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1 **Abstract**

2

3 Unchelated Zn concentrations (Zn') in surface oceanic water are often extremely
4 low, reaching values that are known to limit the growth of laboratory cultures of various
5 marine phytoplankton. Nonetheless, growth limitation by Zn has rarely been observed in
6 field populations, possibly as a result of the reduction in the Zn requirement of
7 phytoplankton by replacement of Zn by Cd and Co. Here we investigate the inter-
8 replacement of Zn, Cd and Co in the cosmopolitan coccolithophore *Emiliana huxleyi* by
9 examining its growth rate, cellular metal quotas and cellular metal uptake rates under
10 different combinations of Zn, Cd and Co concentrations in the medium. Co and Zn can
11 fully replace each other, except perhaps for small individual requirements that are met by
12 minute metal concentrations supplied as contaminants. Zn is used at 75% efficiency by *E.*
13 *huxleyi* compared to Co. In contrast, Cd can only partially replace Zn or Co and is used at
14 66% efficiency compared to Co. Up to 50% of the cellular Zn or Co can be replaced by
15 Cd, once the minimum cellular Zn/Co requirement is fulfilled. The limitation by
16 Zn/Co/Cd and the inter-replacement of the three metals occurs over a range of unchelated
17 metal concentrations –0.2-5 pM—that is relevant to surface oceanic waters. Based on our
18 laboratory results and the published unchelated metal concentrations in surface oceanic
19 water, we calculate that inter-replacement of Zn, Co and Cd should occur in regions
20 where Zn is highly depleted.

21

1 **Introduction**

2

3 Zinc serves as a cofactor for hundreds of metalloproteins involved in all aspects
4 of metabolism, and is known to be an essential micronutrient for marine phytoplankton.
5 Surface depletion and the presence of sub-picomolar concentrations of unchelated Zn in
6 oceanic water suggest that Zn can potentially be a limiting factor in the ocean (Bruland
7 1980, 1989; Bruland et al. 1978; Ellwood 2004; Lohan et al. 2002). Indeed, growth data
8 from pure laboratory cultures show that model species are limited by unchelated Zn
9 concentrations that have been reported for surface seawater (Anderson et al. 1978; Brand
10 et al. 1983; Sunda and Huntsman 1992, 1995). To date, however, only few field
11 enrichment experiments have shown any effect of Zn on phytoplankton productivity in
12 the open ocean and the effect that is seen is far less substantial than that of Fe, a known
13 limiting micronutrient in the oceans (Coale 1991; Coale et al. 2003; Crawford et al. 2003;
14 Ellwood 2004; Leblanc et al. 2005; Lohan et al. 2005). Elucidating the potential role of
15 Zn in the productivity of the oceans clearly requires a better understanding of the Zn
16 physiology of marine phytoplankton.

17

18 Laboratory experiments have shown that oceanic species such as *Thalassiosira*
19 *oceanica* and *Emiliania huxleyi* have much lower Zn requirement for growth than coastal
20 species (Brand et al. 1983; Sunda and Huntsman 1992). A part of this difference may
21 result from the efficiency with which oceanic species replace Zn by other metals for
22 biological functions. Except in marine cyanobacteria which have an absolute Co
23 requirement, Co and Zn are commonly inter-replaceable in marine phytoplankton (Sunda

1 and Huntsman 1995; Yee 1997; Price and Morel 1990; Saito et al. 2002). In the model
2 diatom *T. weissflogii*, a primary role of Zn and Co is to serve as metal cofactors in
3 carbonic anhydrase (CA), which is involved in inorganic carbon acquisition for
4 photosynthesis (Yee and Morel 1996; Lane and Morel 2000b). Cd has also been found
5 to serve as an alternative metal center in CA in diatoms; this is the only documented
6 biochemical function of Cd (Lane and Morel 2000a; Lane et al. 2005). The growth of
7 other phytoplankton, such as the chlorophyte *Tetraselmis maculata*, and the
8 prymnesiophyte *Pleurochrysis carterae*, has also been shown to be promoted by Cd
9 addition (Lee and Morel 1995), but the primary biochemical role of Cd has not yet been
10 identified in these species.

11

12 Cocolithophores are dominant eukaryotic phytoplankton in the open ocean and *E.*
13 *huxleyi* is numerically the most abundant cocolithophore, found in all except the polar
14 oceans (Brown and Yoder 1994; Winter et al. 1994). Several lines of evidence suggest
15 that, as in diatoms, Cd and Co replacement for Zn is a strategy adopted by *E. huxleyi* to
16 reduce its Zn requirement: 1) *E. huxleyi* has relatively high Cd quota compared with
17 diatoms when grown in the presence of Cd under Zn replete conditions (Sunda and
18 Huntsman 2000; Ho et al. 2003); 2) it has an increased Cd uptake rate under Zn limitation
19 (Sunda and Huntsman 2000); 3) it is able to grow in the absence of added Zn when Co is
20 included in the medium and has been found to have a higher maximal growth rate when
21 grown with Co alone than it does with Zn alone (Sunda and Huntsman 1995). However,
22 the available evidence for a beneficial effect of Cd on growth in *E. huxleyi* is ambiguous
23 (Lee and Morel 1995). Further, we do not know the primary role of Zn, Cd or Co in this

1 organism where CA activity is either absent or very low (Nimer et al. 1994; Elzenga et al.
2 2000).

3

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

9

1 **Methods**

2

3 *Cell Culture*

4 *E. huxleyi* strains CCMP373, CCMP374 and CCMP1949 were obtained from the
5 Provasoli-Guillard National Center for Culture of Marine Phytoplankton in Maine, USA,
6 strains DWN53/74/9 and CCMP1516 from the Plymouth culture collection of Marine
7 algae in UK, and strain A-1387 kindly provided by Dr. Larry Brand. Unless a specific
8 strain was indicated, strain CCMP374 was used for the experiments. All the culture
9 experiments were conducted in trace metal ion buffer system in acid-cleaned
10 polycarbonate bottles using 0.2 µm filtered Gulf Stream seawater (Price et al. 1988/1989;
11 Sunda et al. 2005). The culture media were enriched with chelexed and filter sterilized
12 150 µmol L⁻¹ NaNO₃, 10 µmol L⁻¹ PO₄³⁻ or 1 µmol L⁻¹ filter sterilized α-glycerophosphate
13 and 20 µg L⁻¹ thiamine. Free trace metal ion concentrations were controlled by 100 µmol
14 L⁻¹ EDTA, with the total concentrations of 87 nmol L⁻¹ Fe, 19.6 nmol L⁻¹ Cu, 100 or 50
15 nmol L⁻¹ Mn, 10 nmol L⁻¹ Se and varied concentrations of Zn, Co and Cd. Background
16 Zn, Cd and Co concentrations were estimated to be 0.9, 0.01 and 0.01 nmol L⁻¹ from the
17 seawater, respectively (Sunda and Huntsman 2000), and 1.0, 0.03 and 0.02 nmol L⁻¹ from
18 the nutrient stock solutions, respectively. The concentrations of inorganic metal species
19 in the medium were computed from the total metal concentration and the conditional
20 stability constants (Sunda et al. 2005). The cultures were grown at 20°C under continuous
21 light (80-100 µmol quanta m⁻² s⁻¹). Cells were acclimated in the medium without Zn, Cd
22 or Co for a week before transferring into experimental media. Cell numbers were counted

1 daily using a Multisizer II Coulter Counter, and the specific growth rates were then
2 computed during exponential growth.

