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Marie Boye, Mohamed Adjou, Gabriel Dulaquais, Paul Tréguer. Trace metal limitations (Co, Zn) increase PIC/POC ratio in coccolithophore *Emiliana huxleyi*. *Marine Chemistry*, 2017, 192, pp.22-31. 10.1016/j.marchem.2017.03.006 . hal-02336829

**HAL Id: hal-02336829**

**<https://hal.science/hal-02336829>**

Submitted on 29 Oct 2019

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**Trace metal limitations (Co, Zn) increase PIC/POC ratio in  
coccolithophore *Emiliana huxleyi***

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**Boye et al., 2017. *Marine Chemistry*, 192, 22-31**  
<http://dx.doi.org/10.1016/j.marchem.2017.03.006>

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**Keywords:** trace metals (Zn, Co, Cd), *Emiliana huxleyi*, growth/calcification/photosynthesis, PIC/POC ratio, biological carbon pump.

## Abstract

The effect of cobalt (Co), zinc (Zn) or cadmium (Cd) availability on the growth and particulate inorganic (PIC) to organic (POC) carbon ratio of the cosmopolitan coccolithophore *Emiliana huxleyi* was examined using batch cultures. Growth co-limitation by Co and Zn occurred at low free metal ion concentrations below 10-12 pM. The two metals could replace one another in biochemical functions: full replacement of Zn by Co was possible, but the 20% higher growth rate observed with high Co levels in the absence of Zn than observed in the presence of high Zn but no added Co suggested that there was an absolute growth requirement of Co that could only partly be met by Zn. In contrast, Cd had no effect on growth in the absence of Co and Zn, indicating that this strain cannot use Cd under these conditions. The co-limitation by inorganic Co and Zn led to highly calcified cells containing a large quota of nitrogen, but diminished organic carbon content, likely due to a lower photosynthetic production rate. Deficiency of Zn and Co cofactors could have dramatically decreased the activity of carbonic anhydrase, lowering CO<sub>2</sub> availability for photosynthesis. In contrast to the large decreases in carbon fixation rates at low Zn and Co ion concentrations, calcification rates were unaffected at low Zn levels and were only minimally decreased by low Co. As a result the PIC:POC ratios in *E. huxleyi* increased by up to 8-fold under Zn limitation and 3-5-fold under Co limitation to values where coccolithophores act as a source of atmospheric CO<sub>2</sub>. As a result, low Co and Zn availability in seawater could affect the efficiency of the biological carbon pump, and atmospheric CO<sub>2</sub> levels. Thus, the impact of Co and Zn limitation may need to be considered to improve assessments of the impact of *E. huxleyi* and other biogenic calcifiers on oceanic sequestration of CO<sub>2</sub>.

1 **1. Introduction**

2 Our understanding of the effects of zinc (Zn), cobalt (Co) and cadmium (Cd)  
3 on oceanic productivity and the biological carbon pump is still limited, although we  
4 know these trace metals to be essential in many biochemical functions of  
5 phytoplankton (Frausto da Silva and Williams, 2000). They are indeed involved in a  
6 large variety of crucial functions, such as DNA-replication (Zn-fingers) and enzyme  
7 activation, Zn being present in ~300 enzymes (Vallee and Auld, 1990) and Cd in a  
8 native carbonic anhydrase in marine diatoms (Lane et al., 2005). They are also  
9 involved in the structure of vitamins, in which Co acts as a cofactor in vitamin B<sub>12</sub>  
10 (Kobayashi and Shimizu, 1999). Furthermore, the three metals, especially Zn, are linked  
11 to carbon metabolism through their involvement in the functioning and regulation of  
12 the enzyme carbonic anhydrase (CA) for utilization of bicarbonate, which is by far  
13 the most ubiquitous chemical species of inorganic carbon in ambient seawater  
14 (Morel et al., 1994; Yee and Morel, 1996). This enzyme accelerates the otherwise slow  
15 rate of conversion of bicarbonate into carbon dioxide (CO<sub>2</sub>), which is fixed in  
16 photosynthesis by Rubisco (Buitenhuis et al., 1999), and is an essential component of  
17 cellular CO<sub>2</sub> pumps. This enzyme may also be involved in the production of  
18 carbonate (Dreybrodt et al., 1997) used for calcification. Carbonic anhydrase occurs  
19 in most phytoplankton, including the cosmopolitan coccolithophore *Emiliana huxleyi*  
20 (Nimer et al., 1994; Nielsen, 1995; Riebesell et al., 1993).

21 Despite the higher abundance of Zn, Co, and Cd in *E. huxleyi* than in diatoms  
22 (Sunda and Huntsman, 1995, 2000; Ho et al., 2003; Twining and Baines, 2013), little is  
23 known about the effect of these metals on the growth of these calcifying species. For  
24 instance, the proportional biological utilization of dissolved Co and Cd, and the  
25 macronutrient phosphate (P) reported in different oceanic regions (de Baar et al.,  
26 1994; Saito et al., 2010; Boye et al., 2012; Dulaquais et al., 2014a) hints at these metals  
27 playing a key role in bloom-forming phytoplankton, notably in the blooms occupied  
28 seasonally by *Emiliana huxleyi* (Brown and Yoder, 1994). Culture experiments have  
29 also shown that the growth rate of coccolithophores can be limited by low free ion  
30 concentrations of Co and Zn, with maximum growth rates and sensitivities to metal  
31 limitation differing among species and strains (Brand et al., 1983; Sunda and  
32 Huntsman, 1992; Lee and Morel, 1995; Sunda and Huntsman, 1995; Yee and Morel,  
33 1996). *Emiliana huxleyi* requires Co to achieve maximum growth rate but this  
34 requirement is often only partly be met by Zn, an effect that appears to be strain  
35 specific (Xu et al., 2007). In contrast, the inter-replacement of Co and Zn in most  
36 biochemical functions appears to be a general attribute of the *E. huxleyi* species  
37 (Sunda and Huntsman, 1995; Xu et al., 2007). Intriguingly, *E. huxleyi* has a relatively

1 high internal quota of Cd compared with other phytoplankton (Sunda and  
2 Huntsman, 1995, 2000; Ho et al., 2003), whereas there is no identified biochemical  
3 native Cd function in this species. Additions of Cd have been shown to alleviate  
4 growth limitation by Co or Zn, by replacing some biochemical functions in *E. huxleyi*  
5 (Lee and Morel, 1995; Xu et al., 2007). However, there might be a minimum Zn or Co  
6 requirement that Cd cannot replace; hence, substitution of Cd for Zn or Co would  
7 only occur once this minimum requirement was fulfilled (Xu et al., 2007). To elucidate  
8 the effects of potential interplays of Co, Zn and Cd on growth of *E. huxleyi*; we first  
9 need to gain a better understanding of their individual effects.

10         Despite their role in the activation of carbonic anhydrase, only a very few  
11 studies have investigated the interactions between these metals and  
12 coccolithophore carbon metabolism, including the acquisition of inorganic carbon  
13 and production of organic carbon by photosynthesis and calcite by  
14 biomineralization. These studies partially investigated only the effect of Zn in *E. huxleyi*  
15 (Buitenhuis et al., 2003; Schulz et al., 2004), while the impact of Co and Cd on the  
16 carbon quotas and rates of calcification has not been examined. For instance, it has  
17 been suggested that Cd concentrations could modulate the calcification rate of *E.*  
18 *huxleyi* when the primary Zn requirement is met, but this might not be a general trend  
19 in coccolithophores (Xu et al., 2007). Variations in inorganic Zn levels have been  
20 shown to modify the efficiency of bicarbonate utilization by *E. huxleyi*, suggesting a  
21 pivotal role of Zn in modulating the activity of the carbonic anhydrase (Buitenhuis et  
22 al., 2003). Another study reported that low inorganic Zn concentrations led to the  
23 accumulation of calcite in *E. huxleyi* due to the decoupling of the growth limitation  
24 from the calcification rate (which was unaffected), with consequences for oceanic  
25 carbon cycling (Schultz et al., 2004). An increase in calcite content under Zn limitation  
26 can indeed impact the ratio of particulate inorganic to organic carbon export (e.g.  
27 the rain ratio), which is one of the factors determining CO<sub>2</sub> sequestration in the deep  
28 ocean (Archer, 1991; Ridgwell et al., 2009). It can also impact the export of carbon  
29 from the photic zone through ballasting of particulate organic carbon by calcite  
30 (Armstrong et al., 2002; Klaas and Archer, 2002). Some factors that control the  
31 production of organic and inorganic carbon in coccolithophores have been  
32 identified, such as elevated seawater CO<sub>2</sub> (acidification), which can increase overall  
33 photosynthesis (Beardall and Raven, 2004) but reduce calcification (Riebesell et al.,  
34 2000; Findlay et al., 2011; Krug et al., 2011). Another factor is global warming and  
35 consequent macronutrient limitation of phytoplankton growth (Riegman et al., 2000;  
36 Langer et al., 2012; Gerech et al., 2014). However, inorganic carbon acquisition for  
37 photosynthesis and calcification in coccolithophores is still a puzzle, to which the

1 three metals could offer a vital part of the solution through their involvement in  
2 carbonic anhydrase.

