cellular Cd to P ratios increased with increasing Cd' (Fig. 3A,B). The growth enhancement by Cd was most significant when Cd' was below 5 pmol L⁻¹ and cellular Cd concentration below 0.4 mmol mol⁻¹ P (Fig. 3A,B). Further increase in cellular Cd had little effect on growth (Fig. 3A,C). The cellular Cd to P ratio and steady-state Cd uptake rate increased linearly with Cd' in the medium (Fig. 3B,D). These experiments suggest that a cellular Cd quota of 0.4 mmol mol⁻¹ P can fulfill most of the biochemical functions of Cd related to growth. The cellular Zn to P ratio decreased with increasing Cd' and became constant above Cd' = 2 pmol L⁻¹ (Fig. 3E). Correspondingly, the steady-state Zn uptake rate may have decreased upon Cd addition, but the data are noisy (Fig. 3F). The minimum Zn quota of 0.2 mmol mol⁻¹ P observed at high Cd' may correspond to an absolute Zn requirement that cannot be replaced by Cd.

Zn, Cd, and Co interreplacement in different E. huxleyi strains—*E. huxleyi* is a widely distributed species in the ocean with considerable variation in cell physiology and coccolith formation among strains isolated from different regions (Young and Westbroek 1991; Paasche 2001). We examined the growth responses of various *E. huxleyi* strains to Cd and Co additions under Zn-limited conditions (Table 1). The strains tested showed remarkable variations in growth rate and calcite formation under identical growth conditions. Nonetheless, the cellular Zn requirement for growth appeared rather uniform among strains isolated from different regions. Except for A1387, all strains grew at about 45% μ_{max} at Zn' = 0.7 pmol L⁻¹ and corresponding cellular Zn quotas around 0.3–0.5 mmol mol⁻¹ P, and they were all enhanced by Cd addition significantly ($p < 0.05$, t -test) with growth enhancement ranging between 0.2 and 0.4 d⁻¹. For strains CCMP374, CCMP1516, and CCMP1949, cells grown on Co alone yielded similar maximum growth rates as cells grown on Zn alone. For strains CCMP374 naked cells, A1387, and DWN53/74/6, the maximum

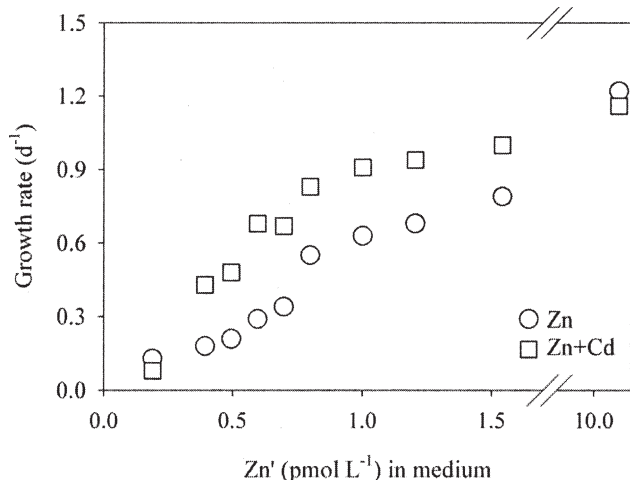


Fig. 2. Specific growth rates of *E. huxleyi* at different Zn' with or without Cd addition (82 pmol L⁻¹ of Cd').