3

4 *Metal quota measurement*

5 Cellular metal quotas were measured as described by Ho et al (2003) and Tang
6 and Morel (2006). Cells were harvested when the cell numbers reached 200,000 cells ml⁻¹.
7 About 100 ml of cell culture was filtered onto 1 µm acid-cleaned polycarbonate
8 membranes under low vacuum (~50 mm Hg) and rinsed five times with chelexed NaCl
9 solution. Filtered samples were then digested in Taflon tubes with 50% HNO₃ (Optima
10 grade, Fisher) at 100°C for 4 hours. After digestion, Milli-Q water was added into the
11 tube to give the final concentration of 5% HNO₃. Cellular concentrations of P, Ca, Zn, Cd
12 and Co were determined by ICPMS (*Element 2*, ThermoFinnigan), and were reported as
13 mmol metal to mol P.

14

1 **Results**

2

3 *Zn, Cd and Co inter-replacement for growth*

4 We examined the effect of Zn, Cd and Co on the growth rate of *E. huxleyi* strain
5 CCMP374 (Fig. 1 and Table 1). The growth rate at a Zn' of 0.8 pmol L⁻¹ in the absence
6 of Cd and Co was reduced to 50% of the maximum rate achieved at Zn' = 15 pmol L⁻¹
7 (0.65 vs. 1.33 d⁻¹). In the presence of 30 pmol L⁻¹ Cd' at 0.8 pmol L⁻¹ Zn', the growth rate
8 was enhanced to 70% of the maximum rate (0.92 vs. 0.65 d⁻¹, t-test, *p*<0.05)
9 demonstrating clearly that Cd is beneficial to the growth of Zn-limited *E. huxleyi*. In the
10 absence of Zn, the growth rate reached its maximum at a Co' of 15 pmol L⁻¹ (1.33 d⁻¹)
11 and additional Zn didn't affect the growth rate, indicating that Co can fully replace Zn for
12 growth. The difference in the effects of Cd and Co on growth suggests that Cd is less
13 efficient than Co as a replacement for Zn (see below).

14

15 We further examined the effect of Cd on growth rate over a range of Zn', from the
16 background level of 0.3 pmol L⁻¹ to 1.6 pmol L⁻¹ (Fig. 2), representing values found in
17 the open ocean (Bruland 1989; Ellwood 2004). With no Zn added to the medium
18 (background Zn_T ~ 1.9 nmol L⁻¹, corresponding to Zn' ~ 0.3 pmol L⁻¹), Cd addition had
19 no effect on the growth rate, indicating that *E. huxleyi* has a minimum Zn requirement
20 that cannot be replaced by Cd (Fig. 2). Cd was beneficial to the growth of Zn-limited
21 cells within the range of Zn addition tested (0.49 to 1.64 pmol L⁻¹ of Zn', Fig. 2) with a
22 growth rate enhancement from 0.2 to 0.4 d⁻¹. The presence of Cd had no significant effect
23 on growth when enough Zn was present to support the maximum growth rate.

1

2 The marked growth enhancement by Cd addition over a range of Zn²⁺ in the
3 medium suggests the presence of one or a few major Zn proteins whose biochemical
4 function(s) can be replaced by Cd-containing proteins. Since the previous experiments
5 had high Cd concentrations we conducted another set of experiments with constant Zn²⁺
6 (0.8 pmol L⁻¹) but various Cd²⁺ in the medium to evaluate the Cd requirement for growth
7 (Fig. 3). The growth rates and cellular Cd to P ratios increased with increasing Cd²⁺ (Fig.
8 3A and 3B). The growth enhancement by Cd was most significant when Cd²⁺ was below 5
9 pmol L⁻¹ and cellular Cd concentration below 0.4 mmol mol⁻¹ P (Fig. 3A and 3B). Further
10 increase in cellular Cd had little effect on growth (Fig. 3A and 3C). The cellular Cd to P
11 ratio and steady state Cd uptake rate increased linearly with Cd²⁺ in the medium (Fig. 3B
12 and 3D). These experiments suggest that a cellular Cd quota of 0.4 mmol mol⁻¹ P can
13 fulfill most of the biochemical functions of Cd related to growth. The cellular Zn to P
14 ratio decreased with increasing Cd²⁺ and became constant above Cd²⁺= 2pmol L⁻¹ (Fig. 3E).
15 Correspondingly the steady state Zn uptake rate may have decreased upon Cd addition
16 but the data are noisy (Fig. 3F). The minimum Zn quota of 0.2 mmol mol⁻¹ P observed at
17 high Cd²⁺ may correspond to an absolute Zn requirement that cannot be replaced by Cd.

18

19 *Zn, Cd and Co inter-replacement in different E. huxleyi strains*

20 *E. huxleyi* is a widely distributed species in the ocean with considerable variation
21 in cell physiology and coccolith morphology among strains isolated from different
22 regions (Young and Westbroek 1991; Vanbleijswijk et al. 1994; Paasche 2001). We
23 examined the growth responses of various *E. huxleyi* strains to Cd and Co addition under

1 Zn limited conditions (Table 1). The strains tested showed remarkable variations in
2 growth rate, Zn uptake rate and calcite formation under identical growth conditions. In
3 general, their growth rates were all limited by $Zn^* = 0.8 \text{ pmol L}^{-1}$ and were all enhanced
4 by Cd addition significantly ($p < 0.05$, t-test). The growth enhancement by Cd addition
5 ranged between 0.2 to 0.4 d^{-1} . For stains CCMP374, CCMP1516 and CCMP1949, cells
6 grown on Co alone yielded similar maximum growth rates as cells grown on Zn alone.
7 For strains CCMP374 naked cells, A1387 and DWN53/74/6, the maximum growth rates
8 were respectively 13%, 8% and 5% higher on Co alone than on Zn alone. Similarly,
9 Sunda and Hunstman found a 34% higher maximum growth rate when grown on Co only
10 compared to Zn only (Sunda and Huntsman 1995). These results clearly indicate that the
11 partial replacement by Cd and the full replacement by Co for Zn is not a strain-specific
12 but a general attribute of *E. huxleyi*.

13

14 The cellular Zn requirement for growth appeared rather uniform among strains
15 isolated from different regions with all cells growing at about 45% μ_{\max} at $Zn^* = 0.8 \text{ pmol}$
16 L^{-1} and corresponding cellular Zn quotas around 0.3-0.5 $\text{mmol mol}^{-1} \text{ P}$.

17

18 *Cellular Zn, Cd and Co requirements for growth and their relative use efficiencies*

19 To further understand the role of metal replacement in *E. huxleyi* physiology and
20 its consequence on the growth of the coccolithophore in the surface ocean, two questions
21 need to be answered: 1) What are the relative use efficiencies of Zn, Cd and Co in *E.*
22 *huxleyi*? 2) How much of the cellular Zn and Co can be replaced by Cd? We explored
23 these two questions by examining the relationship between the growth rate and cellular

1 Zn, Cd and Co quotas in a matrix of experimental Zn, Cd and Co concentrations (Fig. 4
2 and Table 2): two experiments varying Zn' or Co' with no other metal addition (Zn-only
3 and Co-only); an experiment varying Zn' with Cd' = 1.7 pmol L⁻¹ (Zn+Cd); an
4 experiment varying Co' with Cd' = 1.7 pmol L⁻¹ (Co+Cd); and an experiment varying
5 Zn' with Cd' = 1.7 pmol L⁻¹ and Co' = 0.3 pmol L⁻¹ (Zn+Cd+Co). The metal additions of
6 1.7 pmol L⁻¹ Cd' and 0.3 pmol L⁻¹ Co' were chosen to avoid luxury uptake of these
7 metals.