3 In this study, we examined the effect of Zn, Co and Cd on the growth of *E.*  
4 *huxleyi* in culture experiments designed to provide a quantitative basis for  
5 extrapolating to oceanic conditions. The three trace metals were tested separately  
6 to avoid trace metal substitution. We also investigated their impact on cellular  
7 carbon quotas (organic and inorganic) and on photosynthesis and calcification  
8 rates, to further assess their effect on carbon metabolism in *E. huxleyi*.

## 9 **2. Material and Methods**

### 10 **2.1. Cultures**

11 *Emiliana huxleyi* (strain CCMP371, clone 12-1) was obtained from the  
12 Provasoli-Guillard National Center for Marine Algae and Microbiota (Bigelow  
13 Laboratory for Ocean Sciences, West Boothbay Harbour, Maine, USA). This calcifying  
14 and axenic strain originating from the Sargasso Sea was maintained at 17°C in f/50  
15 medium. *Emiliana huxleyi* is a spherical unicellular haptophyte with a diameter  
16 ranging between 4 and 7 µm.

17 Experiments were conducted in batch culture in ultraclean and sterile  
18 conditions. Procedures for the cleaning and sterilisation of bottles and other  
19 equipment were as described in Sunda and Huntsman (1995). Chelexed synthetic  
20 seawater (Aquil) was prepared as described in Price et al. (1988/1989), and used for  
21 the culture medium at f/50 macronutrient concentrations with a mix of vitamin B<sub>12</sub>,  
22 biotin and thiamine added to a final concentration of 0.08 nM, 0.2 nM, and 60 nM,  
23 respectively. Experiments were conducted in duplicate in 2-L polycarbonate  
24 NALGENE® bottles at 15°C at a photon flux density of 200 µE m<sup>-2</sup> s<sup>-1</sup> on a 14/10 hours  
25 light/dark cycle. Ethylenediaminetetraacetic acid (EDTA) was used to control free  
26 trace metal ion concentrations in the media.

27 Cultures were bubbled continuously with filtered ambient air. Before the  
28 experiments were started, cells were conditioned to limiting metal concentrations by  
29 re-inoculating the cultures six times into new medium without Zn, Co and Cd  
30 additions. The cells were then transferred into experimental conditions 3 to 4 times  
31 during exponential growth until they reached a steady growth rate. Once cellular  
32 growth rate was steady, cultures were sampled during the exponential growth phase  
33 in the middle of the daily light period.

34

## 2.2. Trace metal speciation and ranges

In EDTA-containing culture media, dissolved trace metals were present as dissolved inorganic metal species and metal chelates with EDTA. As the metal-EDTA chelates are unavailable for metal uptake in such media, metal uptake and associated physiological effects are determined by the concentration of highly buffered dissolved inorganic metal species or free aquated metal ions. In the present work, we chose to relate our results to the free ion concentrations of the three metals studied (Co, Zn, and Cd) for easy comparison with previous studies.

Concentrations of the free metal ions  $[Zn^{2+}]$ ,  $[Co^{2+}]$  and  $[Cd^{2+}]$  were calculated from the total metal concentrations using an equilibrium complexation model that we adapted from MINEQL+. The stability constants of the metal-EDTA complexes, as well as those of inorganic metals complexes, were defined from the IUPAC database of physical constants (Pettit and Powell, 2001). These constants were corrected for the ion activity coefficients in seawater (Millero and Pierrot, 1998). The computed ratio of free ionic metal to the total concentrations of the metal (mol mol<sup>-1</sup>) was  $10^{-3.97}$  for Zn,  $10^{-3.77}$  for Co, and  $10^{-3.70}$  for Cd. Background metal concentrations in the synthetic seawater cleaned through Chelex were checked using HR-ICP-MS measurements with an Element 2 (Rodushkin and Ruth, 1997), and yielded undetectable values since they were below the detection limit of the analyses (e.g.,  $[Zn] < 0.02$  pM;  $[Co] < 0.03$  pM;  $[Cd] < 0.04$  pM). The free metal ion contribution from the initial media before the addition of EDTA was thus considered negligible.

To avoid artefacts from the potential substitution of one metal for another in certain enzymes, single metal additions were conducted without the two non-tested metals. Six concentrations were then selected for each metal. The trace metal stock solutions were made by diluting atomic absorption standard solutions (SIGMA-ALDRICH) with deionized and distilled water (18 MΩ MilliQ). The EDTA concentration was 100 μM in the final media. The selected range of concentrations for the trace metals encompassed the range of metal concentrations found in surface seawater (Bruland, 1992; Ellwood and van den Berg, 2000; Ellwood and van den Berg, 2001; Ellwood, 2004; Ellwood et al., 2005; Bown et al., 2011; Boye et al., 2012; Dulaquais et al., 2014a) and was based on previous culture experiments using *E. huxleyi* (Sunda and Huntsman, 1995; Buitenhuis et al., 2003). The concentrations tested for  $[Co^{2+}]$  were: 0.05, 0.20, 0.45, 3.05, 11.70 and 63.75 pM; for  $[Zn^{2+}]$ : 0.20, 0.40, 1.00, 6.00, 10.00 and 37.00 pM; and for  $[Cd^{2+}]$ : 0.20, 0.40, 1.00, 6.00, 10.00 and 48.00 pM. A positive control (PC) was set up under replete Zn, Co, and Cd conditions ( $[Co^{2+}] = 15$  pM,  $[Zn^{2+}] = 20$  pM, and  $[Cd^{2+}] = 13$  pM). Additionally, a negative control (NC) was set up

1 to test the impact of Zn, Cd and Co starvation, so *E. huxleyi* was acclimated  
2 gradually to the medium without additions of any of these three metals for more than  
3 four generations. Another three experiments were also conducted to examine the  
4 physiological response of *E. huxleyi* to the direct addition of metals. These three  
5 experiments were made by individually adding one of the three metals at replete  
6 concentration to a culture previously conditioned to grow in media to which none of  
7 the three metals had been added (e.g.; cultures acclimated to deplete conditions).

### 8 **2.3. Growth rate**

9 Cells were counted daily using both a Beckman Coulter (Z2 Coulter Counter) and an  
10 optical microscope. The growth rate ( $\mu$ ; in day<sup>-1</sup>) was then calculated as the slope of  
11 the following linear-equation:

$$12 \quad \text{Ln} (d) = \mu * t + B \quad (1)$$

13 where  $t$  is the time of incubation (in day),  $d$  the cell density (in cells/mL) and B the y-  
14 intercept ( $\sim \text{Ln} (d)$  at  $t_0$ ).

### 15 **2.4. Cellular contents and rates of calcification and photosynthesis**

16 Total particulate carbon (TPC), particulate organic carbon (POC) and  
17 particulate organic nitrogen (PON) were analysed from a sample of 100 to 500 ml  
18 (depending on the cell density) of the culture filtered onto glass fiber filters (Whatman  
19 GF/F) precombusted at 450°C for 4 hours. The filters were stored at -20°C, and dried  
20 for 12 hours at 60°C before analyses. POC and PON were analysed after fuming of  
21 the filter with concentrated HCl for 4 h in a desiccator and drying at 60°C in an oven  
22 as described in Le Moigne et al. (2013). POC and PON were measured using a Carlo  
23 Erba Analyzer 1500. TPC was analysed using the same protocol and method, but  
24 without fuming the filters. The concentrations of particulate inorganic carbon (PIC)  
25 were estimated from the difference between TPC and POC, and represented the  
26 amount of calcium carbonate (CaCO<sub>3</sub>) in *E. huxleyi* (Schulz et al., 2004). Production  
27 rates of calcium carbonate by calcification and of POC by photosynthesis were then  
28 estimated by multiplying the specific growth rate by the cellular quotas of PIC and  
29 POC, respectively. Owing to the experimental approach used in this work, growth  
30 rates and cellular carbon contents did not change significantly over the course of the  
31 experiment, thus allowing such estimates.

32 Chlorophyll-a (Chl-a) was determined after filtration of 50 to 100 ml of culture  
33 on GF/F filters. The filters were placed in 90% (v/v) acetone/water and homogenized  
34 in a cell for a minimum of 4 h followed by a centrifugation. Chl-a level was then



1 measured by fluorescence detection using a TURNER Design 10-AN Fluorimeter. The  
2 detection limit for Chl-a measurement was 0.075 mgL<sup>-1</sup>. Chl-a analyses were  
3 calibrated with pure Chl-a from spinach (Sigma) and concentrations were  
4 calculated according to Lorensen (1966).

## 5 **2.5. Statistical analysis**

6 All experiments were performed in duplicate, and analyses were performed  
7 twice in each replicate bottle. The standard errors reported ( $\pm$  S.E.) are thus based on  
8 an average of four measurements. Paired two-tailed t-tests were used to test the  
9 data for significant differences between pairs of means at the 95% confident interval  
10 ( $p < 0.05$ ).

## 11 **3. Results and discussion**

### 12 **3.1. Effect on growth**

13 We examined the impact of free Zn<sup>2+</sup>, Co<sup>2+</sup>, or Cd<sup>2+</sup> concentrations on the  
14 growth of *E. huxleyi* separately, in the absence of the other two metals (Table 1). The  
15 growth rates ( $\mu$ ) obtained at the various ion concentrations of Zn seemed to fit with a  
16 saturation curve, unlike the growth rates recorded at the various Cd<sup>2+</sup> concentrations,  
17 which remained constant and low (Table 1). The growth rates plotted as a function of  
18 log (Me<sup>2+</sup>) showed a sigmoid curve for Co<sup>2+</sup> (Figure 1), suggesting that the model  
19 saturation equation may actually not fit the data at low Co<sup>2+</sup>. The curvature observed  
20 at these low levels could suggest that there is a minimum Co<sup>2+</sup> requirement for  
21 growth.

