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Zinc-bicarbonate colimitation of *Emiliania huxleyi*

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Abstract

In analogy to the Fe hypothesis, the Zn hypothesis states that Zn may limit primary production in some regions of the world oceans and therefore influence the global carbon cycle. The proposed mechanism is via carbon limitation due to a lack of the cofactor Zn in carbonic anhydrase. In the current conceptual model for the use of inorganic carbon by *E. huxleyi,* carbonic anhydrase in the chloroplast generates CO_2 from HCO_3^- at the site where CO_2 is fixed by ribulose bisphosphate carboxylase oxygenase (Rubisco). The H^+ that is required in this reaction comes from calcification. From this it can be expected that carbonic anhydrase affects the use of HCO_3^- in photosynthesis. First, we grew *E. huxleyi* under Zn^{2+} limitation. The $K_{1/2}$ for growth of *E. huxleyi* is 19 ± 8 pmol L^{-1} Zn^{2+} with a minimum requirement of 9 ± 3 pmol L^{-1}. Additions of both ethylenediaminetetraacetic acid (EDTA) and $ZnCl_2$ show that EDTA is not detrimental to *E. huxleyi* up to a concentration of 200 μmol L^{-1}. Then we grew *E. huxleyi* under Zn^{2+}-HCO_3^- colimitation to test the conceptual model outlined above. The results were partly inconsistent with the model. Contrary to what was expected from the conceptual model, the efficiency of CO_2 use decreased when both Zn^{2+} and HCO_3^- concentrations were low, even though the experiment was conducted at a constant high concentration of CO_2. This shows that Zn^{2+}, and possibly carbonic anhydrase activity, are needed for CO_2 fixation also. In accordance with the model, we found that Zn^{2+} affects the efficiency of HCO_3^- use by *E. huxleyi.* Since the lowest Zn^{2+} concentration in the Northeast Pacific is ~0.4 pmol L^{-1}, Zn limitation of *E. huxleyi* growth may indeed occur.

Emiliania huxleyi is an interesting alga for studying the use of inorganic carbon because it produces both particulate organic carbon (POC) in photosynthesis and particulate inorganic carbon as $CaCO_3$ in calcification and, thus, has an impact on the oceanic cycles of both dissolved inorganic carbon (DIC) and alkalinity. Moreover, *E. huxleyi* occurs over most of the ocean outside the polar regions. Except in tropical and polar regions, it constitutes about 50%–100% of the coccolithophorids by number (McIntyre and Bé 1967). However, coccoliths of *E. huxleyi* are relatively small and fragile. Thus, it is not the dominant contributor to coccolithophorid $CaCO_3$ precipitation (Broerse 2000).

Paasche (1962) suggested that calcification and photosynthesis in *E. huxleyi* are coupled in a manner equivalent to the symbiosis of corals and their associated algae. Later, it was confirmed that there is a direct link between calcification and photosynthesis. The substrate for calcification is HCO_3^- (Paasche 1964), which produces a proton (Eq. 1). This proton can then be used with a second HCO_3^- molecule to produce CO_2 (Eq. 2). The CO_2 can then be fixed in photosynthesis by the enzyme ribulose bisphosphate carboxylase oxygenase (Rubisco) (Eq. 3). Since HCO_3^- is ~100-fold more abundant than CO_2 in seawater, calcification may constitute a viable mechanism for supplying Rubisco with CO_2. The tight coupling between calcification and photosynthesis supports this hypothesis (Buitenhuis et al. 1999).

$$HCO_3^- + Ca^{2+} \rightarrow CaCO_3 + H^+ \quad (1)$$

$$HCO_3^- + H^+ \leftrightarrow CO_2 + H_2O \quad (2)$$

$$CO_2 + H_2O \rightarrow (CH_2)_{organic} + O_2 \quad (3)$$

The uncatalyzed conversion rate of HCO_3^- to CO_2 is very slow (Stumm and Morgan 1981). Thus, to maintain a tight coupling between calcification and photosynthesis, the cells might need the enzyme carbonic anhydrase to catalyze this conversion. Indeed, it has been shown that during exponential growth *E. huxleyi* synthesizes carbonic anhydrase in the chloroplast only (Nimer et al. 1994*b*). Carbonic anhydrase catalyzes the conversion between HCO_3^- and CO_2 in both directions. As an example of the conversion of HCO_3^- into CO_2, carbonic anhydrase is found in the chloroplast of aquatic microorganisms, where it produces CO_2 that is fixed in photosynthesis by Rubisco (*see Fig. 1*). As an example of the conversion of CO_2 into HCO_3^-, it has been suggested that extracellular carbonic anhydrase can supply HCO_3^- to an active uptake transport protein, and in cyanobacteria a putative new type of carbonic anhydrase that is directly linked to such a transporter could have this function (Sültemeyer et al. 1993; Price et al. 2002, and references in these).