8
9 Consistent with our previous results, the growth rate increased as Zn' or Co'
10 increased and the addition of Cd augmented the growth rate considerably (Fig. 4A). To
11 analyze the data, we plotted the growth rate as a function of cellular Zn (Zn-only, Zn+Cd
12 and Zn+Cd+Co, Fig. 4B) and of cellular Co (Co-only and Co+Cd) (Fig. 4C). In all cases,
13 we observed a “hyperbolic” relation between growth rate and Zn or Co quotas with a
14 horizontal displacement toward lower quotas (for a given growth rate) upon addition of
15 Cd or Co. In addition, when the growth rate was limited, cells required a lower cellular
16 quota of Co than of Zn or any combination of Zn, Cd and Co to achieve a given growth
17 rate, showing that they use Co most efficiently for biological functions.

18
19 The “hyperbolic” shapes of the growth curves in Figures 4 B & C, suggested that
20 they may be fitted adequately by the empirical Droop equation (Droop 1983). For
21 quantifying the inter-replacement of the three metals Zn, Cd and Co, we thus attempted to
22 fit the data with the equation

23
$$\mu = \mu_m^* (Q - Q_{\min}) / Q \quad [1],$$

1 where $Q = Q_{Co} + \alpha Q_{Zn} + \beta Q_{Cd}$. In this equation, the specific growth rate (μ , d^{-1}) is given
2 as a function of the cellular concentration (Q , $mmol\ mol^{-1}\ P$) of the combination of Zn,
3 Cd and Co. The parameter μ_m (d^{-1}) is related to the maximum specific growth rate ($\mu_{max} =$
4 $\mu_m * (Q_{max} - Q_{min}) / Q_{max}$) and Q_{min} ($mmol\ mol^{-1}\ P$) is the minimum cellular nutrient
5 concentration necessary for maintaining life. The coefficients α and β for the cellular Zn
6 and Cd quotas reflect how efficiently these metals are used for cell growth compared with
7 Co. The equation using the overall parameter Q cannot be strictly correct since we know
8 that the cells do not grow on Cd in the absence of Zn and Co. But over a concentration
9 range where the minimum Zn/Co quota is satisfied, it should provide a good
10 approximation of the data. We determined what coefficients α and β best fit the data on
11 the basis of the matrix experiment (Table 2).

12

13 With a single metal present (Co-only and Zn-only), the data were adequately
14 fitted with $Q_{min} = 0.15\ mmol\ mol^{-1}\ P$, $\mu_m = 1.76\ d^{-1}$, and $\alpha = 0.75$. The corresponding
15 minimum quota for Zn ($0.15/0.75 = 0.2\ mmol\ mol^{-1}\ P$) is in good agreement with the data
16 obtained earlier (Fig. 3). With two metals present (Zn+Cd and Co+Cd), β was
17 determined as 0.74 using data from Expt. Zn+Cd and as 0.58 using data from Expt.
18 Co+Cd. It is not clear why there should be a difference in the coefficient for Cd use
19 efficiency when the primary requirement for growth is met by Co or by Zn, so to keep the
20 model general, we used 0.66, the mean of 0.74 and 0.58, as β . These coefficients gave an
21 acceptable fit of the data with all three metal present (Zn+Cd+Co) and the growth curves
22 for all experiments in Table 2 superpose each other when plotted as a function of $Q = Q_{Co}$
23 $+ 0.75\ Q_{Zn} + 0.66\ Q_{Cd}$ as shown in Fig 4D. Thus in a first approximation, our data

1 indicate that Zn is used at 75% efficiency compared to Co, and Cd at 66% efficiency
2 once the requirement for minimum cellular Zn or Co is fulfilled. To be more precise, in
3 the range of concentrations where the metals are limiting (and the minimum Zn/Co quota
4 is met), one needs to increase the cellular Zn quota 33% and the Cd quota 50% more than
5 the Co quota to obtain the same increase in growth rate.

6

7 The proportion of Co and Zn replaced by Cd can be calculated as

8 $\beta Q_{Cd}/(Q_{Co}+\beta Q_{Cd})$ and $\beta_{Zn}Q_{Cd}/(Q_{Zn}+\beta_{Zn}Q_{Cd})$, respectively (Table 2). Depending on the
9 cellular Zn and Co concentrations, Cd replaced up to 46% of cellular Zn and 44% of
10 cellular Co in the matrix experiment. These values presumably underestimate the
11 maximum replacement by Cd since Cd' was maintained at 1.7 pmol L^{-1} , below the
12 optimum for growth (see Fig. 3A). In the experiment of Fig. 3, the growth rate increased
13 with the Cd quota up to $0.4 \text{ mmol Cd mol}^{-1} \text{ P}$; this corresponds to 51% of the Q necessary
14 to achieve the maximum growth rate seen on Fig. 4D ($0.4 \times 0.66/0.52 = 0.51$). In the
15 same experiment, the Zn quota decreased to $0.2 \text{ mmol mol}^{-1} \text{ P}$ but the growth rate stayed
16 at 0.9 d^{-1} , about 75 % of μ_{\max} . The minimum Zn quota that supports full growth but
17 cannot be replaced by Cd at high Cd' is thus presumably on the order of $0.2 \times 4/3 = 0.27$
18 $\text{mmol Zn mol}^{-1} \text{ P}$, about 40 % of the Q necessary to achieve the maximum growth rate.
19 Hence, the proportion of Co and Zn that can usefully be replaced by Cd in *E. huxleyi* is
20 around 50-60%.

21

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

1 by the addition of Cd and Co at all (Fig. 5A); this suggests that the variations seen on fig
2 3F may be experimental noise. Further, Zn uptake rates were in general 60-96% of the
3 theoretical limiting rates for the diffusion of dissolved unchelated Zn. The Co uptake rate
4 showed similar pattern and was not affected by the addition of Cd or Zn either (Fig. 5B).
5 Co uptake rates were more than 80% of the theoretical limiting rates for the diffusion of
6 dissolved unchelated Co when Co' was less than 1 pmol L⁻¹. However, the difference
7 between the actual Co uptake rate and the theoretical diffusion rate became greater with
8 increasing Co', and the uptake rate was only 59% of the diffusion rate at Co' = 1.8 pmol
9 L⁻¹, presumably as the result of down-regulation of the Co uptake system. In general, the
10 uptake rates for Zn and Co were comparable, but the Cd uptake rate was about 3-fold
11 lower than those of Zn and Co for the same unchelated metal concentration (about 20%
12 of the computed maximum diffusion rate of unchelated Cd), and was not affected
13 substantially by variations in Zn' or Co'.

14

1 **Discussion**

2

3 *Inter-replacement of Zn, Cd and Co for growth*

4 Our growth experiments confirm that Zn and Co are important micronutrients that
5 can limit the growth of the coccolithophore *E. huxleyi*. These two metals are apparently
6 able to fully replace each other in their biochemical functions, although we cannot rule
7 out that some small individual requirements may be met by minute metal concentrations
8 supplied as contaminants in the cultures. Additions of Cd are able to alleviate Zn/Co
9 limitation on growth, indicating that Cd can replace some biochemical functions of
10 Zn/Co in *E. huxleyi*. However, there is a requirement of minimum cellular Zn or Co that
11 Cd cannot replace, suggesting that this organism uses some essential metalloproteins that
12 can use Zn or Co but not Cd as a cofactor. These Zn/Co proteins account for about 40-
13 50% of the Co/Zn requirement of non-limited cells. Similar inter-replacement of Zn, Cd
14 and Co are observed in a variety of *E. huxleyi* strains isolated from different regions of
15 the ocean. On the basis of our growth data, with strain CCMP374, we estimate that Zn is
16 used 25% less efficiently than Co and that Cd, which is beneficial only when the primary
17 requirement for Zn or Co is met, is used 33% less efficiently than Co.