22 The maximum specific growth rates ( $\mu_{\max}$ ) and the half-saturation constant  
23 values of Me<sup>2+</sup> ( $K_m$ ) obtained when  $\mu = \mu_{\max}/2$  were estimated by using non-linear  
24 regression method of the growth rates as the function of Me<sup>2+</sup> concentrations. The  
25 maximal growth rate of *E. huxleyi* obtained with this method for Co<sup>2+</sup> ( $\mu_{\max-\text{Co}} = 0.71 \text{ d}^{-1}$ )  
26 was reduced by 50% at Co ion concentrations below 0.55 pM ( $K_{m-\text{Co}}$ ). Cell division  
27 was also severely reduced by low free Zn<sup>2+</sup> levels below the  $K_{m-\text{Zn}}$  value of 0.51 pM,  
28 but led to a lower maximum growth rate with replete Zn<sup>2+</sup> conditions above 10–11 pM  
29 ( $\mu_{\max-\text{Zn}} = 0.57 \text{ d}^{-1}$ ) compared with replete Co<sup>2+</sup> levels above 12 pM (Figure 1). The half-  
30 saturation constants obtained for Zn and Co in these experiments are in good  
31 agreement with previous studies using other strains of *E. huxleyi* (Sunda and  
32 Huntsman, 1995; Schulz et al., 2004; Xu et al., 2007). The growth rate of the *E. huxleyi*  
33 strain we used (strain CCMP371) was co-limited by Co<sup>2+</sup> and Zn<sup>2+</sup> since both free  
34 metal ion concentrations needed to be reduced to very low values to limit growth

1 rate to near-zero values. Furthermore, the addition of either  $\text{Co}^{2+}$  or  $\text{Zn}^{2+}$  at a  
2 sufficiently high concentration to cultures of *E. huxleyi* acclimated to deplete  
3 conditions drove a rapid increase (in less than 1-2 days) in the growth rate to values  
4 akin to the maximal values observed in the kinetic growth curves under Co-replete or  
5 Zn-replete conditions (Figure 1). This result suggests that *E. huxleyi* could benefit from  
6 an input in  $\text{Co}^{2+}$  or  $\text{Zn}^{2+}$  for growing, even after a long period of starvation.

7         The 20% higher growth rate observed with high  $\text{Co}^{2+}$  levels in the absence of Zn  
8 than observed in the presence of high  $\text{Zn}^{2+}$  but no added Co (Figure 1) suggests that  
9 there was an absolute growth requirement of Co that could only partly be met by Zn.  
10 Location and possible inter-substitution of Co and Zn in important biomolecules in *E.*  
11 *huxleyi* are reported in Table 2. Because Co and Zn had the ability to substitute for each  
12 other (partially or fully), the co-limitation of the growth by  $\text{Co}^{2+}$  and  $\text{Zn}^{2+}$  was a type II co-  
13 limitation (biochemical substitution co-limitation) according to Saito et al. (2008b). A  
14 similar specific requirement for Co for optimal growth that can only be partly met by Zn  
15 has been previously reported for other strains of *E. huxleyi* (Sunda and Huntsman, 1995;  
16 Jakuba et al., 2008a). For some other strains, however, the maximum growth rate  
17 produced a similar value with either Co or Zn (Xu et al., 2007), suggesting that the use  
18 and requirement of Co and Zn could be strain dependant.

19         In the Cd experiments without added Zn and Co, the growth rate remained  
20 low ( $\mu_{\text{max-Cd}} = 0.07 \text{ d}^{-1}$ ) regardless of the  $\text{Cd}^{2+}$  concentration (Figure 1). This growth rate  
21 was similar to the negative control treatment in which cells were grown without any  
22 of these three metals added to the medium ( $\mu_{\text{NC}} = 0.06 \text{ d}^{-1}$ ; Figure 1). Thus, Cd had no  
23 impact on the growth of the CCMP371 strain in the absence of Zn and Co. This  
24 apparent lack of a Cd requirement for *E. huxleyi* is unexpected since this species has  
25 previously been seen to have a relatively large quota of Cd (Sunda and Huntsman,  
26 2000; Ho et al., 2003). The often nearly linear relationship between dissolved Cd and  
27 phosphate observed in the ocean (Boyle et al., 1976; Frew and Hunter, 1992; Boye et  
28 al., 2012) also suggests that phytoplankton have a biological requirement for Cd, as  
29 has been demonstrated for a diatom for the activation of a Cd-specific carbonic  
30 anhydrase (CDCA1; Lane et al., 2005). However, there is still no *in vivo* evidence of  
31 the use of Cd in a known biochemical function in *E. huxleyi* (Table 2). Nevertheless, a  
32 previous study indicated that Cd can partially substitute for Zn or Co in several *E.*  
33 *huxleyi* strains, once the minimum requirement for Zn or Co has been fulfilled (Xu et  
34 al., 2007). Because of this, we do not exclude the non-viability of some *E. huxleyi* cells  
35 in Cd-experiments due to the absence of subsistence levels of Zn and Co. Our Cd-  
36 experiments suggest that the relatively high cellular Cd quota measured in  
37 coccolithophores (Sunda and Huntsman, 1995, 2000; Ho et al., 2003; Xu et al., 2007)

1 does not necessarily indicate biochemical utilization of Cd. For instance, the  
2 accumulation of Cd could be the effect of Zn-transporters being unable to  
3 differentiate Cd from Zn (and thus transporting Cd into the cell), as has been found  
4 previously in *E. huxleyi* (Sunda and Huntsman 2000).

### 5 **3.2. Effect on cellular organic contents**

6 The cellular content of organic carbon (POC) decreased significantly (t-test,  $p$   
7  $< 0.01$ ,  $n = 4$ ) with decreasing  $\text{Co}^{2+}$  concentration from a plateau (t-test,  $p < 0.05$ ,  $n =$   
8 4) of about  $12.8 \text{ pg C cell}^{-1}$  at roughly  $3 \text{ pM Co}^{2+}$  (Table 1; Figure 2a). It also  
9 decreased significantly (t-test,  $p < 0.01$ ,  $n = 4$ ) with decreasing  $\text{Zn}^{2+}$  concentration  
10 from a maximum of  $18.9 \text{ pg C cell}^{-1}$  at roughly  $6 \text{ pM Zn}^{2+}$ . Although the organic  
11 content also seemed to decrease progressively from this maximum down to  $14.1 \text{ pg}$   
12  $\text{C cell}^{-1}$  with increasing  $\text{Zn}^{2+}$  levels, this decrease was not significant (t-test,  $p > 0.23$ ,  $n =$   
13 4). The change in POC quota at low  $\text{Co}^{2+}$  or  $\text{Zn}^{2+}$  levels was accompanied by a similar  
14 change in POC production (Table 1; Figure 2b). The production of POC indeed  
15 decreased significantly (t-test,  $p < 0.01$ ,  $n = 4$ ), with decreasing  $\text{Co}^{2+}$  or  $\text{Zn}^{2+}$   
16 concentration from maxima of  $8.2$  and  $9.2 \text{ pg C cell}^{-1} \text{ d}^{-1}$  at  $12 \text{ pM Co}^{2+}$  and  $10 \text{ pM}$   
17  $\text{Zn}^{2+}$ , respectively (Figure 2b). Hence, the observed decrease in cellular POC content  
18 was due to an impact on production rate of POC by photosynthesis, whereby slow  
19 growing cells became empty of organic carbon under these  $\text{Co}^{2+}$  or  $\text{Zn}^{2+}$  deplete  
20 conditions. Light limitation can similarly lead to a decrease in organic production by  
21 *E. huxleyi*, resulting, for example, in a decline of its cellular lipid content, which  
22 amounts to 45 to 60% of cellular POC, by a factor of 2.5 (Fernandez et al., 1996). The  
23 effect of low  $\text{Zn}^{2+}$  and  $\text{Co}^{2+}$  on organic carbon production suggests that these metals  
24 could play a key role in the photosynthesis process. It is unlikely that this role is due to  
25 a change in chlorophyll-a synthesis since similar Chl-a levels ( $\sim 0.13 \text{ pg cell}^{-1}$ ) were  
26 detected independently of these metal concentrations (Table 1). Among the  
27 important biomolecules in *E. huxleyi* in which Zn and Co are involved and/or  
28 substitute each other (Table 2), it has been suggested that a significant portion of the  
29 cellular quota of Zn and Co in eukaryotic phytoplankton can actually serve as a  
30 cofactor for carbonic anhydrase (CA) (Saito et al., 2008b). The CA in the chloroplasts  
31 of *E. huxleyi* (Nimer et al., 1994) converts  $\text{HCO}_3^-$  into  $\text{CO}_2$  at the site of  $\text{CO}_2$  fixation by  
32 Rubisco, the first enzyme involved in assimilation of  $\text{CO}_2$  in the Calvin cycle of  
33 photosynthesis. Because Rubisco has a low affinity for  $\text{CO}_2$  and because dissolved  
34  $\text{CO}_2$  ordinarily represents less than 1% of the total dissolved inorganic carbon (DIC) in  
35 seawater, the binding of  $\text{CO}_2$  to Rubisco can often constitute the limiting step in  
36 photosynthesis. On the other hand,  $\text{HCO}_3^-$  represents about 90% of the DIC in  
37 seawater and the conversion of  $\text{HCO}_3^-$  to  $\text{CO}_2$  by CA can maintain high rates of