Since Zn^{2+} is the cofactor of carbonic anhydrase, we hypothesize that under Zn^{2+} limitation the coupling between calcification and photosynthesis becomes less efficient. More specifically, we hypothesize that under Zn^{2+} limitation (1) the efficiency with which HCO_3^- is used in photosynthesis would be lowered (Eq. 2, Fig. 1), while (2) the efficiency with which CO_2 is used in photosynthesis would be unaf-

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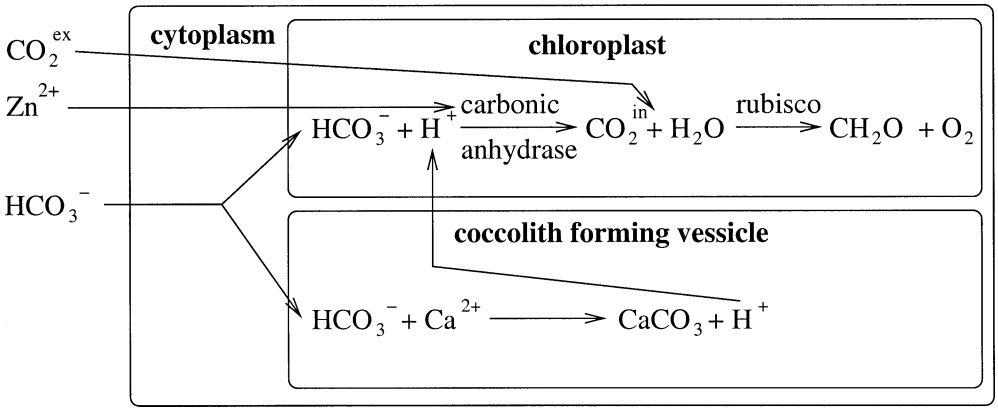

Fig. 1. Uptake of inorganic carbon by *Emiliania huxleyi.* Adapted after Buitenhuis et al. (1999).

fected (Eq. 3, CO_2^{ex} in Fig. 1). To test this hypothesis we measured the effect of Zn^{2+} limitation and Zn^{2+}-HCO_3^- colimitation on the specific growth rate. In the Zn^{2+} limitation experiment the growth rate was measured both at a constant Zn_T concentration and at a constant ethylenediaminetetraacetic acid (EDTA) concentration to prove unequivocally that the decrease in growth rate is due to a change in the availability of Zn ion and not to direct toxicity of EDTA. To exclude the effect of CO_2 on the photosynthetic rate, we have performed the Zn^{2+}-HCO_3^- colimitation experiments at constant CO_2. We review to what extent our results are consistent with the current conceptual model of inorganic carbon use by *E. huxleyi.*

Materials and methods

The growth medium was prepared from low-nutrient seawater. The concentration of Zn was reduced by the addition of 50 μmol L^{-1} particulate MnO_2. After stirring for 24 h the water was filtered over two connected filters of 0.2-μm and 0.07-μm pore size to remove the particulate MnO_2. We then added 250 μmol L^{-1} NO_3^-, 25 μmol L^{-1} PO_4^{3-}, vitamins according to Paasche (1964), and 50 nmol L^{-1} $FeCl_2$. All handling was done in a trace metal clean laminar flow hood. *Emiliania huxleyi* strain Ch24–90 (morphotype A, van Bleijswijk et al. 1991, 1994), a normally calcifying strain with a typical calcification/photosynthesis ratio of 0.6 (Buitenhuis et al. 1999), was grown in acid-cleaned (1 mol L^{-1} HCl) polycarbonate square bottles, in a suite of volumes from 30 to 1,000 ml to generate a dilution series with relatively constant biomass concentrations. The dilution of the inoculum in the first bottle of the dilution series was at least 10,000 fold from a culture with high trace metal concentrations (~600 nmol L^{-1} total Zn). Bottles were incubated at 15°C on a rotating bench (120 rpm) at saturating light (300 μmol quanta m^{-2} s^{-1}).