18

19 In the only previous study to examine the effect of Cd on the growth of Zn-
20 limited *E. huxleyi*, only a very subtle effect was seen (0.59 d^{-1} at $0.16 \text{ pmol L}^{-1} \text{ Zn} + 4.6$
21 $\text{ pmol L}^{-1} \text{ Cd}$ VS. 0.52 d^{-1} at $0.16 \text{ pmol L}^{-1} \text{ Zn}$) and no firm conclusion could be drawn
22 (Lee and Morel 1995). The reason for the differences between our results and those of
23 this previous study are not clear but they may result from differences in the growth

1 conditions or in the strains that were tested. Differences in trace metal physiology among
2 strains are clearly documented in the results of Table 1, particularly in the fact that some
3 Co-replete cells grow at higher rate than Zn-replete cells, while others do not. Sunda and
4 Huntsman reported a higher maximum growth rate on Co than on Zn in the *E. huxleyi*
5 strain they tested (Sunda and Huntsman 1995).

6

7 It is notable that in the experiment reported in Fig. 2, the growth rates obtained at
8 various Zn' with a high Cd addition are less than μ_{max} . At $Zn' = 0.8 \text{ pmol L}^{-1}$, the growth
9 rate is in fact lower than seen in Fig. 3 for a more modest Cd addition. In retrospect, it
10 appears that the addition of enough Cd to achieve $Cd' = 82 \text{ pmol L}^{-1}$ in the culture
11 medium in this particular experiment is much higher than can be used by the cells and is
12 likely partially toxic to *E. huxleyi*.

13

14 *Metal use efficiency*

15 To verify that the Droop equation with a combined cellular quota for Zn, Cd and
16 Co is generally applicable, we calculated the growth rate based on the cellular Zn and Cd
17 when Cd' is below 5 pmol L^{-1} for the experiments presented in Fig 3. The calculated
18 growth rate is always between 95% and 120% of the measured growth rate, showing that
19 the Droop equation gives a reasonable description of the growth data and should thus
20 provide a fairly robust estimate of the relative use efficiencies for the three metals in
21 strain CCMP374. When Cd' is above 5 pmol L^{-1} , the calculated growth rate is 50 to 60%
22 higher than the measured growth rate, suggesting that there indeed may be a toxic effect
23 of Cd on growth at high level. However, we have to bear in mind that the model is

1 empirical and the actual values for the coefficients α and β may vary among strains.
2 Indeed, the strains tested in this study showed different Zn use efficiency (i.e. they grew
3 at different growth rates for similar concentrations of cellular Zn; see Table 1), and they
4 responded to different extent to Cd additions.

5
6 In contrast to *E. huxleyi*, diatoms like *T. oceanica* and *T. pseudonana* use Co less
7 efficiently than Zn (Sunda and Huntsman 1995). The difference in maximum growth rate
8 between Zn and Co is less pronounced in the oceanic diatom *T. oceanica* (μ_{\max} 25%
9 lower on Co than on Zn) than in the coastal diatom *T. pseudonana* (μ_{\max} 40% lower on
10 Co than on Zn). In terms of the actual metal use efficiency, the oceanic species *T.*
11 *oceanica* and *E. huxleyi* use Zn and Co more efficiently than the coastal species *T.*
12 *pseudonana*, but *T. oceanica* has higher Zn and Co use efficiencies than *E. huxleyi*
13 (Sunda and Huntsman 1995 and this study). For example, *T. oceanica* requires 0.04
14 mmol Zn mol⁻¹ P or 0.09 mmol Co mol⁻¹ P to achieve a growth rate of 0.9 d⁻¹ (Sunda and
15 Huntsman 1995), whereas *E. huxleyi* requires 0.4 mmol Zn mol⁻¹ P or 0.3 mmol Co mol⁻¹
16 P (this study). However, according to our data with strain CCMP374, *E. huxleyi* has
17 faster Zn and Co uptake rates than *T. oceanica* allowing it to accumulate higher cellular
18 quotas of these metals at a given concentration in the medium. The net result is that *E.*
19 *huxleyi* and *T. oceanica* grow at approximately the same rate at the same limiting ambient
20 concentration of Co or Zn (Fig. 6). We note, however, that Sunda and Huntsman
21 observed a slower metal uptake rate in their *E. huxleyi* strain (Sunda and Huntsman 1995),
22 resulting in a lower growth rate than *T. oceanica* under similar Co/Zn limitation.

23

1 Similar to *E. huxleyi*, there is a minimum Zn requirement that Cd cannot replace
2 in the diatoms *T. pseudonana* and *T. weissflogii* (Lee and Morel 1995). Compared with *E.*
3 *huxleyi*, *T. weissflogii* seems to use Cd more efficiently. For example, 0.1 mmol Cd mol⁻¹
4 P (8 amol Cd cell⁻¹) at Zn' of 3.2 pmol L⁻¹ could support 25% of μ_{\max} in *T. weissflogii*
5 (calculated from Lee et al. (1995)), whereas 0.4 mmol Cd mol⁻¹ P at Zn' of 0.5 pmol L⁻¹
6 support 23% of μ_{\max} in *E. huxleyi* (this study). The difference in metal usage between the
7 oceanic and coastal species and between different oceanic phytoplankton taxa is not only
8 reflected in a different requirement for a given metal to support growth (Brand et al. 1983;
9 Sunda and Huntsman 1992, 1995), but also in different relative metal use efficiencies.
10 And the availability of metals in different environmental regimes can act as a selective
11 force on cell physiology to determine the distribution and abundance of different
12 phytoplankton species.

13

14 *Uptake system and regulation*

15 It has been suggested that under Zn limitation, Cd and Co may be taken up by the
16 same transport system as Zn, or by a transport system that is negatively regulated by Zn
17 (Sunda and Huntsman 1995, 1998b, 2000). Our steady state uptake data obtained at
18 limiting concentrations of Zn, Cd or Co, when the transport systems are far from
19 saturation, showed no competition between the metals and no evidence of down-
20 regulation of the transport system of one metal upon increase in the concentration of
21 another. This is consistent with the results of Sunda and Hunstman (1995, 2000). Also
22 consistent with the results of Sunda and Huntsman (1995, 2000) are the fast metal uptake
23 rates that we observed: Zn and Co uptake rates within 60%-90% of the calculated

1 maximum rates for diffusion of unchelated Zn and Co and Cd uptake rate within 15-25%
2 of the maximum diffusion rate. These are remarkably fast rates since the diffusion
3 calculation is based on a zero concentration at the cell surface. Hudson and Morel (1993)
4 have calculated that, to approach the diffusion limit, cells must possess an extremely high
5 concentration of transport molecules and that ca 80% of the maximum diffusion rate is a
6 practical maximum for uptake. Although it seems unlikely, one may wonder if *E. huxleyi*
7 may be able to acquire some metals directly from the EDTA complexes. The relatively
8 low rate of Cd uptake, compared to Zn and Co, may reflect the different extent of
9 complexation of these metals by inorganic ligands in seawater: while Zn^{2+} and Co^{2+} are
10 present largely as hydrated cations, Cd^{2+} is almost entirely bound to Cl^- (97% as $CdCl^+$,
11 $CdCl_2$, $CdCl_3^-$ and $CdCl_4^{2-}$ (Morel and Hering 1993)). The negative charge of bound
12 chloride may slow down the diffusion of the complexes to a negatively charged cell
13 surface, or the dissociation of Cd^{2+} from $CdCl_x$ may slow down the binding to uptake
14 molecules. Our results support the notion that in trace metal-limited oceanic water,
15 phytoplankton species have optimized their physiology to achieve metal uptake rates
16 close to their maximum physical limits (Hudson and Morel 1993; Sunda and Huntsman
17 1995).