1 inorganic carbon fixation by Rubisco, even under the low dissolved CO<sub>2</sub>  
2 concentrations in seawater. This mechanism, which would involve activation of CA by  
3 a cofactor to supply CO<sub>2</sub> for photosynthesis, was first hypothesized by Buitenhuis et al.  
4 (2003) who tested Zn and bicarbonate co-limitation on *E. huxleyi*. The decline we  
5 observed in the production of POC at low Zn and Co concentrations can, therefore,  
6 be conceivably due to low availability of CO<sub>2</sub> for photosynthesis, since the deficiency  
7 of Zn and Co cofactor would dramatically decrease the CA activity. Interestingly,  
8 higher organic carbon content and production rate were generally found with Zn  
9 rather than with Co (Figure 2a-b), suggesting an overall higher CA affinity for Zn in  
10 strain CCMP371. Furthermore, the absence of Cd effect on cellular POC production  
11 in the absence of Zn and Co (Table 1) suggests that this strain did not have a CA  
12 homologous to that of diatom CDCA1, which is known to use Cd (Lane et al., 2005).  
13 The absence of CDCA1-like has been also observed in another four strains of *E.*  
14 *huxleyi* (Park et al. 2007). Our result also suggests that Cd cannot replace Zn or Co in  
15 the CA, in line with the DNA sequencing of  $\delta$ -EhCA1 in *E. huxleyi* (Soto et al., 2006)  
16 that is a close homologue of TWCA1 found in diatoms and known to use Zn or Co as  
17 a cofactor (Roberts et al., 1997).

18 Contrary to organic carbon, nitrogen accumulation was observed in the cells  
19 (t-test,  $p < 0.01$ ) at low concentrations of Co<sup>2+</sup> and Zn<sup>2+</sup> (Figure 2c). Indeed, the  
20 amount of organic nitrogen per cell (PON) increased by about 3 times when Co<sup>2+</sup>  
21 and Zn<sup>2+</sup> decreased from 3 and 1 pM to 0.05 and 0.2 pM, respectively. The  
22 decoupling of growth (Figure 1) from organic nitrogen synthesis likely led to this  
23 accumulation under Co and Zn deplete conditions. The acquisition of nitrogen was  
24 probably not affected by growth limitation by Co and Zn. Similar accumulation of  
25 organic nitrogen was previously observed in *E. huxleyi* grown under low Zn conditions  
26 (Schulz et al., 2004).

27 As a result of PON accumulation and POC decline, the organic N to C ratio  
28 increased significantly (t-test,  $p < 0.01$ ) when growth was Co<sup>2+</sup> and Zn<sup>2+</sup> limited (Figure  
29 2d). Severe limitation by Co<sup>2+</sup> and Zn<sup>2+</sup> indeed led to a ~6 fold increase in PON/POC  
30 ratio from 0.05–0.06 mol N per mol C up to 0.37 mol N per mol C (Table 1). The N/C  
31 ratios of *E. huxleyi* reported in the literature range broadly from 0.07 to 0.27 mol N per  
32 mol C, depending on the study conditions and strain (Fernandez et al., 1996;  
33 Biddanda and Benner, 1997; Buitenhuis et al., 1999; Ho et al., 2003; Leonardos and  
34 Geider, 2005). Our experiments demonstrate a significant shift in the N/C  
35 stoichiometry of *E. huxleyi* with metal availability, due to a change in C metabolism.

36

### 3.3. Effect on cellular inorganic carbon and PIC/POC ratio

The amount of inorganic carbon per cell (PIC) increased significantly (t-test,  $p < 0.01$ ,  $n = 4$ ) at low concentrations of either  $Zn^{2+}$  or  $Co^{2+}$ , rising from  $\sim 10$  pg C cell<sup>-1</sup> at 1 pM  $Zn^{2+}$  and 0.45 pM  $Co^{2+}$  to 38 pg C cell<sup>-1</sup> and 25 pg C cell<sup>-1</sup> at 0.2 pM  $Zn^{2+}$  and 0.05 pM  $Co^{2+}$ , respectively (Figure 3a). Scanning electron microscope pictures showed that the increase in PIC per cell at low  $Co^{2+}$  and  $Zn^{2+}$  concentrations resulted from an increase in the number of coccoliths per cell, which accumulated around the coccosphere (Figure 4). The increase in the amount of calcite per cell observed at low  $Zn^{2+}$  concentrations was consistent with the previous findings of Schulz et al. (2004).

Contrary to the cellular PIC content, the production rate of calcite was significantly lower (t-test,  $p < 0.01$ ,  $n = 4$ ) from 6.5 to 3 pg C cell<sup>-1</sup> d<sup>-1</sup> under reduced  $Co^{2+}$  availability, while the effect of  $Zn^{2+}$  concentrations on the calcification rate was statistically not significant (t-test,  $p > 0.1$ ,  $n = 6$ ) (Figure 3b). Therefore, *E. huxleyi* was producing less calcite under  $Co^{2+}$  limitation, whereas this species became simultaneously more heavily calcified under this condition (Figure 3b). The severe decline of growth under low  $Co^{2+}$  conditions (Figure 1) exceeded the  $\sim 2$  fold limiting effect on calcite production (Figure 3b), which likely led to the accumulation of inorganic carbon under  $Co^{2+}$  limitation (Figure 3a). The decline of inorganic carbon production rate under limiting  $Co^{2+}$  conditions suggested a direct or indirect role of Co in the calcification process. Physiological studies are necessary to understand this potential role, especially to examine whether Co could be involved in the production of carbonate ( $CO_3^{2-}$ ) used for calcification through the activation of a carbonic anhydrase (Dreybrodt et al., 1997).

Because calcite accumulated while cellular organic carbon decreased with decreasing metal availability, the PIC/POC ratio was much higher (ratio  $> 1-2$ ) under  $Zn^{2+}$  and  $Co^{2+}$  limitation, compared with replete conditions (ratio  $< 1$ ) (Figure 3c; Table 1). Coccolithophores act as a source of atmospheric  $CO_2$  at a cellular PIC/POC ratio above 1 (Balch et al., 1991; Buitenhuis et al., 1996; Rost and Riebesell, 2004), and limitation by  $Zn^{2+}$  and  $Co^{2+}$  could, therefore, lead to a positive feedback on rising atmospheric  $CO_2$ . Increasing  $CO_2$  concentration (i.e., decreasing pH) led to the opposite trend in *E. huxleyi* PIC/POC ratio compared with metal limitation, due to an overall decrease in calcification (Riebesell et al., 2000; Zondervan et al., 2001, 2002). Warming can also decrease this ratio in coccolithophores to values lower than 1 by reducing calcification (Gerecht et al., 2014). However, the effect of pH and probably temperature is likely not a general trend for all species and morphotypes of *E. huxleyi* (Langer et al., 2006, 2009; Iglesias-Rodriguez et al., 2008). Such individual responses of

1 coccolithophores to trace metal availabilities still need to be assessed. For instance,  
2 the rather high PIC/POC ratio (>5) obtained in the absence of both Zn and Co (Table  
3 1) could hint at strong sensitivity of this strain to the supply of these metals, especially  
4 for photosynthesis.

#### 5 **4. Possible oceanic implications**

6 Culture experiments would suggest that coccolithophores tend to primarily  
7 require Co rather than Zn to attain high growth rates and would therefore be more  
8 abundant in oceanic waters with high Co concentrations and Co/Zn ratios (Sunda  
9 and Huntsman, 1995). However, the limitation of *E. huxleyi* by Co and Zn may be  
10 difficult to demonstrate in the field, partly because these two metals may inter-  
11 replace each other in cellular components. In addition, the chemical speciation of  
12 these two trace metals is still poorly documented in seawater (Tables 3 and 4),  
13 although organic complexation is thought to dominate their chemical speciation  
14 (Ellwood and van den Berg, 2000, 2001; Saito and Moffett, 2001; Bown et al., 2012).  
15 The bioavailability of the different dissolved chemical forms in seawater is also  
16 unknown, complicating the extrapolation of results obtained in EDTA-containing  
17 media to the natural system. Despite these limitations, comparison of the half-  
18 saturation constant for Co<sup>2+</sup>-limited growth obtained in this study (0.55 pM) with the  
19 free ion concentrations of cobalt (Co<sup>2+</sup>) that have been estimated in the surface  
20 waters in different biogeochemical domains of the oceans (Table 3) indicated the  
21 potential for Co<sup>2+</sup> availability to strongly limit the growth of *E. huxleyi* in the subtropical  
22 Atlantic, especially at its south-western and north-eastern sides (sub-femtomolar Co<sup>2+</sup>  
23 levels), and possibly in the southern branch of the Antarctic Circumpolar Current  
24 (ACC) south of the Polar Front (femtomolar Co<sup>2+</sup> levels). Likewise, the reduction of the  
25 growth observed below 10 pM Zn<sup>2+</sup> (Figure 1) compared with the free ion  
26 concentrations of zinc measured in surface oceanic waters (Table 4) maintains the  
27 possibility for Zn<sup>2+</sup> limitation in the subtropical waters of the north Atlantic (< 10 pM)  
28 and the subantarctic waters of the Pacific (< 1 pM). In addition, growth limitation by  
29 Zn likely occurs in the subarctic Pacific, since Zn additions stimulated phytoplankton  
30 growth in deck incubation experiments run in these waters, and had the largest  
31 effect on the growth of *E. huxleyi* and other coccolithophores compared with other  
32 phytoplankton types tested (Crawford et al., 2003). Further studies examining the  
33 organic speciation of Co and Zn would be required to confirm and extend these  
34 trends. However, based on the observations available to date, the low levels of free  
35 ion concentrations of both Co and Zn in subtropical waters (generally < 10 pM)  
36 suggest that *E. huxleyi* could be severely co-limited at these latitudes, notably  
37 because Co and Zn inter-replacement would be too low to support its growth. Similar