The cells were counted with a Coulter XL flowcytometer (Buitenhuis et al. 1999), starting after 3 d of adaptation to the medium over a 4- to 12-d period. Specific growth rates (μ) were calculated as the slope of ln(cell counts) against time (in days). Calculation of K_m, μ_{max}, and Zn^{2+}_{min} was done by curve fitting of the growth rates to Eqs. 5 to 8 (Buitenhuis et al. 1999).

We performed three sets of growth experiments: one Zn^{2+}-limitation experiment and two Zn^{2+}-HCO_3^- colimitation experiments. In all three experiments the availability of Zn was decreased by the addition of the metal ion chelator EDTA. Since the Zn-EDTA complex is not available to the algae, the available concentration of Zn^{2+} can be reduced to concentrations in the low pmol L^{-1} range while the total Zn concentration (Zn_T) is in the low nmol L^{-1} range by addition of 6 to 300 μmol L^{-1} EDTA.

In the Zn^{2+}-limitation experiment, in order to make sure that the cells were limited by Zn^{2+}, we added either Zn^{2+} or Co^{2+} to the bottles with the highest EDTA concentrations. We tested for the conceivable detrimental effect of EDTA itself on growth by performing additional incubations with a constant EDTA concentration of 200 μmol L^{-1} in which Zn^{2+} was varied by additions of $ZnCl_2$. The total concentration of Zn (Zn_T) was between 4 and 200 nmol L^{-1}. The DIC speciation was not controlled or measured in the Zn^{2+} limitation experiment, but at an alkalinity of ~2,540 μeq kg^{-1} and a typical fCO_2 in the laboratory of ~500 μatm, concentrations of 2,161 μmol L^{-1} HCO_3^- and 19 μmol L^{-1} CO_2 can be calculated.

In the two Zn^{2+}-HCO_3^- colimitation experiments the HCO_3^- concentrations were varied at a constant CO_2 concentration of 16.7 μmol L^{-1}. This was done by first manipulating the alkalinity and then fixing the CO_2 concentration. The alkalinity was lowered by addition of 10 mol L^{-1} HCl (quartz distilled, Zn_T undetectable) or increased by addition of 1 mol L^{-1} $NaHCO_3$ (129 nmol L^{-1} Zn_T in stock solution, no significant change of Zn_T in growth medium). The CO_2 concentration was fixed by bubbling overnight at 15°C with air from a gas cylinder. This gas cylinder had a slightly elevated CO_2 content relative to atmospheric air corresponding to an fCO_2 of 444.6 μatm. The alkalinity of the medium was calculated from DIC and fCO_2 with the dissociation constants of Roy et al. (1993), using a program by Lewis and Wallace (http://cdiac.esd.ornl.gov/oceans/co2rprt.html). DIC was determined according to DOE (1994); fCO_2 in the gas cylinder was calculated from the gas mixing ratio xCO_2 according to Weiss (1974). xCO_2 was determined by infrared gas analysis (IRGA, LICOR 6252) as described in Buitenhuis et al. (1996).

Total Zn (Zn_T) was measured by flow injection analysis

Table 1. Conversion of HCO_3^- to CO_2 in the chloroplast (Eq. 2).

(A) Rate of uncatalyzed conversion from HCO_3^- to CO_2		
$H^+_{chloroplast}$ (mol L^{-1})	1.3×10^{-8}	Anning et al. (1996)
HCO_3^- (mol L^{-1})	5.0×10^{-4}	Brownlee et al. (1995) determined in whole cells
k_1 (s^{-1})	1.2×10^{4}	Wolf-Gladrow and Riebesell (1997)
$V_{chloroplast}$ (L $cell^{-1}$)	1.4×10^{-14}	estimated from van der Wal et al. (1985)
Light period (s (16 h)$^{-1}$)	5.8×10^{4}	this study
$HCO_3^- \rightarrow CO_2$ (mol L^{-1} s^{-1})	7.4×10^{-8}	$\delta[CO_2]/\delta t = H^+_{chloroplast} \times HCO_3^- \times k_1$
CO_2 generation (mol $cell^{-1}$ d^{-1})	6.0×10^{-17}	$V_{chloroplast} \times$ light period $\times \delta[CO_2]/\delta t$
(B) Minimum concentration of Zn^{2+} as cofactor in carbonic anhydrase (CA) required for catalyzed conversion at given calcification rate		
$pH_{chloroplast}$	7.9	Anning et al. (1996)
$k_{cat}(pH_{chloroplast})$ (s^{-1})	1.0×10^{6}	Pocker and Ng (1973), Pocker and Miksch (1978)
K_m (mol L^{-1})	3.4×10^{-2}	Pocker and Miksch (1978)
Calcification rate (mol $cell^{-1}$ d^{-1})	2.1×10^{-12}	Buitenhuis et al. (1999) (at fCO_2 = 444.6 μatm, and pH = 8)
Cell concentration (L^{-1})	1.0×10^{8}	this study
CA required (mol $cell^{-1}$)	2.5×10^{-20}	calcification rate $\times$ $(HCO_3^- + K_m)(k_{cat} \times HCO_3^-)^{-1} \times$ light period^{-1}
Zn^{2+} in CA (mol L^{-1})	2.5×10^{-12}	CA required $\times$ cell concentration