18

19 *Speculation on the biochemical mechanisms for different Zn, Cd and Co use efficiencies*

20 The underlying biochemical mechanisms for the inter-replacement of Zn, Cd and
21 Co in *E. huxleyi* and the differences in the use efficiency of these metals are not clear.

22 There are two possible ways for a metal to replace another: direct metal cofactor
23 substitution and protein replacement. For example, in Zn deficient diatom *T. weissflogii*,

1 Co may be used to substitute Zn directly in the metal center for TWCA1, whereas a Cd-
2 specific CdCA1 is induced as the replacement for TWCA1 (Yee and Morel 1996; Lane
3 and Morel 2000a, 2000b; Lane et al. 2005). We have no way at this point to distinguish
4 between these two mechanisms in *E. huxleyi*.

5
6 Co substitution for Zn in vitro almost always results in enzymatically active
7 proteins although the magnitude of the activity may be either increased or decreased
8 (Table 3) (Vallee and Galdes 1984; Vallee and Falchuk 1993). Furthermore, in vivo Co-
9 substituted RNA polymerase in Zn-deficient *E. coli* cells and Co-substituted carbonic
10 anhydrase in the diatom *T. weissflogii* are fully active (Rosenbusch and Weber 1971; Yee
11 and Morel 1996). It appears that in *E. huxleyi*, Co and Zn can replace each other in the
12 metalloproteins that account for the bulk of the Zn/Co requirement of the cell. The
13 background concentration of Zn or Co is apparently enough to meet the Zn or Co demand
14 of the metalloproteins that require Zn or Co exclusively, and which must represent only a
15 minor fraction of the corresponding metal quotas.

16
17 In contrast to Co, only some in vitro Cd-substituted proteins retain enzymatic
18 activities and their activities are almost always reduced relative to their Zn forms (Table 3;
19 more examples in Vallee and Ulmer (1972) Table 4 and Table 5). In vivo Cd-substituted
20 aspartate transcarbamylase remains most of its activity in Zn-deficient *E. coli* cells
21 (Speckhard et al. 1977). Therefore, in *E. huxleyi*, Cd replacement for Zn/Co in the major
22 Zn/Co metalloproteins probably has two opposite effects: a beneficial effect when the Cd
23 form of the protein retains part of its activity; a toxic effect when the Cd form of the

1 protein loses activity. It is likely that the Cd-irreplaceable primary Zn/Co requirement in
2 *E. huxleyi* includes proteins involved in nucleotide synthesis, such as DNA and RNA
3 polymerase since DNA synthesis is blocked in S/G₂ in Zn deficient *Euglena gracilis*, and
4 Cd addition can not initiate DNA synthesis (Falchuk et al. 1975). The most interesting
5 question is that of biochemical basis for the beneficial effect of Cd in *E. huxleyi*, which
6 reflects presumably one or more proteins that are active with Cd and which account for
7 about 50% of the Zn/Co requirement in this organism. Since *E. huxleyi* does not possess
8 or require high CA activity (Nimer et al. 1994; Elzenga et al. 2000; Brownlee and Taylor
9 2004), it is possible that its use of Cd may represent a yet unknown Cd protein,

10

11 *Implication for the ocean*

12 We performed our culture experiments with *E. huxleyi* over a range of unchelated
13 concentrations of Zn, Cd and Co that correspond to those found in the oceans. We can
14 thus estimate whether Zn, Cd and Co inter-replacement may indeed take place in the
15 ocean on the basis of published data on trace metal concentrations and speciation in
16 oceanic surface water. Here we use *E. huxleyi* as the model oceanic eukaryotic
17 phytoplankton, since it has similar growth rate as *T. oceanica* does at the same level of
18 unchelated Zn and Co. If no other nutrient is limiting the unchelated Zn concentrations in
19 the North Pacific and North Atlantic oceans, should allow *E. huxleyi* to at least grow at
20 half its maximum rate, indicating a modest Zn limitation (Table 4). When Zn is depleted,
21 Cd should enhance the growth rate by 7 to 42% of the maximum rate (Table 4). The
22 potential enhancement of growth by Co is difficult to estimate since the concentration of
23 unchelated Co is poorly known. Nonetheless, it seems likely that Cd and Co replace Zn to

1 support near maximal growth of *E. huxleyi* in the Zn-depleted waters of the North Pacific
2 and North Atlantic oceans. In contrast, the subantarctic surface water east of New
3 Zealand has the lowest Zn concentration ever reported (Ellwood 2004), which, by itself,
4 shouldn't support the growth of *E. huxleyi* at all. Accounting for the presence of Cd, we
5 calculate that *E. huxleyi* could only achieve at most 13% of its maximum growth rate
6 (Table 4). Surprisingly, no response of phytoplankton to Zn amendment has been
7 observed there (Ellwood 2004). However, with the presence of 2 pmol L⁻¹ of total
8 dissolved Co, it is very likely that Cd and Co replacement for Zn occurs in this region.

9

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

15

16

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- 18
- 19

Table 1. Effect of Zn, Co and Cd on growth rates and cellular Zn quotas in different *E. huxleyi* strains. n=2

Strain	Location of Isolation	Ca/P (mol mol P ⁻¹)	Growth rates (d ⁻¹) and cellular Zn quotas (in parenthesis, mmol mol ⁻¹ P)			
			0.8 pM Zn'	0.8 pM Zn' 20 pM Cd'	15 pM Zn'	15 pM 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
AI387	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.

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 ($\beta_{Zn}Q_{Cd}$ for Zn, $\beta_{Zn} = 0.77$; $\beta_{Co}Q_{Cd}$ for Co, $\beta_{Co}=0.66$); % of Zn or Co replaced by Cd (% of Zn= $\beta_{Zn}Q_{Cd}/(Q_{Zn}+\beta_{Zn}Q_{Cd})\times 100\%$, % of Co= $\beta_{Co}Q_{Cd}/(Q_{Co}+\beta_{Co}Q_{Cd})\times 100\%$) (see text). (β_{Zn} was determined as the best fit value when the growth curve of Zn+Cd superposes that of Zn.)