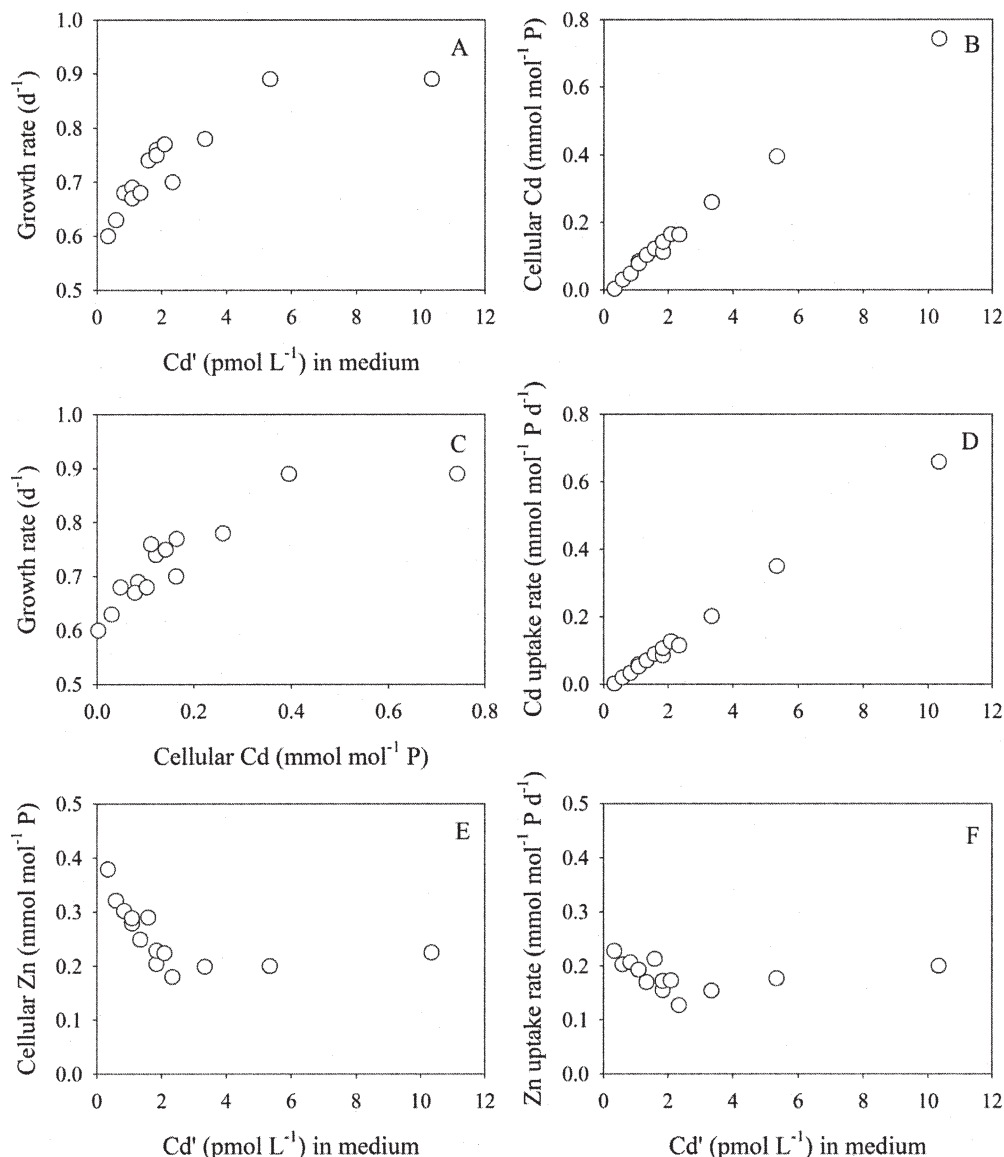


Fig. 3. (A, B, D) Specific growth rate, cellular Cd:P ratio, and cellular Cd uptake rate of *E. huxleyi* as function of Cd'. (C) Specific growth rate as function of cellular Cd:P ratio. (E, F) Cellular Zn:P ratio and cellular Zn uptake rate as function of Cd'. Zn' = 0.7 pmol L⁻¹ for all treatments.

growth rates were, respectively, 13%, 8%, and 5% higher on Co alone than on Zn alone. Similarly, Sunda and Huntsman (1995) found a 34% higher maximum growth rate when grown on Co only compared with Zn only. These results clearly indicate that the partial replacement by Cd and the full replacement by Co for Zn is not a strain-specific but a general attribute of *E. huxleyi*.

Cellular Zn, Cd, and Co requirements for growth and their relative use efficiencies—To better understand the physiology of metal replacement in *E. huxleyi*, two questions need to be answered: (1) What are the relative use efficiencies of Zn, Cd, and Co in *E. huxleyi*? (2) How much of the cellular Zn and Co can be replaced by Cd? We explored these two questions by examining the relationship between the

growth rate and cellular Zn, Cd, and Co quotas in a matrix of experimental Zn, Cd, and Co concentrations (Fig. 4; Table 2): two experiments varying Zn' or Co' with no other metal addition (Zn only and Co only); an experiment varying Zn' with Cd' = 1.8 pmol L⁻¹ (Zn + Cd); an experiment varying Co' with Cd' = 1.8 pmol L⁻¹ (Co + Cd); and an experiment varying Zn' with Cd' = 1.8 pmol L⁻¹ and Co' = 0.3 pmol L⁻¹ (Zn + Cd + Co). The metal additions of 1.8 pmol L⁻¹ Cd' and 0.3 pmol L⁻¹ Co' were chosen to maintain some degree of metal limitation.

Consistent with our previous results, the growth rate increased as Zn' or Co' increased, and the addition of Cd augmented the growth rate considerably (Fig. 4A). To analyze the data, we plotted the growth rate as a function of cellular Zn (Zn only, Zn + Cd, and Zn + Cd + Co,

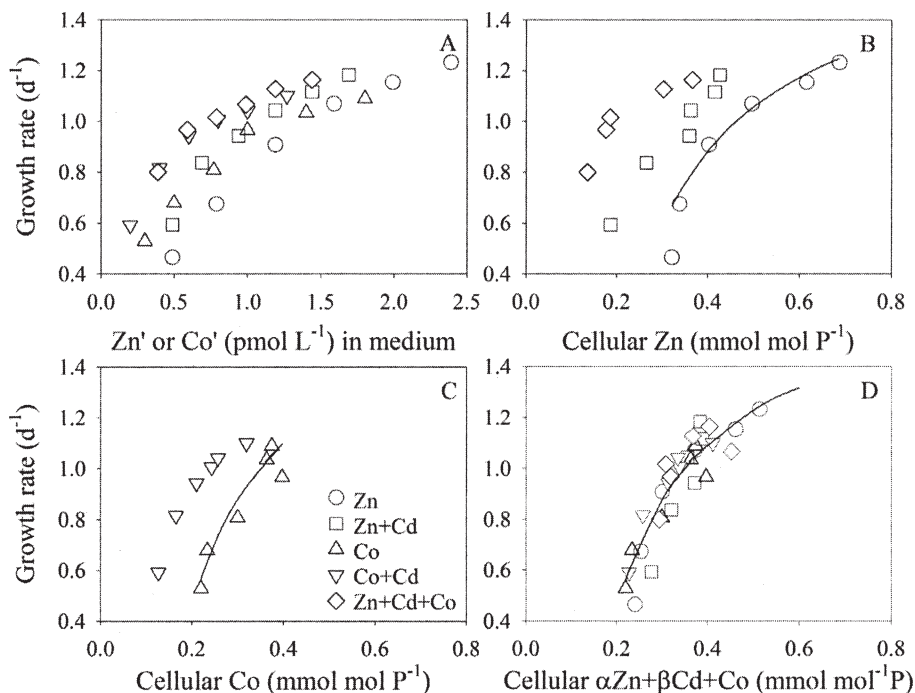


Fig. 4. (A) Specific growth rate of *E. huxleyi* as function of Zn' or Co'. (B, C, D) Specific growth rate as function of cellular Zn:P ratio, cellular Co:P ratio, and a combination of cellular Zn, Cd, and Co ($\alpha\text{Zn} + \beta\text{Cd} + \text{Co}$):P ratio ($\alpha = 0.75$, $\beta = 0.66$, see text). The lines show the fit of the Droop model with $\mu_m = 1.76 \text{ d}^{-1}$ and $Q_{\min} = 0.15 \text{ mmol mol}^{-1} \text{ P}$. Symbols for each treatment are shown on panel C.

Fig. 4B) and of cellular Co (Co only and Co + Cd) (Fig. 4C). In all cases, we observed a “hyperbolic” relation between growth rate and Zn or Co quotas with a horizontal displacement toward lower quotas (for a given growth rate) upon addition of Cd or Co. In addition, when the growth rate was limited, cells required a lower cellular quota of Co than of Zn or any combination of Zn, Cd, and Co to achieve a given growth rate, showing that they use Co most efficiently for biological functions.