followed by fluorometric detection (Nolting et al. 2000). The Zn^{2+} concentration was calculated with the MINEQL program, in which the DIC dissociation constants were changed to those determined by Roy et al. (1993).

Results

Conversion of HCO_3^- to CO_2—We calculated the uncatalyzed rate of dehydration of HCO_3^- to CO_2 and the amount of carbonic anhydrase (CA) that is needed to catalyze this dehydration from literature values and our culture conditions (Table 1). The $k_{cat}^{HCO_3^-}$ of carbonic anhydrase was extrapolated from pH 7.62 (Pocker and Miksch 1978) to pH 7.9 by using the Haldane relationship:

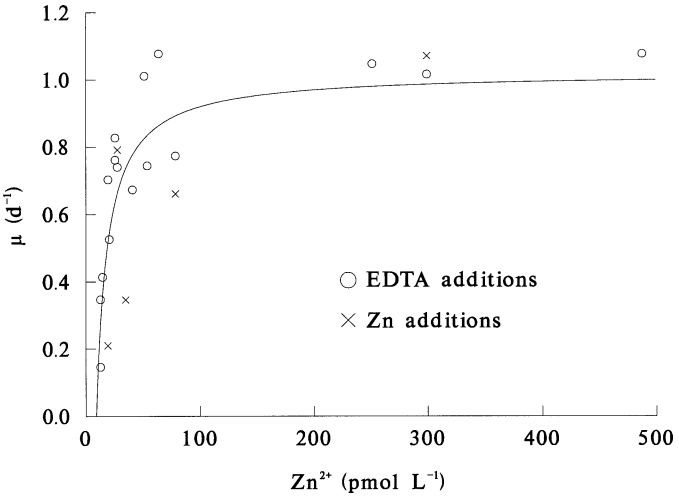

Fig. 2. Growth rate of *Emiliania huxleyi* as a function of Zn^{2+}. Circles represent measurements made at a constant Zn_T concentration (either 3.7 or 10.0 nmol L^{-1}); crosses represent measurements at a constant EDTA concentration (200 μmol L^{-1}). *See text* for fitted equation (Eq. 5) and parameter values.

$$\frac{k_{cat}^{CO_2} K_m^{HCO_3^-}}{k_{cat}^{HCO_3^-} K_m^{CO_2}} = \frac{K_1}{[H^+]} \quad (4)$$

The value of K_1 was calculated for those values for which both $k_{cat}^{HCO_3^-}/K_m^{HCO_3^-}$ (Pocker and Miksch 1978) and $k_{cat}^{CO_2}/K_m^{CO_2}$ (Pocker and Ng 1973) were available. While the average value of pK_1 was 6.05, compared to a value of 6.03 calculated from Roy et al. (1993, at 25°C and 1 psu), the value of pK_1 decreased by 0.4 per pH unit. We used this correlation with pH to get a smooth transition from the measured points of $k_{cat}^{HCO_3^-}/K_m^{HCO_3^-}$ to the extrapolated value using $k_{cat}^{CO_2}/K_m^{CO_2}$ and K_1 in Eq. 4, which we have deemed justifiable for the small extrapolation range (from pH 7.62 to 7.9).

From these calculations (Table 1), it can be seen that the uncatalyzed CO_2 generation is 4.5 orders of magnitude too small to support the use of the protons that are generated at the measured calcification rate. The minimum cellular requirement of Zn in carbonic anhydrase can then be calculated to be 2.5 pmol L^{-1} at our experimental cell concentration.