Exp.	M' concentration in medium (pmol L ⁻¹)			Growth rate (d ⁻¹)	Cellular metal quotas (mmol mol ⁻¹ P)			Cellular metal uptake rate (mmol mol ⁻¹ P d ⁻¹)			Amount of Zn or Co replaced by Cd (mmol/mol ⁻¹ P)	% of Zn or Co replaced by Cd
	[Zn']	[Cd']	[Co']		μ	Zn:P	Cd:P	Co:P	ρ_{Zn}	ρ_{Cd}		
Zn	0.6			0.47	0.32			0.15				
	0.9			0.67	0.34			0.23				
	1.3			0.91	0.40			0.37				
	1.7			1.07	0.50			0.53				
	2.1			1.15	0.62			0.71				
	2.5			1.23	0.69			0.85				
Zn+Cd	0.6	1.7		0.59	0.19	0.21		0.11	0.12		0.16	46
	0.8	1.7		0.84	0.27	0.19		0.22	0.16		0.14	35
	1.0	1.7		0.94	0.36	0.16		0.34	0.15		0.12	25
	1.3	1.7		1.04	0.36	0.13		0.38	0.13		0.099	21
	1.5	1.7		1.12	0.42	0.12		0.46	0.13		0.091	18
	1.8	1.7		1.18	0.43	0.098		0.51	0.12		0.075	15
Co			0.3	0.53			0.22			0.12		

		0.5	0.68		0.23		0.16			
		0.75	0.81		0.30		0.24			
		1	0.97		0.34		0.38			
		1.4	1.03		0.36		0.38			
		1.8	1.10		0.37		0.41			
Co+Cd	1.7	0.2	0.59	0.15	0.13	0.091	0.075	0.10		44
	1.7	0.4	0.82	0.14	0.17	0.12	0.14	0.094		36
	1.7	0.6	0.94	0.16	0.21	0.15	0.20	0.10		33
	1.7	0.8	1.02	0.14	0.24	0.15	0.24	0.095		28
	1.7	1	1.04	0.12	0.26	0.12	0.27	0.078		23
	1.7	1.25	1.10	0.14	0.32	0.15	0.35	0.092		22
Zn+Cd+Co	0.5	1.7	0.3	0.80	0.14	0.14	0.10	0.11	0.11	0.082
	0.7	1.7	0.3	0.97	0.18	0.15	0.088	0.17	0.15	0.085
	0.9	1.7	0.3	1.02	0.19	0.13	0.083	0.19	0.13	0.084
	1.1	1.7	0.3	1.07	0.40	0.11	0.083	0.42	0.12	0.088
	1.3	1.7	0.3	1.13	0.30	0.11	0.068	0.34	0.13	0.077
	1.5	1.7	0.3	1.16	0.37	0.096	0.066	0.43	0.11	0.077

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>E. coli</i>	N	Y (-)	1
δ-Aminolevulinate dehydratase	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. wessflogii</i>	Y #	Y (-) *	5, 6, 7, 12, 13
Carboxypeptidase A	Bovine	Y (-)	Y (+)	1, 2
Carboxypeptidase B	Porcine	Y	Y	1
Carboxypeptidase P	<i>S. griseus</i>		Y	1
b-lactmase	<i>B. fragilis</i>	Y (-)	Y (-)	8
Leucine aminopeptidase	Bovine		Y (+)	1, 9
Methionyl aminopeptidase	<i>E. coli</i>		Y	8
Neutral protease	<i>B. subtilis</i>		Y	1
RNA polymerase	<i>E. coli</i>		Y (-)	1, 10
Superoxide dismutase	Bovine	Y (-)	Y (-)	1, 11
Thermolysin	<i>B. thermoproteolyticus</i>	N	Y (+)	1, 2

* In vivo experiments # Cd specific CA

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 2000; 8 - Coleman 1998; 9 - Thompson and Carpenter 1976; 10 - Speckhard et al. 1977; 11 - Beem et al. 1974; 12 - Lane and Morel 2000a; 13 - Lane et al. 2005

Table 4. Estimated growth rate (d^{-1}) of *E. huxleyi* based on Zn, Cd and Co concentrations in surface oceanic 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 ⁽⁴⁻⁶⁾	7.9 – 28 ⁽⁷⁾	40-106 ⁽⁸⁻¹⁰⁾	5-100 ⁽¹¹⁾	5-131 ⁽¹¹⁻¹³⁾	6-17 ⁽¹⁴⁾	4-7 ⁽¹⁴⁾	2-15 ⁽¹⁴⁾
M' (pmol L⁻¹) *	0.8-3	0.6-12		0.8-2.12	1.5-30		0.26-1.11 ⁽¹⁴⁾	0.29–0.85 ⁽¹⁴⁾	
Cellular M (mmol mol⁻¹ P)	0.31-0.81	0.039-0.39 [#]		0.31-0.61	0.11-0.39 [#]		0.18-0.38	0.015-0.059	
Growth rate (d⁻¹)	Zn only	Zn+Cd		Zn only	Zn+Cd		Zn only	Zn+Cd	
Minimum Zn	0.61	0.71-1.18		0.61	0.86-1.18		0	0-0.18	
$\Delta\mu/\mu'_{\max}$ (%)^{&}		7-42%			19-42%			0-13%	
Maximum Zn	1.32	1.34-1.44		1.18	1.25-1.37		0.82	0.85-0.92	
$\Delta\mu/\mu'_{\max}$ (%)^{&}		1-9%			5-14%			2-7%	

* Assuming 98% of total dissolved Zn is complexed by organic ligands (Bruland 1989) and 70% of Cd complexed by organic ligands (Bruland 1992).

[#] If Cd' is higher than 5 pM, 5 pM is used to calculate cellular Cd concentration since *E. huxleyi* cells have luxury uptake when Cd' is higher than 5 pM.

[&] $\Delta\mu/\mu'_{\max}$ (%) reflects the enhancement of Cd on growth; $\Delta\mu = \mu_{Zn+Cd} - \mu_{Zn}$ and μ'_{\max} (apparent maximum growth rate) = 1.35 d^{-1} .
 1 – Lohan et al. 2002; 2 – Bruland 1980; 3 – Bruland 1989; 4 – Bruland et al. 1978; 5 – Bruland 1980; 6 – Bruland 1992; 7 – Martin et al. 1989; 8 – Brand et al. 1983; 9 – Bruland and Franks 1983; 10 – Ellwood and Van Den Berg 2000; 11 – Kremling and Streu 2001; 12 – Saito and Moffett 2002; 13 - Ellwood and Van Den Berg 2001; 14 – Ellwood 2004

Figure legend:

Fig 1. Growth curves for *E. huxleyi* cells grown with different metal additions in the media. Low Zn (\circ): 0.8 pmol L^{-1} of Zn' and no Co added; Low Zn+Cd (\blacksquare): 0.8 pmol L^{-1} of Zn' + 30 pmol L^{-1} of Cd' and no Co added; High Zn (\square): 15 pmol L^{-1} of Zn' and no Co added; High Co (\blacktriangle): 15 pmol L^{-1} of Co' and no Zn added.

Fig 2. Specific growth rates of *E. huxleyi* at different Zn' levels with (\circ) or without (\blacktriangle) 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' in media. 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' in media. Zn' = 0.8 pmol L^{-1} for all the treatments.

Fig 4. A. Specific growth rate of *E. huxleyi* as function of Zn' or Co' in media. B. Specific growth rate as function of cellular Zn : P ratio for Expt. Zn-only, Zn+Cd and Zn+Cd+Co. C. Specific growth rate as function of cellular Co : P ratio for Expt. Co-only and Co+Cd. D. Specific growth rate as function of combination of cellular α Zn : P ratio, cellular β Cd : P ratio and cellular Co : P ratio. ($\alpha = 0.75$, $\beta = 0.66$ in Expt. Zn+Cd, Co+Cd and Zn+Cd+Co, see text). The line shows the predicted growth rate by our Droop model.

Fig 5. A, B, C. Cellular uptake rate of Zn, Co and Cd in *E. huxleyi* as function of Zn' or Co' in media. Dotted lines gives the computed limiting rate for diffusion of Zn', Co' and Cd' to the cell surface, respectively. The maximum diffusion rate was calculated from the equation $\rho = 4\pi rD[M']$ (Hudson and Morel 1990). r , the cell radius, was calculated from the mean cell volume as 2.3 μm . 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 dissolved inorganic species.

Fig 6. A and B. Specific growth rate as function of cellular Zn : P ratio or cellular Co : P ratio. C and D. Specific growth rate as function of Zn' or Co' in media. E and F. cellular Zn or Co uptake rate as function of Zn' or Co' in media.