The “hyperbolic” shapes of the growth curves in Fig. 4B,C suggested that they may be fitted adequately by the empirical Droop equation (Droop 1983). For quantifying the interreplacement of the three metals Zn, Cd, and Co, we thus attempted to fit the data with the equation

$$\mu = \mu_m \times (Q - Q_{\min})/Q \quad (1)$$

where $Q = Q_{\text{Co}} + \alpha Q_{\text{Zn}} + \beta Q_{\text{Cd}}$. In this equation, the specific growth rate (μ , d^{-1}) is given as a function of the cellular concentration (Q , $\text{mmol mol}^{-1} \text{ P}$) of the combination of Zn, Cd, and Co. The parameter μ_m (d^{-1}) is related to the maximum specific growth rate ($\mu_{\max} = \mu_m \times (Q_{\max} - Q_{\min})/Q_{\max}$), and Q_{\min} ($\text{mmol mol}^{-1} \text{ P}$) is the minimum cellular nutrient concentration necessary for maintaining life. The coefficients α and β for the cellular Zn and Cd quotas reflect how efficiently these metals are used for cell growth compared with Co. The equation using the overall parameter Q cannot be strictly correct, since we know that the cells do not grow on Cd in the absence of Zn and Co. But over a concentration range where the minimum Zn or Co quota is satisfied, it should provide a good approxima-

tion of the data. We determined what coefficients α and β best fit the data on the basis of the matrix experiment (described in Table 2 and Fig. 4).

With a single metal present (Co only and Zn only), the data were adequately fitted with $Q_{\min} = 0.15 \text{ mmol mol}^{-1} \text{ P}$, $\mu_m = 1.76 \text{ d}^{-1}$, and $\alpha = 0.75$ (Fig. 4C). The corresponding minimum quota for Zn ($0.15/0.75 = 0.2 \text{ mmol mol}^{-1} \text{ P}$) is in good agreement with the data obtained earlier (Fig. 4B). With two metals present (Zn + Cd and Co + Cd), β was determined as 0.74 using data from the Zn + Cd experiment and as 0.58 using data from the Co + Cd experiment. It is not clear why there should be a difference in the coefficient for Cd use efficiency when the primary requirement for growth is met by Co or by Zn, so to keep the model general, we used 0.66, the mean of 0.74 and 0.58, as β . These coefficients gave an acceptable fit of the data with all three metals present (Zn + Cd + Co), and the growth curves for all experiments in Table 2 superpose each other when plotted as a function of $Q = Q_{\text{Co}} + 0.75Q_{\text{Zn}} + 0.66Q_{\text{Cd}}$, as shown in Fig. 4D. Thus, in a first approximation, our data indicate that Zn is used at 75% efficiency compared with Co, and Cd at 66% efficiency once the requirement for minimum cellular Zn or Co is fulfilled. To be more precise, in the range of concentrations where the metals are limiting (and the minimum Zn or Co quota is met), one needs to increase the cellular Zn quota 33% and the Cd quota 50% more than the Co quota to obtain the same increase in growth rate.

The proportion of Co and Zn replaced by Cd can be calculated as $\beta Q_{\text{Cd}}/(Q_{\text{Co}} + \alpha Q_{\text{Zn}} + \beta Q_{\text{Cd}})$ (Table 2).

Table 2. Effect of variations in Zn', Cd', and Co' (pmol L⁻¹) on specific growth rate (μ , d⁻¹); cellular Zn : P, Cd : P, and Co : P ratios (mmol mol⁻¹ P); steady-state cellular Zn, Cd, and Co uptake rates (ρ_{Zn} , ρ_{Cd} , and ρ_{Co} , mmol mol⁻¹ P d⁻¹); amount of Zn or Co replaced by Cd (βQ_{Cd}); and percentage of Zn or Co replaced by Cd ($\beta Q_{Cd}/(Q_{Co} + \alpha Q_{Zn} + \beta Q_{Cd}) \times 100\%$) (see text for definitions and values of α and β).

Experiment	<i>M'</i> concentration in medium (pmol L ⁻¹)			μ growth rate (d ⁻¹)	Cellular metal quotas (mmol mol ⁻¹ P)			Cellular metal uptake rate (mmol mol ⁻¹ P d ⁻¹)			βQ_{Cd} amount of Zn or Co replaced by Cd (mmol mol ⁻¹ P)	% of Zn or Co replaced by Cd
	Zn'	Cd'	Co'		Zn : P	Cd : P	Co : P	ρ_{Zn}	ρ_{Cd}	ρ_{Co}		
Zn	0.5			0.47	0.32			0.15				
	0.8			0.67	0.34			0.23				
	1.2			0.91	0.40			0.37				
	1.6			1.07	0.50			0.53				
	2.0			1.15	0.62			0.71				
	2.4			1.23	0.69			0.85				
Zn+Cd	0.5	1.8		0.59	0.19	0.21		0.11	0.12		0.18	50
	0.7	1.8		0.84	0.27	0.19		0.22	0.16		0.16	38
	0.9	1.8		0.94	0.36	0.16		0.34	0.15		0.14	28
	1.2	1.8		1.04	0.36	0.13		0.38	0.13		0.11	24
	1.4	1.8		1.12	0.42	0.12		0.46	0.13		0.10	20
	1.7	1.8		1.18	0.43	0.098		0.51	0.12		0.086	17
Co			0.3	0.53			0.22			0.12		
			0.5	0.68			0.23			0.16		
			0.8	0.81			0.30			0.24		
			1.0	0.97			0.34			0.38		
			1.4	1.03			0.36			0.38		
			1.8	1.10			0.37			0.41		
Co+Cd		1.8	0.2	0.59		0.15	0.13		0.091	0.075	0.10	44
		1.8	0.4	0.82		0.14	0.17		0.12	0.14	0.094	36
		1.8	0.6	0.94		0.16	0.21		0.15	0.20	0.10	33
		1.8	0.8	1.02		0.14	0.24		0.15	0.24	0.095	28
		1.8	1.0	1.04		0.12	0.26		0.12	0.27	0.078	23
		1.8	1.3	1.10		0.14	0.32		0.15	0.35	0.092	22
Zn+Cd+Co	0.4	1.8	0.3	0.80	0.14	0.14	0.10	0.11	0.11	0.082		
	0.7	1.8	0.3	0.97	0.18	0.15	0.088	0.17	0.15	0.085		
	0.8	1.8	0.3	1.02	0.19	0.13	0.083	0.19	0.13	0.084		
	1.0	1.8	0.3	1.07	0.40	0.11	0.083	0.42	0.12	0.088		
	1.2	1.8	0.3	1.13	0.30	0.11	0.068	0.34	0.13	0.077		
	1.4	1.8	0.3	1.16	0.37	0.096	0.066	0.43	0.11	0.077		