1 co-limitation by Zn and Co could also occur in the subarctic Pacific, since Zn  
2 additions were seen to stimulate the growth of coccolithophores in these waters  
3 (Crawford et al., 2003). Likewise, the replacement of  $\text{Co}^{2+}$  ( $< 0.006 \text{ pM}$ ) by  $\text{Zn}^{2+}$  ( $> 44$   
4  $\text{pM}$ ) in the southern ACC, and the substitution of  $\text{Zn}^{2+}$  ( $< 0.5 \text{ pM}$ ) by  $\text{Co}^{2+}$  ( $3\text{--}17 \text{ pM}$ ) in  
5 subantarctic waters could alleviate the limitation by either  $\text{Co}^{2+}$  or  $\text{Zn}^{2+}$  in the Southern  
6 Ocean. The ability to replace the limiting metal would give a competitive advantage  
7 to the taxa (Sunda and Hunstman, 1995), with consequences for phytoplankton  
8 community structure and productivity. For instance, *E. huxleyi* would be severely  
9 limited in low Zn and Co environments in our experiments, whereas diatoms that can  
10 commonly replace Zn by Cd would still benefit from high Cd concentrations (Lane  
11 and Morel, 2000), such as in the Antarctic Circumpolar Current (Boye et al., 2012).

12 In addition to the low  $\text{Co}^{2+}$  and  $\text{Zn}^{2+}$  concentrations observed in the subtropics  
13 (Tables 3-4), these oligotrophic waters are characterized by extremely low  
14 concentrations of macronutrients (Moore et al., 2008). There, the N/P ratio can be  
15 below the Redfield ratio, suggesting that nitrogen can become limiting before  
16 phosphorus (Dulaquais et al., 2014b). Because *E. huxleyi* tends to accumulate PON  
17 under low  $\text{Co}^{2+}$  and  $\text{Zn}^{2+}$  availability by continuing to use dissolved N substrates (e.g.,  
18 nitrate) to synthesize organic nitrogen (Figure 2), its limitation by N could therefore be  
19 intensified in subtropical waters due to  $\text{Co}^{2+}$  and  $\text{Zn}^{2+}$  limitation. Similarly, warming of  
20 sea-surface waters can intensify P-limitation of coccolithophores due to a higher P-  
21 requirement for maintaining growth and POC production rates (Gerecht et al., 2014).  
22 Growth limitation by Co and Zn could, on the other hand, inhibit the biological  
23 utilization of organic phosphorus in oligotrophic waters by decreasing the activity of  
24 Zn/Co alkaline phosphatase enzymes (Shaked et al., 2006; Jakuba et al., 2008a). The  
25 likelihood of changes in metal input and macronutrient supply in the changing ocean  
26 can in turn alleviate or exacerbate these multiple and additional limitations. Long  
27 time-series of atmospheric and oceanic observations will be very important for  
28 examining such changes in the future. This will help to determine the patterns of  
29 abundance and occurrence of this major phytoplankton group.

30 The trends of PIC/POC observed at limiting conditions of  $\text{Co}^{2+}$  and  $\text{Zn}^{2+}$  can  
31 also impact the carbon cycle in extremely low  $\text{Co}^{2+}$  and  $\text{Zn}^{2+}$  areas such as  
32 subtropical waters and likely subarctic waters, by inducing an increase in the  $\text{CaCO}_3$   
33 content and a decrease in the POC content of *E. huxleyi*, therefore altering the ratio  
34 of  $\text{CaCO}_3$  to POC. The overall effect would be a decrease in the  $\text{CO}_2$  storage  
35 capacity of the upper ocean by the lowering of surface water alkalinity and the  
36 driving of a  $\text{CO}_2$  efflux back to the atmosphere. Furthermore, an increase in the  
37 PIC/POC ratio can induce shifts in the calcite ballasting effect (Klaas and Archer,

1 2002) by increasing the density of sinking cellular particles at low  $Zn^{2+}$  and  $Co^{2+}$  but, at  
2 the same time, lowering the amount of POC per PIC transported to depth.  
3 Intensification of metal inputs to limited areas would in turn promote photosynthesis of  
4 *E. huxleyi* by increasing the activity of carbonic anhydrase. Its PIC/POC ratio would  
5 thus decrease and the atmospheric  $CO_2$  fixed by photosynthesis would increase,  
6 both constituting a negative feedback on rising atmospheric  $CO_2$ . Since low metal  
7 availability would act against high  $CO_2$  in surface waters (i.e., acidification) for  
8 atmospheric  $CO_2$  sequestration by *E. huxleyi*, the removal of metal limitation due, for  
9 instance, to increasing inputs in the changing ocean, would act in concert, and  
10 possibly amplify, the effect of acidification for atmospheric  $CO_2$  sequestration.  
11 However, the higher seawater  $CO_2$  concentrations reached under acidification  
12 could actually alleviate the limiting step of  $CO_2$  binding to Rubisco for photosynthesis,  
13 therefore lowering the need for Zn and Co in order to activate a carbonic anhydrase.  
14 Thus, change in these metal inputs may need to be studied together with  
15 acidification to further highlight the synergic effect of climate change on the  
16 PIC/POC ratio of this major producer of pelagic calcite, and to better anticipate  
17 feedbacks of the biological carbon pump on rising atmospheric  $CO_2$ .

## 18 **5. Conclusions**

19 Shifts in  $Co^{2+}$  and  $Zn^{2+}$  availability could cause changes not only in growth but  
20 also in cellular contents of organic and inorganic carbon in *Emiliania huxleyi*. The  
21 metabolic effect of Co, and especially Zn, could be to modulate the activity of the  
22 carbonic anhydrase (CA) in the chloroplast and, thus, the supply of  $CO_2$  for Rubisco,  
23 which is used in photosynthesis to produce organic carbon. The availability of  $Co^{2+}$   
24 could additionally modulate the calcification, either directly or indirectly, but other  
25 studies are necessary in order to understand the potential underlying process. The  
26 effect of limitation by  $Co^{2+}$  and  $Zn^{2+}$  was an increase of the PIC/POC ratio in *E.*  
27 *huxleyi*, which in turn decreases the efficiency of the ocean's biological  $CO_2$  pump.  
28 Reciprocally, the alleviation of these limitations promoted growth and photosynthesis  
29 of *E. huxleyi* and decreased its PIC/POC ratio, which would likely stimulate the  
30 biological  $CO_2$  pump. The likelihood of change in metal inputs into the surface ocean  
31 may thus need to be considered when assessing future change in the efficiency of  
32 the biological carbon pump due to this major phytoplankton producer of pelagic  
33 calcite. Metal speciation and its evolution in the changing ocean will also need to be  
34 considered in order to improve our understanding of changes in metal bioavailability  
35 and potential feedbacks on the PIC/POC ratio. Finally, since Co and Zn limitation  
36 could lead to opposite trends in the PIC/POC ratio of *E. huxleyi* to that of ocean  
37 acidification, the sensitivity of *E. huxleyi* to the combined effects of acidification and



1 changes in Co and Zn inputs to surface waters will also need to be addressed. For  
2 instance, it is possible that the co-limitation by  $\text{Co}^{2+}$  and  $\text{Zn}^{2+}$  is alleviated at higher  
3 partial pressure of  $\text{CO}_2$  due to higher availability of  $\text{CO}_2$  for Rubisco, but this question  
4 requires further investigation.

5

## 6 **Acknowledgements**

7 We gratefully acknowledge Annick Masson (LEMAR) for her assistance with the CHN-  
8 analyses. This work was supported by the CAMAÏEUD project (carbon and metals  
9 interactions with *E. huxleyi* and diatoms) funded by the French National LEFE-CYBER  
10 program of INSU-CNRS. The University of Western Brittany supported M. Adjou's PhD  
11 fellowship within the European Network of Excellence EUR-OCEANS and G. Dulaquais'  
12 PhD fellowship with the Brittany Region within LABEX-MER.

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## TABLES

**Table 1.** Effect of free metal ion concentrations ( $\log[\text{Zn}^{2+}]$ ,  $\log[\text{Co}^{2+}]$ ,  $\log[\text{Cd}^{2+}]$ ) on the growth rate ( $\mu$ ) of *E. huxleyi* and its cellular concentrations of organic carbon (POC), organic nitrogen (PON), inorganic carbon (PIC) and Chl-a, and on cellular ratios of inorganic to organic carbon (PIC/POC) and organic nitrogen to carbon (PON/POC). The standard deviations of the measurements were calculated on replicate analyses (as explained in the text).