Ehux (Sunda) (●) and *T. oceanica* (▲): data for *T. oceanica* and *E. huxleyi* are calculated from Sunda and Huntsman (1995). Ehux (Xu) (□): data for *E. huxleyi* are from this study.

Fig. 1 Xu et al 2006

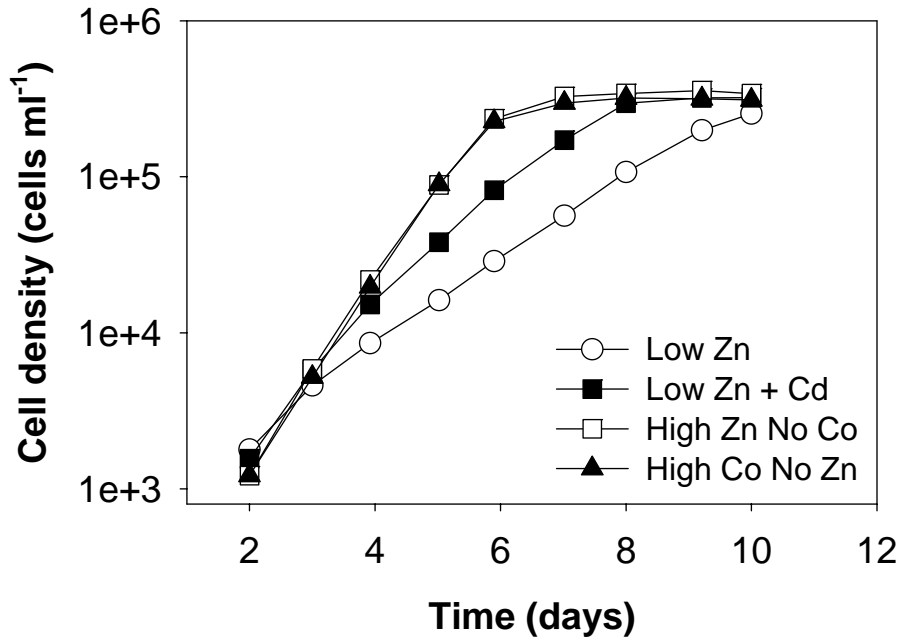


Fig. 2 Xu et al 2006