Depending on the cellular Zn and Co concentrations, Cd replaced up to 50% of cellular Zn and 44% of cellular Co in the matrix experiment. These values presumably underestimate the maximum replacement by Cd since Cd' was maintained at 1.8 pmol L⁻¹, below the optimum for growth (see Fig. 3A). In the experiment of Fig. 3, the growth rate increased with the Cd quota up to 0.4 mmol Cd mol⁻¹ P; this corresponds to 51% of the *Q* necessary to achieve the maximum growth rate seen in Fig. 4D ($0.4 \times 0.66/0.52 = 0.51$). In the same experiment, the Zn quota decreased to 0.2 mmol mol⁻¹ P but the growth rate stayed at 0.9 d⁻¹, about 75% of μ_{max} . The minimum Zn quota that supports full growth but cannot be replaced by Cd at high Cd' is thus presumably on the order of $0.2 \times 4/3 = 0.27$ mmol Zn mol⁻¹ P, about 40% of the *Q* necessary to achieve the maximum growth rate. Hence, the proportion of Co and Zn that can usefully be replaced by Cd in *E. huxleyi* is around 50–60%.

The steady-state uptake rates of Zn, Cd, or Co were calculated for the matrix experiment (Fig. 5). The Zn uptake rate increased linearly with Zn' and was not

affected by the addition of Cd and Co at all (Fig. 5A); this suggests that the variations seen in Fig. 3F may be experimental noise. Further, Zn uptake rates were in general 60–90% of the theoretical limiting rates for the diffusion of dissolved unchelated Zn. The Co uptake rate showed a similar pattern and was not affected by the addition of Cd or Zn either (Fig. 5B). Co uptake rates were more than 70% of the theoretical limiting rates for the diffusion of dissolved unchelated Co when Co' was less than 1 pmol L⁻¹. However, the difference between the actual Co uptake rate and the theoretical diffusion rate became greater with increasing Co', and the uptake rate was only 59% of the diffusion rate at Co' = 1.8 pmol L⁻¹, presumably as the result of down-regulation of the Co uptake system. In general, the uptake rates for Zn and Co were comparable, but the Cd uptake rate was about threefold lower than those of Zn and Co for the same unchelated metal concentration (13–20% of the computed maximum diffusion rate of unchelated Cd) and was not affected substantially by variations in Zn' or Co'.

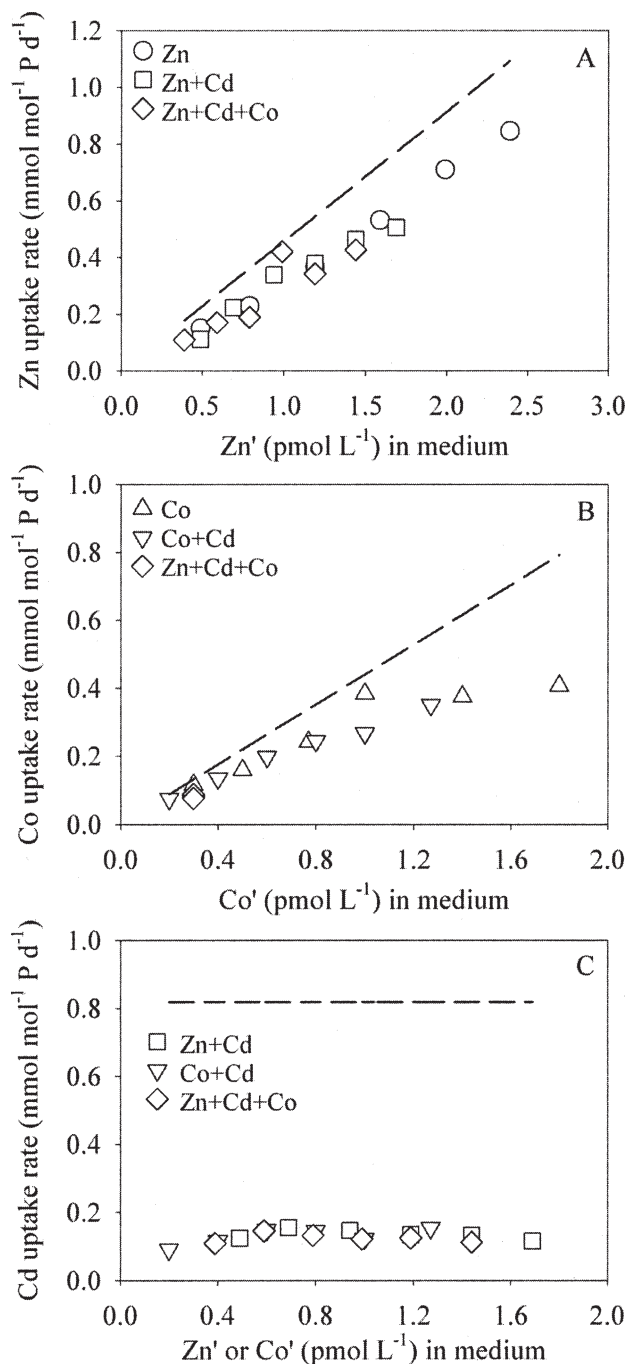


Fig. 5. (A, B, C) Cellular uptake rate of Zn, Co, and Cd in *E. huxleyi* as function of Zn' or Co' . Dashed lines give the computed limiting rates for diffusion of Zn' , Co' , and Cd' to the cell surface, respectively. The maximum diffusion rate was calculated from the equation $\rho = 4\pi rDM'$. The cell radius, r , was calculated from the mean cell volume as $2.3 \mu\text{m}$. D is the diffusion rate constant for inorganic species at 20°C ($6.41 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ for Zn, $6.38 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ for Cd, and $6.17 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ for Co; Li and Gregory 1974). M' is the concentration of unchelated metal.