Experiment	$\log[\text{Zn}^{2+}]$	$\log[\text{Co}^{2+}]$	$\log[\text{Cd}^{2+}]$	$\mu$ ( $\text{d}^{-1}$ )	POC ( $\mu\text{gC}$ $\text{cell}^{-1}$ )	PIC ( $\mu\text{gC}$ $\text{cell}^{-1}$ )	PON ( $\mu\text{gN}$ $\text{cell}^{-1}$ )	PIC/POC ( $\text{mol C}$ $\text{mol C}^{-1}$ )	PON/POC ( $\text{mol N}$ $\text{mol C}^{-1}$ )	Chl-a ( $10^{-1}\mu\text{g}$ $\text{cell}^{-1}$ )
Zn	-12.70	Negl.	Negl.	$0.15 \pm 0.01$	$6.74 \pm 0.54$	$38.04 \pm 3.19$	$2.91 \pm 0.33$	$5.64 \pm 0.02$	$0.37 \pm 0.01$	$1.35 \pm 0.02$
	-12.40	Negl.	Negl.	$0.29 \pm 0.01$	$12.11 \pm 1.53$	$23.27 \pm 5.03$	$2.08 \pm 0.09$	$1.92 \pm 0.66$	$0.15 \pm 0.03$	$1.35 \pm 0.05$
	-12.00	Negl.	Negl.	$0.37 \pm 0.01$	$16.64 \pm 1.15$	$11.92 \pm 0.59$	$1.06 \pm 0.03$	$0.72 \pm 0.09$	$0.05 \pm 0.01$	$1.39 \pm 0.06$
	-11.22	Negl.	Negl.	$0.49 \pm 0.01$	$18.85 \pm 2.19$	$13.55 \pm 0.77$	$1.10 \pm 0.11$	$0.72 \pm 0.04$	$0.05 \pm 0.00$	$1.31 \pm 0.06$
	-11.00	Negl.	Negl.	$0.53 \pm 0.01$	$16.94 \pm 3.26$	$14.56 \pm 2.10$	$1.22 \pm 0.06$	$0.86 \pm 0.04$	$0.06 \pm 0.01$	$1.29 \pm 0.04$
	-10.43	Negl.	Negl.	$0.57 \pm 0.01$	$14.08 \pm 1.61$	$10.43 \pm 0.54$	$0.95 \pm 0.01$	$0.74 \pm 0.12$	$0.06 \pm 0.01$	$1.34 \pm 0.01$
Co	Negl.	-13.33	Negl.	$0.13 \pm 0.01$	$6.68 \pm 0.46$	$24.56 \pm 2.11$	$2.41 \pm 0.57$	$3.68 \pm 0.06$	$0.31 \pm 0.04$	$1.39 \pm 0.02$
	Negl.	-12.63	Negl.	$0.20 \pm 0.01$	$6.68 \pm 0.55$	$18.08 \pm 1.63$	$1.42 \pm 0.14$	$2.71 \pm 0.02$	$0.18 \pm 0.03$	$1.34 \pm 0.02$
	Negl.	-12.33	Negl.	$0.30 \pm 0.02$	$8.91 \pm 0.24$	$9.01 \pm 1.91$	$1.04 \pm 0.10$	$1.01 \pm 0.24$	$0.10 \pm 0.01$	$1.29 \pm 0.06$
	Negl.	-11.52	Negl.	$0.48 \pm 0.01$	$12.83 \pm 0.63$	$10.91 \pm 0.62$	$0.81 \pm 0.10$	$0.85 \pm 0.01$	$0.05 \pm 0.00$	$1.34 \pm 0.01$
	Negl.	-10.93	Negl.	$0.67 \pm 0.03$	$12.23 \pm 0.39$	$10.03 \pm 2.16$	$0.75 \pm 0.06$	$0.82 \pm 0.20$	$0.05 \pm 0.01$	$1.31 \pm 0.00$
	Negl.	-10.20	Negl.	$0.71 \pm 0.02$	$11.84 \pm 1.03$	$8.80 \pm 1.03$	$0.69 \pm 0.00$	$0.74 \pm 0.15$	$0.05 \pm 0.00$	$1.34 \pm 0.02$
Cd	Negl.	Negl.	-12.70	$0.06 \pm 0.00$	$4.14 \pm 0.38$	$27.11 \pm 3.91$	$1.44 \pm 0.05$	$6.55 \pm 0.35$	$0.30 \pm 0.03$	1.32
	Negl.	Negl.	-12.40	$0.07 \pm 0.00$	$3.49 \pm 0.40$	$27.90 \pm 3.11$	$1.51 \pm 0.07$	$7.99 \pm 1.82$	$0.37 \pm 0.03$	1.27
	Negl.	Negl.	-12.00	$0.07 \pm 0.01$	$3.64 \pm 0.35$	$25.56 \pm 2.88$	$1.26 \pm 0.39$	$7.02 \pm 0.12$	$0.30 \pm 0.10$	1.30
	Negl.	Negl.	-11.22	$0.07 \pm 0.00$	$4.25 \pm 0.07$	$20.71 \pm 1.53$	$1.34 \pm 0.07$	$4.87 \pm 0.44$	$0.27 \pm 0.01$	1.31
	Negl.	Negl.	-11.00	$0.07 \pm 0.01$	$4.23 \pm 0.61$	$22.13 \pm 0.22$	$1.27 \pm 0.02$	$5.23 \pm 0.81$	$0.26 \pm 0.03$	1.34
	Negl.	Negl.	-10.32	$0.07 \pm 0.00$	$4.45 \pm 0.10$	$25.83 \pm 2.46$	$1.26 \pm 0.03$	$5.80 \pm 0.69$	$0.24 \pm 0.01$	1.31
Positive control (PC)	-10.70	-10.82	-10.89	$0.71 \pm 0.01$	$12.89 \pm 1.28$	$9.41 \pm 0.31$	$1.05 \pm 0.08$	$0.73 \pm 0.10$	$0.07 \pm 0.00$	$1.31 \pm 0.03$
Negative control (NC)	Negl.	Negl.	Negl.	$0.06 \pm 0.00$	$6.48 \pm 0.56$	$36.38 \pm 0.73$	$2.65 \pm 0.19$	$5.61 \pm 0.37$	$0.35 \pm 0.01$	n.d.
Zn addition to NC	-10.70	Negl.	Negl.	$0.52 \pm 0.01$	$12.94 \pm 0.06$	$10.04 \pm 0.63$	$1.12 \pm 0.09$	$0.78 \pm 0.05$	$0.07 \pm 0.01$	n.d.
Co addition to NC	Negl.	-10.82	Negl.	$0.69 \pm 0.02$	$12.32 \pm 0.35$	$9.82 \pm 0.32$	$1.15 \pm 0.08$	$0.80 \pm 0.00$	$0.08 \pm 0.01$	n.d.
Cd addition to NC	Negl.	Negl.	-10.89	$0.07 \pm 0.00$	$4.66 \pm 0.75$	$26.44 \pm 1.69$	$1.85 \pm 0.20$	$5.67 \pm 1.30$	$0.34 \pm 0.09$	n.d.

**Note:** Negl.: negligible concentration ( $[\text{Zn}^{2+}] < 0.02 \text{ pM}$ ;  $[\text{Co}^{2+}] < 0.03 \text{ pM}$ ;  $[\text{Cd}^{2+}] < 0.04 \text{ pM}$ ). n.d.; not determined.

**Table 2.** Location and substitution of Co, Zn and Cd in important biomolecules of *E. huxleyi*.

Biomolecules \ Metals	Zn	Co	Cd
Carbonic Anhydrase (CA)	Cofactor of $\delta$ -EhCA1 <sup>(a)</sup> (closely homologous to TWCA1 <sup>(b)</sup> )	Substitution for Zn in $\delta$ -EhCA1 <sup>(a)</sup>	No role (not homologous to CDCA1 <sup>(c)</sup> in <i>E. huxleyi</i> <sup>(d)</sup> )
Alkaline Phosphatase (AP)	Cofactor of AP <sup>(e,f)</sup>	Substitution for Zn in AP <sup>(f)</sup>	No known role
Zinc Fingers	DNA- and RNA-binding proteins* <sup>(g,h)</sup>	No known role	No known role
Cyanocobalamin (vitamine B <sub>12</sub> )	No role (exclusively Co-containing vitamin)	Central atom of B <sub>12</sub> synthesized by coccolithophorids <sup>(i)</sup>	No role (exclusively Co-containing vitamin)

<sup>(a)</sup> Soto et al. 2006; <sup>(b)</sup> Roberts et al. 1997; <sup>(c)</sup> Lane et al.2005; <sup>(d)</sup> Park et al. 2007; <sup>(e)</sup> Shaked et al. 2006;

<sup>(f)</sup> Jakuba et al. 2008a; <sup>(g)</sup> Vallee and Auld, 1990; <sup>(h)</sup> Berg and Shi, 1996; <sup>(i)</sup> Carlucci and Bowes, 1970.

\* for eukaryotes

**Table 3.** Organic speciation of cobalt in the worldwide surface ocean: concentrations of dissolved cobalt (DCo), inorganic cobalt (Co'), free cobalt ions (Co<sup>2+</sup>), organic cobalt-binding ligand (L<sub>Co</sub>), and conditional stability constant of CoL complexes (K'<sub>CoL</sub>).