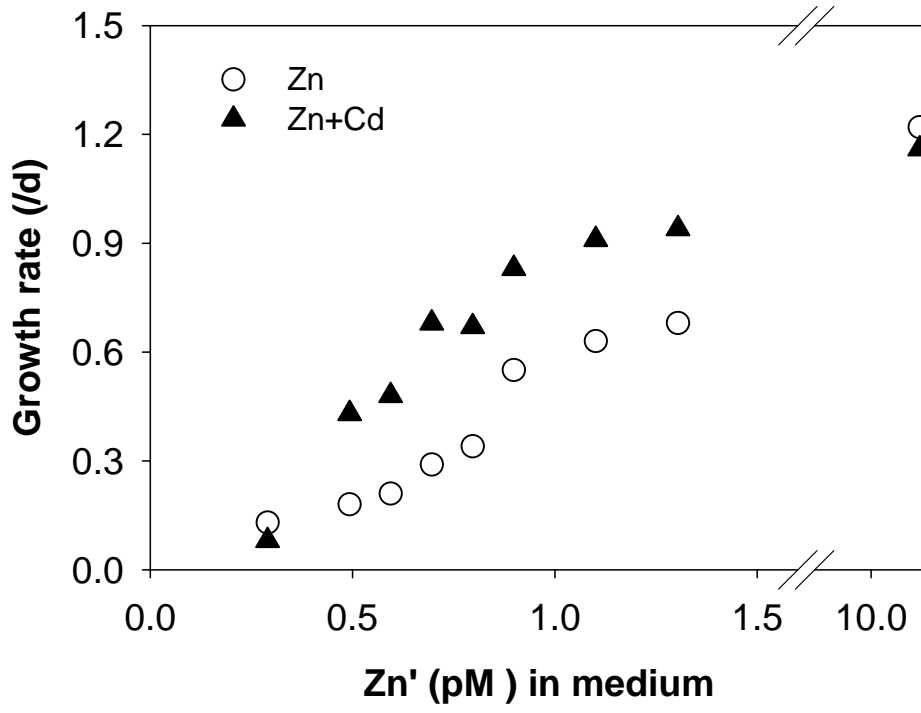


Fig. 3 Xu et al 2006

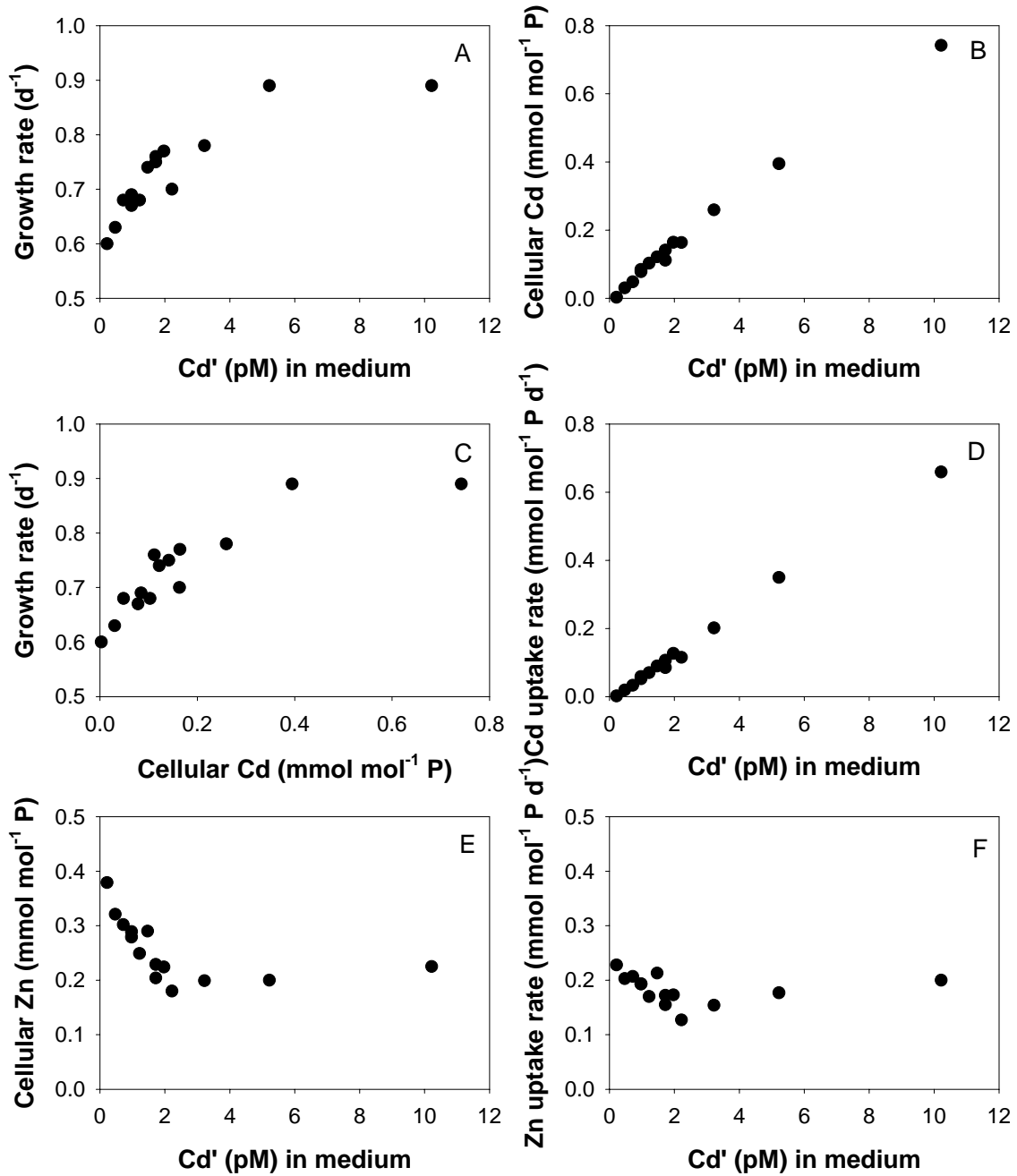


Fig. 4 Xu et al 2006

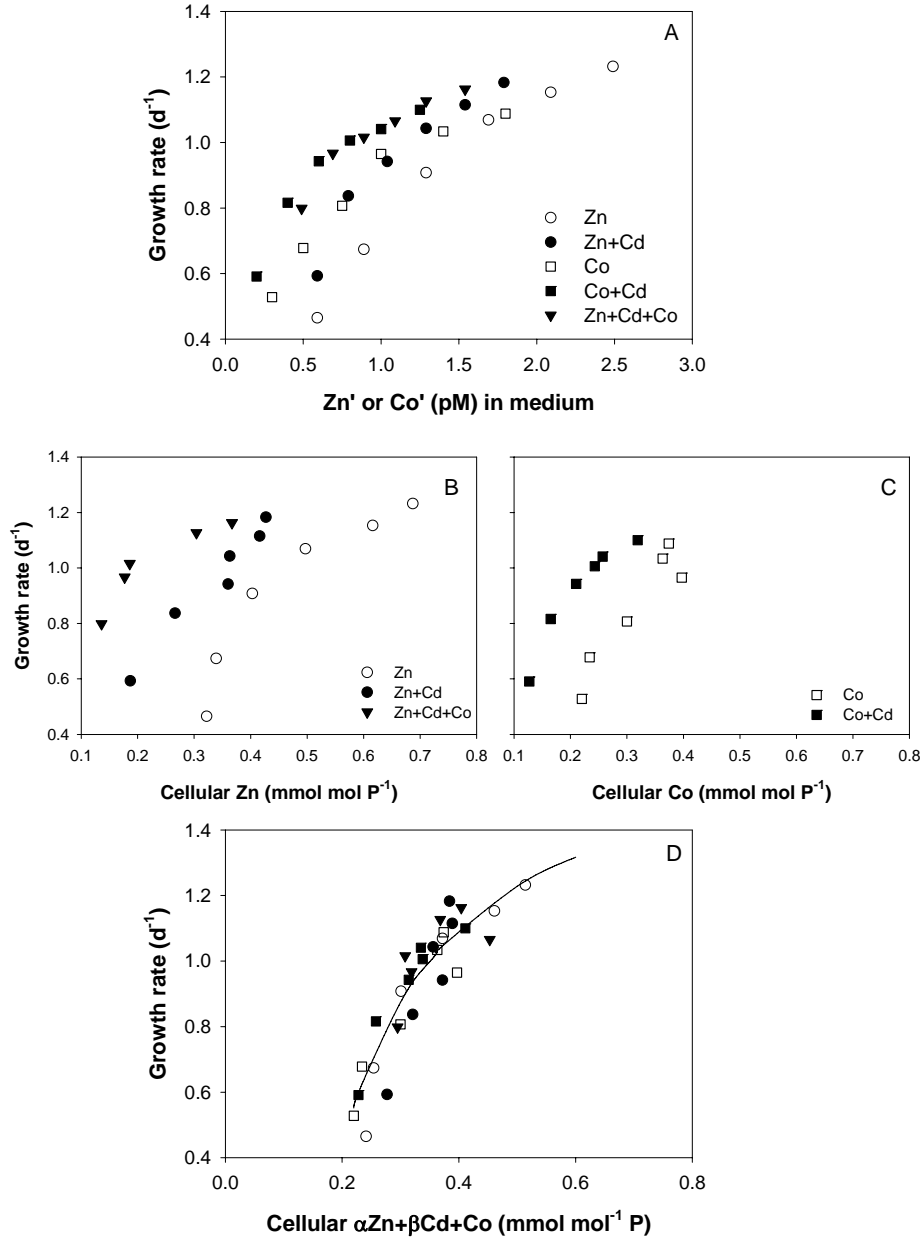


Fig. 5 Xu et al 2006

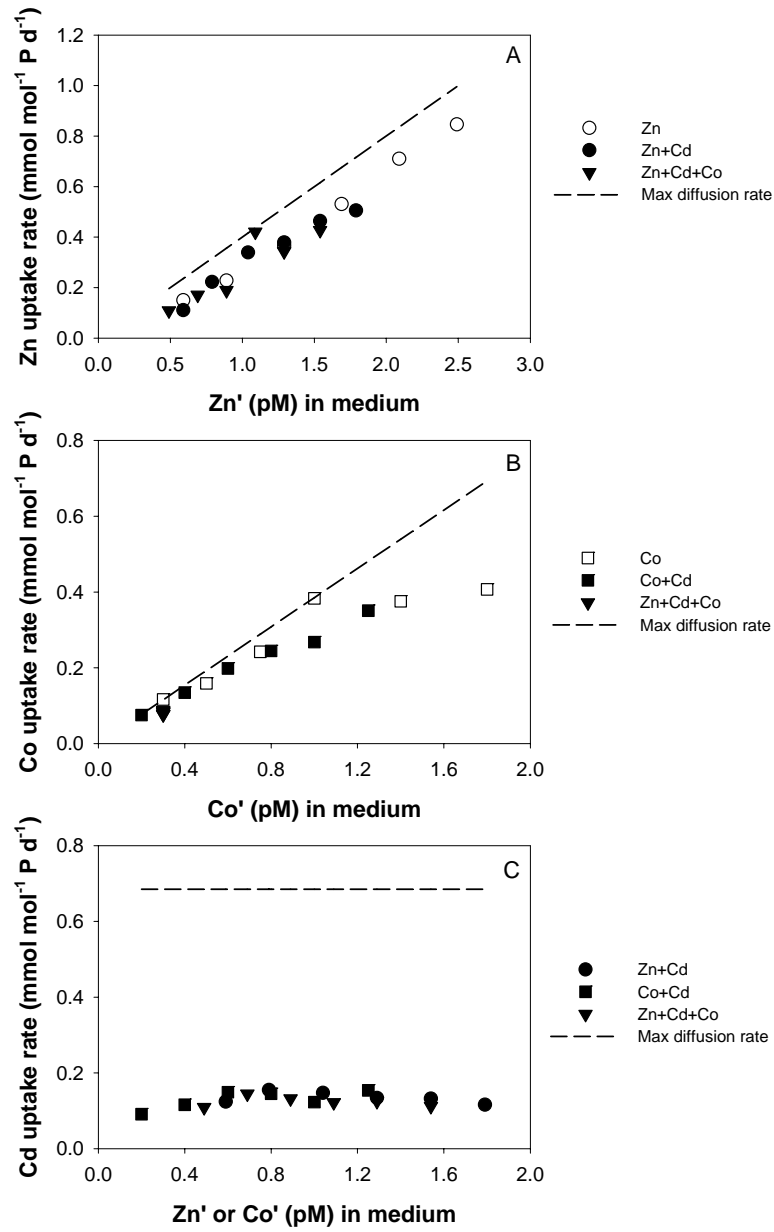


Fig. 6 Xu et al 2006