Discussion

Interreplacement of Zn, Cd, and Co for growth—Our results with *E. huxleyi* cultures amended with various concentrations of Zn and Co are in good agreement with studies by Sunda and Huntsman (1995) and Timmermans et al. (2001) and extend them to several *E. huxleyi* strains. Zn and Co are apparently able to fully replace each other in their biochemical functions, although we cannot rule out that some small individual requirements may be met by minute metal concentrations supplied as contaminants in the cultures. Half the maximum growth rate was obtained at a Zn' of 0.7 pmol L^{-1} ($[Zn^{2+}] = 0.46 \text{ pmol L}^{-1}$) in the absence of Cd and Co, which is consistent with previous studies by Sunda and Huntsman (1995; 50% μ_{max} at $0.56 \text{ pmol L}^{-1} Zn^{2+}$) and Schulz et al. (2004; 50% μ_{max} at $0.5 \text{ pmol L}^{-1} Zn^{2+}$), but a bit lower than observed by Timmermans et al. (2001; 50% μ_{max} at $0.8 \text{ pmol L}^{-1} Zn^{2+}$). Additions of Cd are able to alleviate Zn or Co limitation on growth, indicating that Cd can replace some biochemical functions of Zn or Co in *E. huxleyi*. However, there is a requirement of minimum cellular Zn or Co that Cd cannot replace, suggesting that this organism uses some essential metalloproteins that can use Zn or Co but not Cd as a cofactor. These Zn or Co proteins account for about 40–50% of the Zn or Co requirement of nonlimited cells. Similar interreplacement of Zn, Cd, and Co are observed in a variety of *E. huxleyi* strains isolated from different regions of the ocean. On the basis of our growth data, with strain CCMP374, we estimate that Zn is used 25% less efficiently than Co. When the primary requirement for Zn or Co is met (ca. $0.2 \text{ mmol Zn or Co mol}^{-1} \text{ P}$), Cd is used 34% less efficiently than Co.

In the only previous study to examine the effect of Cd on the growth of Zn-limited *E. huxleyi*, only a very subtle effect was seen (0.59 d^{-1} at $0.16 \text{ pmol L}^{-1} Zn' + 4.6 \text{ pmol L}^{-1} Cd'$ vs. 0.52 d^{-1} at $0.16 \text{ pmol L}^{-1} Zn'$) and no firm conclusion could be drawn (Lee and Morel 1995). The reasons for the differences between our results and those of this previous study are not clear, but they may result from differences in the growth conditions or in the strains that were tested. Differences in trace metal physiology among strains are clearly documented in the results of Table 1, particularly in the fact that some Co-replete cells grow at higher rates than Zn-replete cells, while others do not. Sunda and Huntsman (1995) reported a higher maximum growth rate on Co than on Zn in the *E. huxleyi* strain they tested.

It is notable that in the experiment reported in Fig. 2, the growth rates obtained at various Zn' with a high Cd addition are less than μ_{max} . At $Zn' = 0.7 \text{ pmol L}^{-1}$, the growth rate is in fact lower than seen in Fig. 3 for a more modest Cd addition. In retrospect, it appears that the addition of enough Cd to achieve $Cd' = 82 \text{ pmol L}^{-1}$ in the culture medium in this particular experiment is much higher than can be used by the cells and is likely partially toxic to *E. huxleyi*.

Schulz et al. (2004) reported that the calcification rate is not affected by Zn limitation in *E. huxleyi*. We have also examined the calcification rate under different metal

conditions with various strains (data not shown). Except DWN53/74/6 and CCMP1949, all strains calcified at insignificant rates during exponential growth, and no consistent trend could be observed. However, a significantly higher calcification rate was found in strain DWN53/74/6 grown with $0.7 \text{ pmol L}^{-1} \text{ Zn}'$ and $20 \text{ pmol L}^{-1} \text{ Cd}'$ than in the absence of Cd ($0.7 \text{ pmol L}^{-1} \text{ Zn}'$, $15 \text{ pmol L}^{-1} \text{ Zn}'$, or $15 \text{ pmol L}^{-1} \text{ Co}'$).

Metal use efficiency—To verify that the Droop equation with a combined cellular quota for Zn, Cd, and Co is generally applicable, we calculated the growth rate based on the cellular Zn and Cd when Cd' is below 5 pmol L^{-1} for the experiments presented in Fig. 3. The calculated growth rate is always between 95% and 120% of the measured growth rate, showing that the Droop equation gives a reasonable description of the growth data and should thus provide a fairly robust estimate of the relative use efficiencies for the three metals in strain CCMP374. When Cd' is above 5 pmol L^{-1} , the calculated growth rate is 50% to 60% higher than the measured growth rate, suggesting that there indeed may be a toxic effect of Cd on growth at high concentration. However, we have to bear in mind that the model is empirical and the actual values for the coefficients α and β may vary among strains. Indeed, the strains tested in this study showed different Zn use efficiency (i.e., they grew at different growth rates for similar concentrations of cellular Zn; see Table 1), and they responded to a different extent to Cd additions.