Ocean	Region	[DCo] (pM)	[Co'] (pM)	[Co <sup>2+</sup> ]* (pM)	[L <sub>Co</sub> ] (pM)	Log K' <sub>CoL</sub>	References
Atlantic	Temperate North East	33.5–55.2	2–11	1.1–6.3	30–39	15.7–16.2	Ellwood and van Den Berg, 2001
	Temperate North West	27	16.3	9.4	10.7	17.6	Dulaquais et al., in prep.
	Subtropical North East	14.4–26.0	< 0.005	<0.003	22–32	15.6–16.1	Ellwood and van Den Berg, 2001 ; Baars and Croot, 2014
	Subtropical South East	15.6–29.7	< 0.01	<0.006	34.9–71.9	16.6–19.2	Bown et al., 2012
	Subtropical North West	23.0–30.7	5.5–14	3.2–8.1	8.0–24.3	16–18.3	Saito and Moffett, 2001 ; Dulaquais et al., in prep.
	Subtropical South West	15.2–17.8	0.000001	0.0000006	18.9–31.2	17.3–18.7	Dulaquais et al., in prep.
	Equatorial North West	20.5	0.9	0.5	19.6	18.9	Dulaquais et al., in prep.
	Equatorial South West	8–31.7	0.00001	0.000006	19.7–35.2	18.1–19.0	Noble et al., 2012 ; Dulaquais et al., in prep.
	Equatorial South East	33–135	1.7–24	1–13.8	n.d	n.d	Noble et al., 2012
	Central Equatorial South	6–48.5	0–1	0–0.6	n.d	n.d	Noble et al., 2012
	Angola upwelling	185.7–202.7	19.8–101.3	11.4–58.3	n.d	n.d	Noble et al., 2012
	English channel	61.3–103.0	12–19	6.9–10.9	29–60	15.6–15.8	Ellwood and van den Berg, 2001
Southern Ocean	Subantarctic eastern Atlantic sector	5.7–27.3	< 0.01	<0.006	35.6–49.0	18.2–19.4	Bown et al., 2012
	Subantarctic western Atlantic sector	23.0–52.2	5.7–30.0	3.3–17.3	8.3–22.2	18.1–19.6	Dulaquais et al., in prep.
	North of Polar Front in Atlantic sector	21–43.5	6–16	3.4–9.2	15–35.5	15.7–18.8	Ellwood et al., 2005 ; Bown et al., 2012
	South of Polar Front in Atlantic sector	12–40	< 0.01	<0.006	12–40	15.8	Ellwood et al., 2005
	Southern ACC in Atlantic sector	36.7–51.3	< 0.01	<0.006	42.7–52.0	18.1–19.1	Bown et al., 2012
Arctic	Subarctic gyre in Atlantic sector	57.0–68.0	26.6–47.7	15.3–27.4	9.2–40.2	17.3–18.6	Dulaquais et al., in prep.
Pacific	Costa Rica upwelling	45–93	0–37	0–21.3	n.d	n.d	Saito et al., 2005
	Equatorial Eastern	27–55	< 0.001–25	<0.0006–14.4	n.d	n.d	Saito et al., 2004
	Peru upwelling	55–315	10–200	5.7–115.1	n.d	n.d	Saito et al., 2004
	Hawaiian Island	6–26	< 0.01	<0.006	n.d	n.d	Noble et al., 2008

\* [Co<sup>2+</sup>] is calculated by dividing [Co'] by the inorganic side reaction coefficient of cobalt at pH 8.2 (log  $\alpha_{Co'} = 0.24$  ; Turner et al., 1981)

**Table 4.** Organic speciation of zinc in the worldwide surface ocean: concentrations of dissolved zinc (DZn), inorganic zinc (Zn'), free zinc ions (Zn<sup>2+</sup>), organic zinc-binding ligand (L<sub>Zn</sub>), and conditional stability constant of ZnL complexes (K'<sub>ZnL</sub>).

Ocean	Region	[DZn] (nM)	[Zn'] (pM)	[Zn <sup>2+</sup> ]* (pM)	[L <sub>Zn</sub> ] (nM)	Log K' <sub>ZnL</sub>	References
Atlantic	Temperate North East	0.3–0.6	10–25	4.6–11.4	1.3–2.3	10.2–10.4	Ellwood & van Den Berg 2000
	Subtropical North East	0.3–0.5	6.5–6.8	3.0–3.1	2.4–2.5	10.3–10.6	Ellwood & van Den Berg 2000
	Sargasso Sea	0.2–0.4	6–10	2.7–4.6	0.6–1.2	9.8–10.5	Jakuba et al., 2008b
	English Channel	0.6–1.5	151–205	69.0–93.7	0.4–1.4	10.2	Ellwood & van Den Berg 2000
Pacific	Western North Pacific gyre	0.24–0.43	17–108	7.8–49.4	0.7–1.2	9.6–10.2	Kim et al., 2015a
	Sea of Japan	0.88	257	117.5	1.3	9.1	Kim et al., 2015a
	Sea of Okhotsk	2.11	234	107	2.6	9.5	Kim et al., 2015a
	Temperate North East	0.1	5	2.3	1.1	10	Jakuba et al., 2012
	Temperate North	0.2–0.9	2–7	0.9–3.2	1.1–1.4	10.5	Jakuba et al., 2012 ; Lohan et al., 2005 ; Donat and Bruland, 1990
	Temperate North West	0.78	33	15.1	1.4	9.8	Jakuba et al., 2012
	Subtropical North West	0.07–0.18	20–54	9.1–24.7	0.2–0.3	9.1–9.7	Kim et al., 2015a
	Subtropical North East	0.15–0.3	2–8	0.9–3.7	1–1.4	10.7–10.9	Bruland, 1989
Southern Ocean	Subantarctic in the Pacific sector	0.01–0.03	0.3–1.1	0.1–0.5	1.2–2.4	10.0–10.8	Ellwood, 2004
	Atlantic sector	0.8–3.5	97–2800	44.3–1279.8	1.4–5.3	9.1–10.4	Baars & Croot, 2011
Arctic	Bering Sea	0.89	71	32.4	3.6	9.3	Jakuba et al., 2012
Indian Ocean	North	0.26	47	21.5	0.5	10	Kim et al., 2015b
Black Sea	West	1.2–5.5	29.0–110.0	13.2–50.3	6.3–7.5	9.4–10.3	Muller et al., 2001

\* [Zn<sup>2+</sup>] is calculated by dividing [Zn'] by the inorganic side reaction coefficient of zinc at pH 8.2 (log α<sub>Co</sub>' = 0.34 ; Turner et al., 1981)

## FIGURE CAPTIONS

**Figure 1.** Growth rates ( $\mu$ ; in day<sup>-1</sup>) of *E. huxleyi* as a function of logarithmic values of [Co<sup>2+</sup>], [Zn<sup>2+</sup>] and [Cd<sup>2+</sup>] present in f/50 Aquil growth media. The replete metal conditions are as following: [Co<sup>2+</sup>] = 15 pM ; [Zn<sup>2+</sup>]= 20 pM ; [Cd<sup>2+</sup>] =13 pM.

**Figure 2.** Cellular organic carbon content POC (a), organic carbon production rate (b), cellular nitrogen PON (c), and nitrogen cellular to organic carbon ratio PON/POC (d) of *E. huxleyi* as a function of logarithmic values of [Co<sup>2+</sup>] and [Zn<sup>2+</sup>].

**Figure 3.** Cellular inorganic carbon content PIC (a), inorganic carbon production per cell (b), and inorganic to organic carbon ratio PIC/POC (c) of *E. huxleyi* as a function of logarithmic values of [Co<sup>2+</sup>] and [Zn<sup>2+</sup>].

**Figure 4.** Scanning electron microscope pictures of *E. huxleyi* grown in replete (left) and limiting (right) Zn<sup>2+</sup>-Co<sup>2+</sup> conditions.