Zn^{2+} limitation experiment—In the first experiment, the Zn^{2+} requirement of *Emiliania huxleyi* was determined. The growth rate as a function of Zn^{2+} clearly follows a saturation curve (Fig. 2). At the highest EDTA concentration (300 μmol L^{-1}, 8 pmol L^{-1} Zn^{2+}), the growth rate was negative (not shown) and increased after addition of either $ZnCl_2$ (data not shown) or $CoCl_2$. Addition of Co to a concentration of 6 pmol L^{-1} Co^{2+} gave a half-maximum growth rate (0.51 d^{-1}). The response of the growth rate was the same when Zn_T was varied at a constant EDTA concentration (crosses in Fig. 2). Since it was obvious that the growth curve did not go through the origin, the results were fitted to a modified Michaelis–Menten equation (Michaelis and Menten 1913) with a minimum Zn^{2+} concentration

$$\mu = \frac{([Zn^{2+}] - [Zn^{2+}_{min}])\mu_{max}}{[Zn^{2+}] - [Zn^{2+}_{min}] + K_m} \quad (5)$$

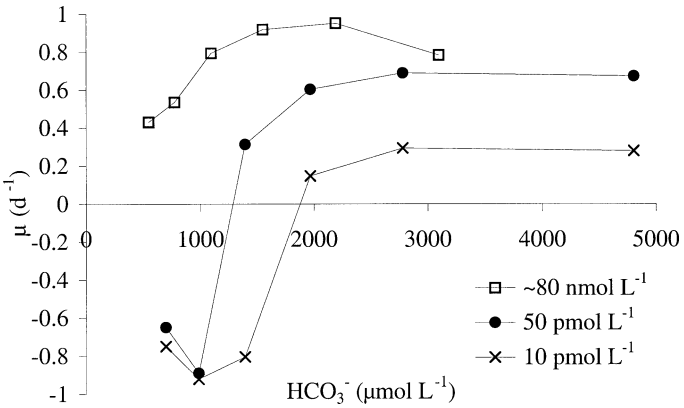

Fig. 3. Growth rate of *Emiliania huxleyi* as a function of HCO_3^- at 10 and 50 pmol L^{-1} Zn^{2+}, and a constant CO_2 concentration of 16.7 μmol L^{-1}. Zn^{2+} decreases by 5% with the increasing HCO_3^- due to a change in Zn speciation with pH. For reference, the growth rates at constant CO_2 (13.8 μmol L^{-1}) and ~80 nmol L^{-1} Zn^{2+} are included (Buitenhuis et al. 1999).

The best fit through all the positive growth rates gives a minimum Zn^{2+} concentration $[Zn^{2+}_{min}] = 9 \pm 3$ pmol L^{-1}, a maximum growth rate $\mu_{max} = 1.03 \pm 0.07$ d^{-1}, and a half-saturation constant $K_{1/2} = K_m + [Zn^{2+}_{min}] = 19 \pm 8$ pmol L^{-1}. The maximum growth rate is not significantly different from the maximum growth rate of 1.1 ± 0.1 d^{-1} determined by Buitenhuis et al. (1999) on the same strain of *E. huxleyi*. The half-saturation constant is the same order of magnitude as the calculated minimum cell quota of Zn in carbonic anhydrase.

Zn^{2+}-HCO_3^- colimitation experiments—In the second experiment, *E. huxleyi* was grown in a range of HCO_3^- concentrations at two Zn^{2+} concentrations (10 and 50 pmol L^{-1}). At both Zn^{2+} concentrations the cells were dying at low HCO_3^- concentrations (Fig. 3), even though the CO_2 concentration was uniformly high (17 μmol L^{-1}) in all cultures. The cells were dying even at a concentration of 50 pmol L^{-1} Zn^{2+}, a concentration at which growth rate was almost maximal at a higher HCO_3^- concentration in the Zn^{2+} limitation experiment (Fig. 2). This shows that Zn^{2+} was needed to use the abundant supply of CO_2. This fact is not accounted for in the currently accepted conceptual model of inorganic carbon use by *E. huxleyi* (Fig. 1). The higher HCO_3^- concentration that is needed for cells to grow at the lower Zn^{2+} concentration of 10 pmol L^{-1} shows that Zn^{2+} also affects the efficiency with which HCO_3^- is used, as is expected if carbonic anhydrase converts HCO_3^- to CO_2 for fixation in photosynthesis.