In contrast to *E. huxleyi*, diatoms like *T. oceanica* and *Thalassiosira pseudonana* use Co less efficiently than Zn (Sunda and Huntsman 1995), and the diatom *Chaetoceros calcitrans* cannot use Co at all (Timmermans et al. 2001). The difference in maximum growth rate between Zn and Co is less pronounced in the oceanic diatom *T. oceanica* (μ_{max} 25% lower on Co than on Zn) than in the coastal diatom *T. pseudonana* (μ_{max} 40% lower on Co than on Zn). In terms of the actual metal use efficiency, the oceanic species *T. oceanica* and *E. huxleyi* use Zn and Co more efficiently than the coastal species *T. pseudonana*, but *T. oceanica* has higher Zn and Co use efficiencies than *E. huxleyi* (Sunda and Huntsman 1995 and this study). For example, *T. oceanica* requires $0.04 \text{ mmol Zn mol}^{-1} \text{ P}$ or $0.09 \text{ mmol Co mol}^{-1} \text{ P}$ to achieve a growth rate of 0.9 d^{-1} (Sunda and Huntsman 1995), whereas *E. huxleyi* requires $0.4 \text{ mmol Zn mol}^{-1} \text{ P}$ or $0.3 \text{ mmol Co mol}^{-1} \text{ P}$ (this study). However, according to our data with strain CCMP374, *E. huxleyi* has faster Zn and Co uptake rates than *T. oceanica*, allowing it to accumulate higher cellular quotas of these metals at a given concentration in the medium. For example, at the growth rate of 0.9 d^{-1} , the metal uptake rate is $0.37 \text{ mmol Zn mol}^{-1} \text{ P d}^{-1}$ or $0.28 \text{ mmol Co mol}^{-1} \text{ P d}^{-1}$ in *E. huxleyi*, which is much higher than that in *T. oceanica* ($0.036 \text{ mmol Zn mol}^{-1} \text{ P d}^{-1}$ or $0.084 \text{ mmol Co mol}^{-1} \text{ P d}^{-1}$). The net result is that *E. huxleyi* and *T. oceanica* grow at approximately the same rate at the same limiting ambient concentration of Zn or Co (Fig. 6). We note, however, that Sunda and Huntsman (1995) observed a slower metal uptake rate in their *E. huxleyi* strain,

resulting in a lower growth rate than *T. oceanica* under similar Zn or Co limitation.

Similar to *E. huxleyi*, there is a minimum Zn requirement that Cd cannot replace in the diatoms *T. pseudonana* and *T. weissflogii* (Lee and Morel 1995). Compared with *E. huxleyi*, *T. weissflogii* seems to use Cd more efficiently. For example, $0.1 \text{ mmol Cd mol}^{-1} \text{ P}$ ($8 \text{ amol Cd cell}^{-1}$) at Zn' of 3.2 pmol L^{-1} could support 25% of μ_{max} in *T. weissflogii* (calculated from Lee et al. 1995), whereas $0.4 \text{ mmol Cd mol}^{-1} \text{ P}$ at Zn' of 0.5 pmol L^{-1} support 23% of μ_{max} in *E. huxleyi* (this study). The difference in metal usage between the oceanic and coastal species and between different oceanic phytoplankton taxa is not only reflected in a different requirement for a given metal to support growth (Brand et al. 1983; Sunda and Huntsman 1995), but also in different relative metal use efficiencies. And the availability of metals in different environmental regimes can act as a selective force on cell physiology to determine the distribution and abundance of different phytoplankton species.

Uptake system and regulation—It has been suggested that under Zn limitation, Cd and Co may be taken up by the same transport system as Zn, or by a transport system that is negatively regulated by Zn (Sunda and Huntsman 1995, 2000). Our steady-state uptake data obtained at limiting concentrations of Zn, Cd, or Co, when the transport systems are far from saturation, showed no competition between the metals and no evidence of down-regulation of the transport system of one metal upon increase in the concentration of another. This is consistent with the results of Sunda and Huntsman (1995, 2000). Also consistent with the results of Sunda and Huntsman (1995, 2000) are the fast metal uptake rates that we observed: Zn and Co uptake rates within 60–90% of the calculated maximum rates for diffusion of unchelated Zn and Co, and Cd uptake rate within 13–20% of the maximum diffusion rate. These are remarkably fast rates, since the diffusion calculation is based on a zero concentration at the cell surface. Hudson and Morel (1993) have calculated that, to approach the diffusion limit, cells must possess an extremely high concentration of transport molecules and that ca. 80% of the maximum diffusion rate is a practical maximum for uptake. Although it seems unlikely, one may wonder whether *E. huxleyi* may be able to acquire some metals directly from the EDTA complexes, or whether small concentrations of metal complexes with natural ligands might be “bioavailable.” Similarly, Hudson (1998) observed that Cu uptake rate is greater than the limit for diffusion of the unchelated metal in some diatoms and *E. huxleyi*, and thus suggested that some Cu-EDTA complexes may be available. In addition, Lohan et al. (2005) observed a decrease in the concentrations of both the Zn-binding ligand and the total dissolved Zn in incubation experiments with natural phytoplankton assemblage, suggesting that the Zn-ligand complex may be available to the cells. The relatively low rate of Cd uptake, compared with Zn and Co, may reflect the different extent of complexation of these metals by inorganic ligands in seawater: while Zn^{2+} and Co^{2+} are present largely as hydrated cations, Cd^{2+} is