Figure 1

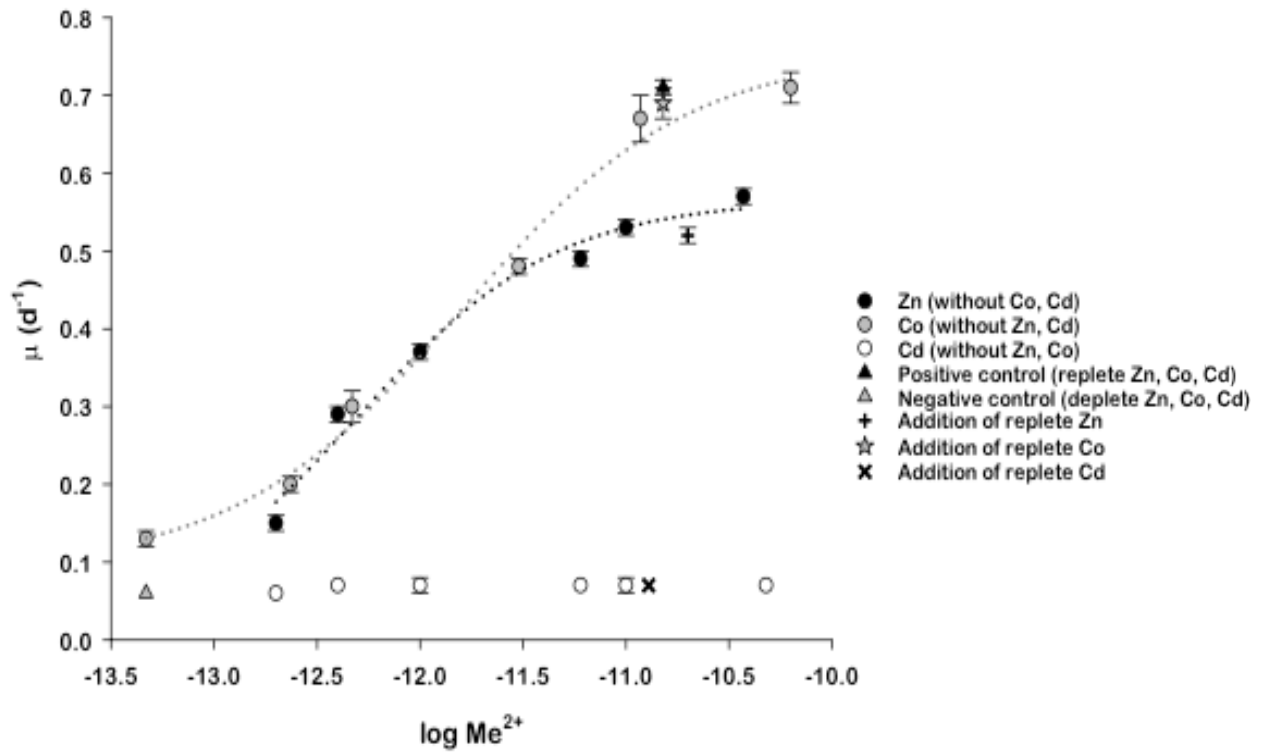


Figure 2

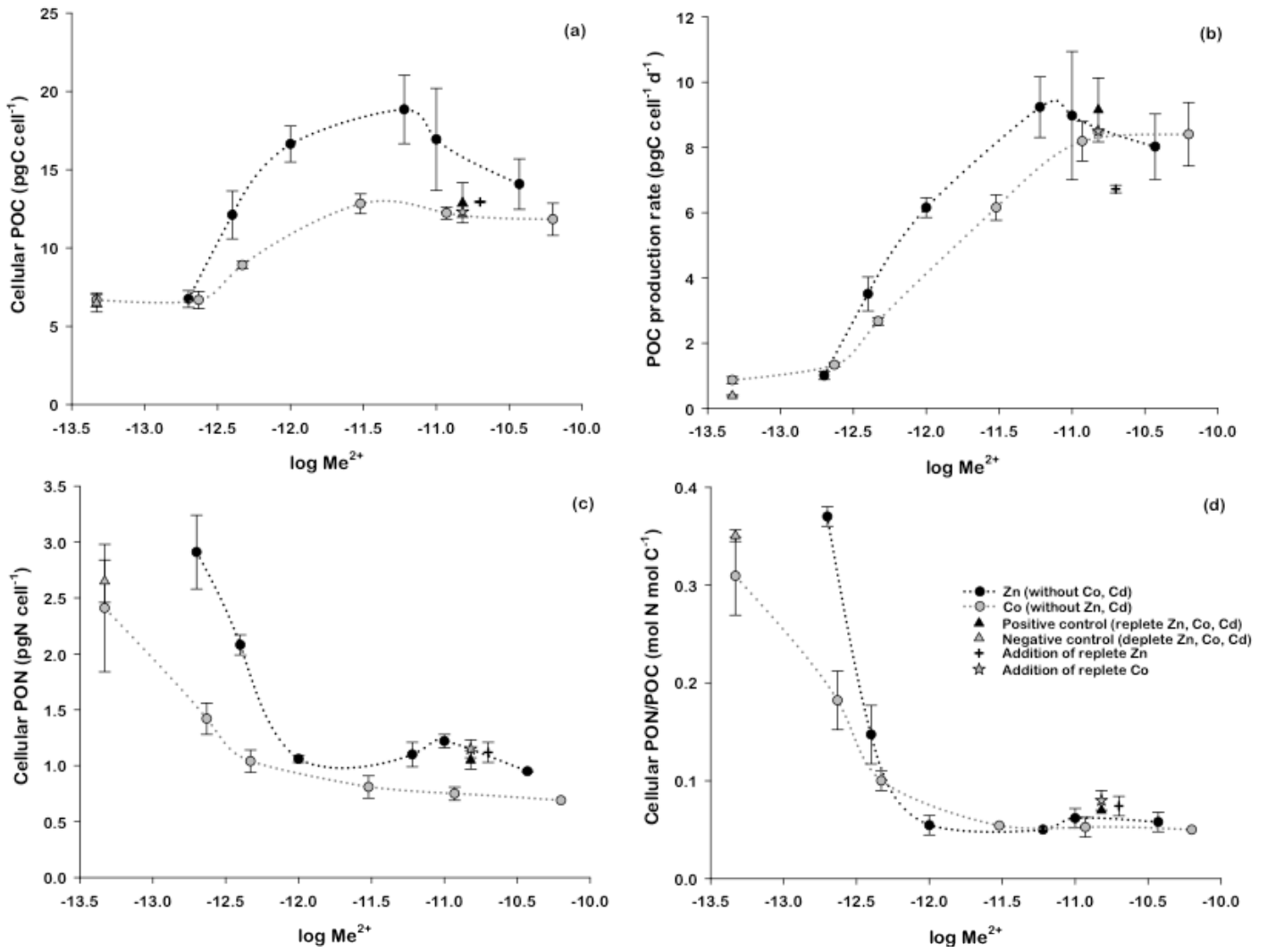


Figure 3

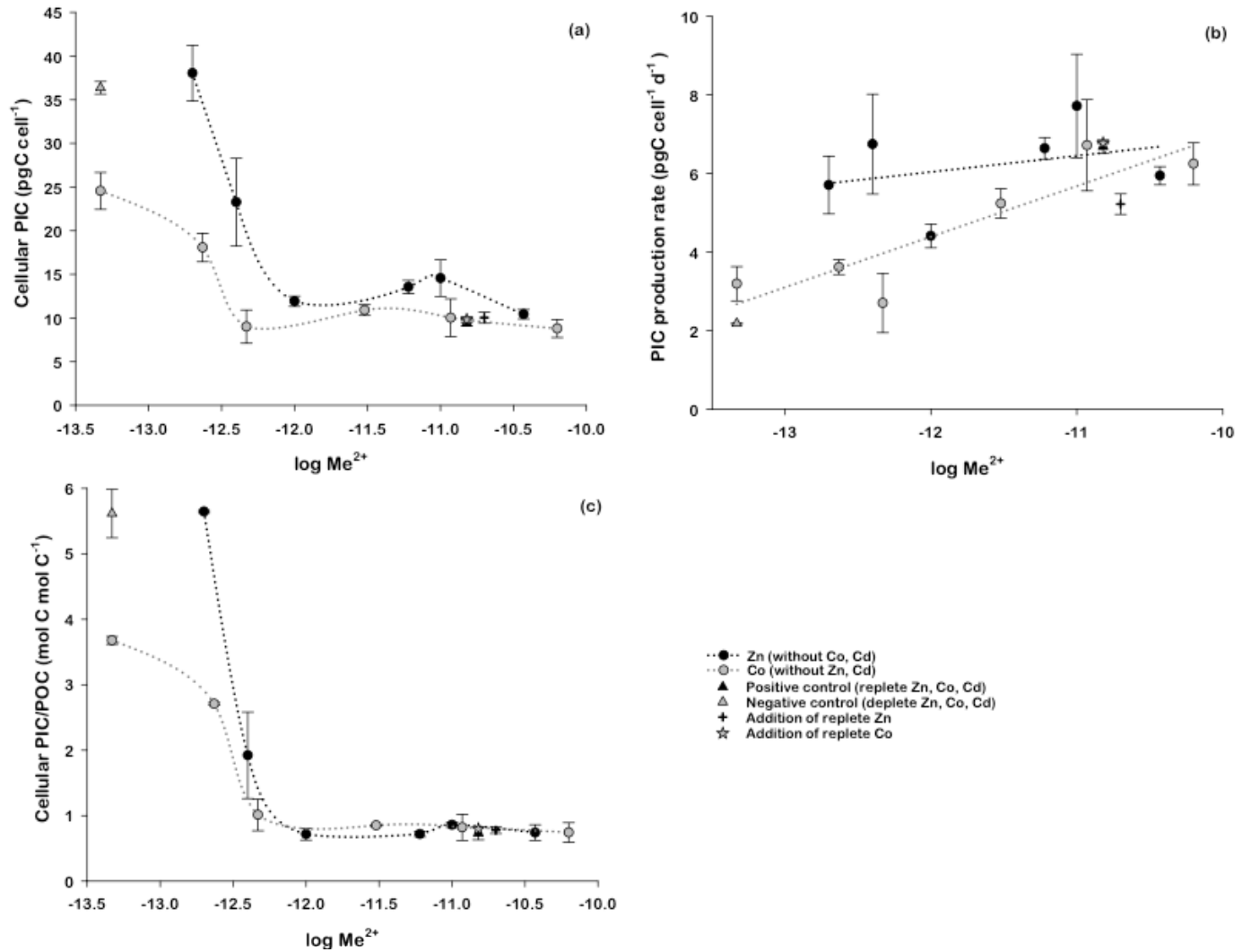


Figure 4