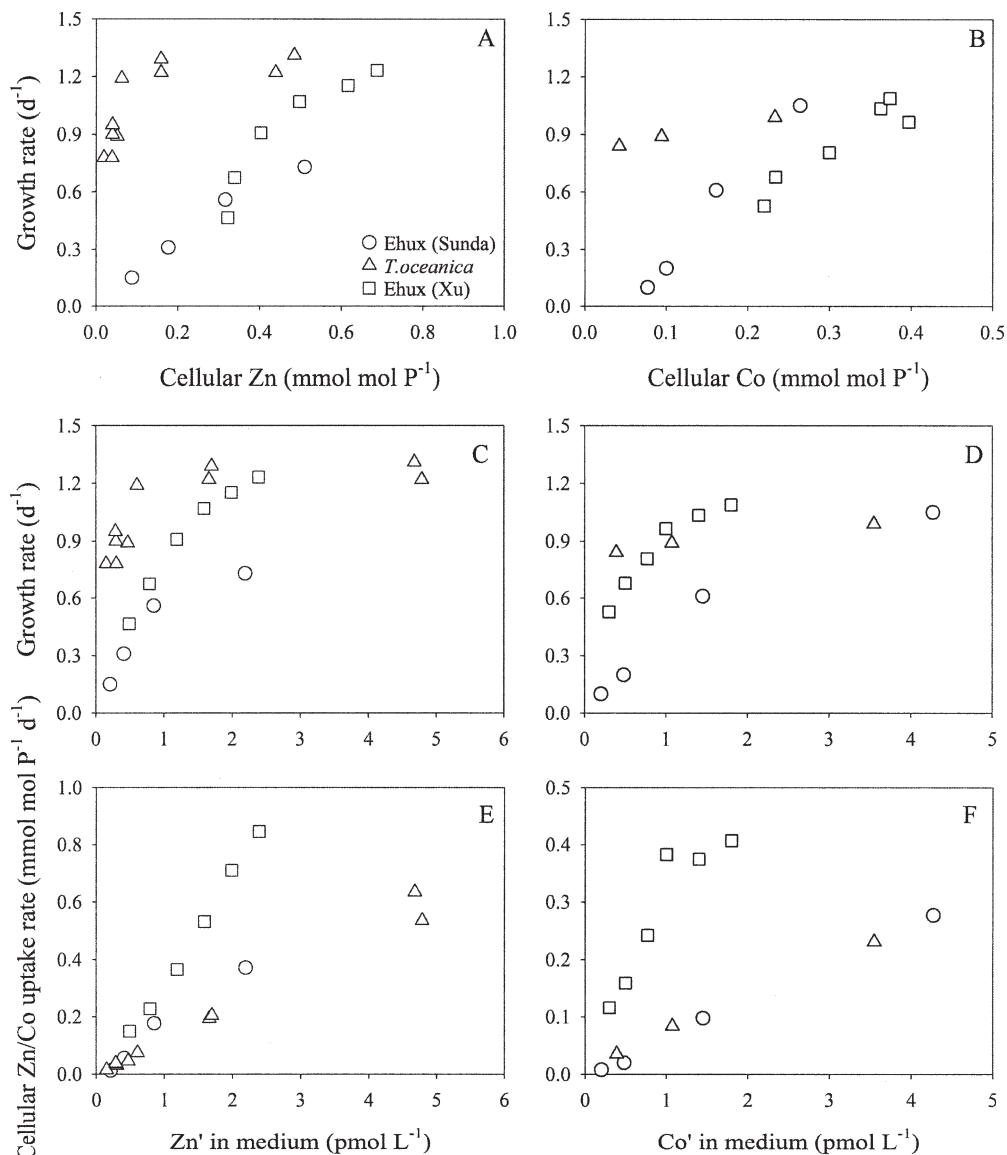


Fig. 6. (A, B) Specific growth rate as function of cellular Zn : P ratio or cellular Co : P ratio. (C, D) Specific growth rate as function of Zn' or Co'. (E, F) cellular Zn or Co uptake rate as function of Zn' or Co'. Symbols for each study are shown in panel A: Ehux (Sunda) and *T. oceanica* from Sunda and Huntsman (1995); Ehux (Xu) from this study.

almost entirely bound to Cl^- (97% as CdCl^+ , CdCl_2 , CdCl_3^- , and CdCl_4^{2-} ; Morel and Hering 1993). The negative charge of bound chloride may slow down the diffusion of the complexes to a negatively charged cell surface, or the dissociation of Cd^{2+} from CdCl_x may slow down the binding to uptake molecules. Our results support the notion that in trace metal-limited oceanic water, phytoplankton species have optimized their physiology to achieve metal uptake rates close to their maximum physical limits (Hudson and Morel 1993; Sunda and Huntsman 1995).

Biochemical basis of metal use efficiencies—The underlying biochemical mechanisms for the interreplacement of Zn, Cd, and Co in *E. huxleyi* and the differences in the

use efficiency of these metals are not clear. There are two possible ways for a metal to replace another: direct metal cofactor substitution and protein replacement. For example, in Zn-deficient diatom *T. weissflogii*, Co may be used to substitute Zn directly in the metal center for TWCA1, whereas a Cd-specific CdCA1 is induced as the replacement for TWCA1 (Yee and Morel 1996; Lane and Morel 2000a; Lane et al. 2005). We have no way at this point to distinguish between these two mechanisms in *E. huxleyi*.

Co substitution for Zn in vitro almost always results in enzymatically active proteins, although the magnitude of the activity may be either increased or decreased (Table 3; Vallee and Galde 1984; Vallee and Falchuk 1993). Furthermore, in vivo Co-substituted RNA polymerase in Zn-deficient *Escherichia coli* cells and Co-substituted CA in

Table 3. Effect of Co and Cd substitution on Zn metalloenzyme activities. Y = activity retained (+, more active than the original Zn form; -, less active); N = activity lost. Unless indicated, all enzymatic activities are from in vitro substitution experiments. (Part of the table is modified from table 6 in Vallee and Galdes 1984).

Enzyme	Source	Effect of metal substitution on enzyme activity		Reference*
		Cd	Co	
Alcohol dehydrogenase	Horse	Y (-)	Y (-)	1, 2
Aldolase	Yeast	N	Y (-)	1
Alkaline phosphatase	<i>Escherichia coli</i>	N	Y (-)	1
$\bar{\alpha}$ -Aminolevulinatase	Bovine	Y	N	1, 3
AMP aminohydrolase	Rabbit		Y	1
Angiotensin converting enzyme			Y (-)	1
Aspartate transcarbamylase	<i>E. coli</i>	Y†	Y	1, 4
Carbonic anhydrase	Bovine	N	Y (-)	1, 2
Carbonic anhydrase	<i>T. weissflogii</i>	Y‡	Y (-)†	5, 6, 7, 8
Carboxypeptidase A	Bovine	Y (-)	Y (+)	1, 2
Carboxypeptidase B	Porcine	Y	Y	1
Carboxypeptidase P	<i>Streptomyces griseus</i>		Y	1
b-lactmase	<i>Bacteroides fragilis</i>	Y (-)	Y (-)	9
Leucine aminopeptidase	Bovine		Y (+)	1
Methionyl aminopeptidase	<i>E. coli</i>		Y	9
Neutral protease	<i>Bacillus subtilis</i>		Y	1
RNA polymerase	<i>E. coli</i>		Y (-)	1, 10
Superoxide dismutase	Bovine	Y (-)	Y (-)	1
Thermolysin	<i>Bacillus thermoproteolyticus</i>	N	Y (+)	1, 2

* (1) Vallee and Galdes (1984); (2) Parkin (2004); (3) Bevan et al. (1980); (4) Rosenbusch and Weber (1971); (5) Morel et al. (1994); (6) Yee and Morel (1996); (7) Lane and Morel (2000a); (8) Lane et al. (2005); (9) Coleman (1998); (10) Speckhard et al. (1977).

† In vivo experiments.

‡ Cd-specific CA.

the diatom *T. weissflogii* are fully active (Rosenbusch and Weber 1971; Yee and Morel 1996). It appears that in *E. huxleyi*, Co and Zn can replace each other in the metalloproteins that account for the bulk of the Zn or Co requirement of the cell. The background concentration of Zn or Co is apparently enough to meet the Zn or Co demand of the metalloproteins that require Zn or Co exclusively and that must represent only a minor fraction of the corresponding metal quotas.

In contrast to Co, only some in vitro Cd-substituted proteins retain enzymatic activities, and their activities are almost always reduced relative to their Zn forms (Table 3; more examples in Vallee and Ulmer 1972, Table 4 and Table 5). In vivo Cd-substituted aspartate transcarbamylase

retains most of its activity in Zn-deficient *E. coli* cells (Speckhard et al. 1977). Therefore, in *E. huxleyi*, Cd replacement for Zn or Co in the major Zn or Co metalloproteins probably has two opposite effects: a beneficial effect when the Cd form of the protein retains part of its activity; a toxic effect when the Cd form of the protein loses activity. It is likely that the Cd-irreplaceable primary Zn or Co requirement in *E. huxleyi* includes proteins involved in nucleotide synthesis, such as DNA and RNA polymerase, since DNA synthesis is blocked in *S/G₂* in Zn-deficient *Euglena gracilis*, and Cd addition cannot initiate DNA synthesis (Falchuk et al. 1975). The most interesting question is that of the biochemical basis for the beneficial effect of Cd in *E. huxleyi*, which reflects presumably one or

Table 4. Estimated growth rates (d^{-1}) of *E. huxleyi* based on Zn, Cd, and Co concentrations in surface water in different regions.

Location	N. Pacific			N. Atlantic			Subantarctic		
	Zn	Cd	Co	Zn	Cd	Co	Zn	Cd	Co
Total M ($pmol L^{-1}$)	40–150 ^{(1-3)*}	2–40 ⁽⁴⁻⁵⁾	7.9–28 ⁽⁶⁾	40–106 ⁽⁷⁻⁹⁾	5–100 ⁽¹⁰⁾	5–131 ⁽¹⁰⁻¹²⁾	6–17 ⁽¹³⁾	4–7 ⁽¹³⁾	2–15 ⁽¹³⁾
M' ($pmol L^{-1}$)†	0.8–3	0.6–12		0.8–2.12	1.5–30		0.26–1.11 ⁽¹³⁾	0.29–0.85 ⁽¹³⁾	
Cellular M ($mmol mol^{-1} P$)	0.33–0.83	0.034–0.40‡		0.33–0.63	0.11–0.40‡		0.21–0.40	0.010–0.054	
Growth rate (d^{-1}) for minimum Zn	Zn only 0.68	Zn+Cd 0.76–1.21		Zn only 0.68	Zn+Cd 0.90–1.21		Zn only 0.03	Zn+Cd 0.1–0.32	

* (1) Lohan et al. (2002); (2) Bruland (1980); (3) Bruland (1989); (4) Bruland (1980); (5) Bruland (1992); (6) Martin et al. (1989); (7) Brand et al. (1983); (8) Bruland and Franks (1983); (9) Ellwood and Van Den Berg (2000); (10) Kremling and Streu (2001); (11) Saito and Moffett (2002); (12) Ellwood and Van Den Berg (2001); (13) Ellwood (2004).

† Assumes 98% of Zn and 70% of Cd are complexed by organic ligands (Bruland 1989, 1992).

‡ If Cd' is higher than 5 $pmol L^{-1}$, 5 $pmol L^{-1}$ is used to calculate cellular Cd concentration.

more proteins that are active with Cd and that account for about 50% of the Zn or Co requirement in this organism. Since *E. huxleyi* does not possess or require high CA activity (Nimer et al. 1994; Elzenga et al. 2000), it is possible that its use of Cd may represent a yet unknown Cd protein.

Implication for the ocean—We performed our culture experiments with *E. huxleyi* over a range of unchelated concentrations of Zn, Cd, and Co that correspond to those found in the oceans. We can thus estimate whether Zn, Cd, and Co interreplacement may indeed take place in the ocean on the basis of published data on trace metal concentrations and speciation in oceanic surface water. If no other nutrient is limiting, the minimum unchelated Zn concentrations in the North Pacific and North Atlantic oceans should allow *E. huxleyi* to grow at about half its maximum rate (Table 4). Under these conditions, Cd should significantly enhance the growth rate, up to 90% of the maximum (Table 4). The potential enhancement of growth by Co is difficult to estimate since the concentration of unchelated Co is poorly known. Nonetheless, it seems likely that Cd and Co replace Zn to support near maximal growth of *E. huxleyi* in the Zn-depleted waters of the North Pacific and North Atlantic oceans. In contrast, the subantarctic surface water east of New Zealand has the lowest Zn concentration ever reported (Ellwood 2004), which, by itself, should not support the growth of *E. huxleyi* at all. Accounting for the presence of Cd, we calculate that *E. huxleyi* may achieve a respectable growth rate, up to 20% of the maximum (Table 4). Surprisingly, no response of phytoplankton to Zn amendment has been observed there (Ellwood 2004). However, with the presence of 2 pmol L⁻¹ of total dissolved Co, it is very likely that Cd and Co replacement for Zn occurs in this region.

There is a paucity of field data on the biological roles of trace metals other than Fe in the open ocean. We also do not yet understand the biochemical mechanisms underlying the interreplacement of Zn, Cd, and Co in *E. huxleyi*. Nonetheless, it now clearly appears that the availability of these three metals should play a role in the ecology of this cosmopolitan coccolithophore.

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