To quantify this interconnection, in the third experiment *E. huxleyi* was grown at a range of HCO_3^- and Zn^{2+} concentrations. At low HCO_3^- concentrations and intermediate Zn^{2+} concentrations (~150 pmol L^{-1}), the growth rate of *E. huxleyi* was reduced. At high HCO_3^- concentrations and intermediate Zn^{2+} concentrations, the growth rate was unaffected (Fig. 4). This shows that we do not observe a simple Zn^{2+} limitation but that we observe a Zn^{2+}-HCO_3^- colimitation. While this does not prove that the observed reduction in growth rate is caused by a lowered efficiency of the carbonic anhydrase as it is shown in Fig. 1, it does show that Zn^{2+} affected the efficiency with which HCO_3^- is used.

We considered three equations expressing growth rate as a function of two nutrient concentrations. We used the maximum growth rate as determined from the Zn^{2+} limitation experiment, $\mu_{max} = 1.03$ d^{-1}, because the maximum growth rate was poorly constrained by the data and to minimize the number of parameters to be fitted. The positive growth rates ($n = 61$) were fitted to the following functions:

- Multiplication of two Michaelis–Menten type saturation curves (Eq. 6, Fig. 4A)

$$\mu = \frac{[HCO_3^-]}{[HCO_3^-] + K_{m,HCO_3^-}} \times \frac{[Zn^{2+}]}{[Zn^{2+}] + K_{m,Zn^{2+}}} \times \mu_{max} \quad (6)$$

This is mathematically the simplest functional equation in which the two nutrients act independently on growth rate. Minimizing the sum of the residual squares gives $K_{m,HCO_3^-} = 1{,}189 \pm 331$ μmol L^{-1} and $K_{m,Zn^{2+}} = 38 \pm 12$ pmol L^{-1}. The mean residual on μ is 0.018 d^{-1} (where the mean residual is $\sqrt{\hat{\sigma}^2/n} = \sqrt{(\text{sum of squared residuals})/n}$).

- The minimum of two Michaelis–Menten type saturation curves (Eq. 7, Fig. 4B)

$$\mu = \text{MIN}\left(\frac{[HCO_3^-]\mu_{max}}{[HCO_3^-] + K_{m,HCO_3^-}}, \frac{[Zn^{2+}]\mu_{max}}{[Zn^{2+}] + K_{m,Zn^{2+}}}\right) \quad (7)$$

For independent nutrients, this has been shown to be the best representation (Zonneveld 1996), whereby the most limiting nutrient controls growth rate, while the supply of the non-limiting nutrient is adequate at this lower growth rate. Minimizing the sum of the residual squares gives $K_{m,HCO_3^-} = 2{,}458$ μmol L^{-1} and $K_{m,Zn^{2+}} = 62$ pmol L^{-1}. No error estimates for the parameter values were derived. The mean residual on μ is 0.020 d^{-1}.

- Affinity for HCO_3^- depends on Zn^{2+} (Eq. 8, Fig. 4C)

$$\mu = \frac{[HCO_3^-]\mu_{max}}{[HCO_3^-] + ([Zn^{2+}] + K_m)\mu_{max}/([Zn^{2+}]\alpha_{max,HCO_3^-})} \quad (8)$$

This equation has been derived from a simple Michaelis–Menten equation, by substituting $K_{m,HCO_3^-} = \mu_{max}/\alpha_{HCO_3^-}$ and assuming that the affinity for HCO_3^- ($\alpha_{HCO_3^-}$) is a saturating function of the Zn^{2+} concentration $\alpha_{HCO_3^-} = Zn^{2+} \times \alpha_{max,HCO_3^-}/(Zn^{2+} + K_{m,Zn})$. Minimizing the sum of the residual squares gives $\alpha_{max,HCO_3^-} = 0.00165 \pm 0.00002$ L (μmol d)$^{-1}$ (that is, $\alpha^{-1}_{max,HCO_3^-} = 606$ μmol d L^{-1}), and $K_{m,Zn^{2+}} = 317 \pm 45$ pmol L^{-1}. The mean residual on μ is 0.020 d^{-1}.

Discussion

Conversion of HCO_3^- to CO_2—It is possible to calculate whether *Emiliania huxleyi* needs carbonic anhydrase by comparing the rate at which protons are generated in calcification with the rate of the uncatalyzed conversion of HCO_3^- into CO_2. Most of the values in Table 1 have considerable uncertainties. The pH in the chloroplast was measured as 7.9 ± 0.6 (Anning et al. 1996). At present it is unclear whether *E. huxleyi* has a carbon concentrating mechanism (Sekino and Shiraiwa 1994) or not (Nimer et al. 1994*b*),

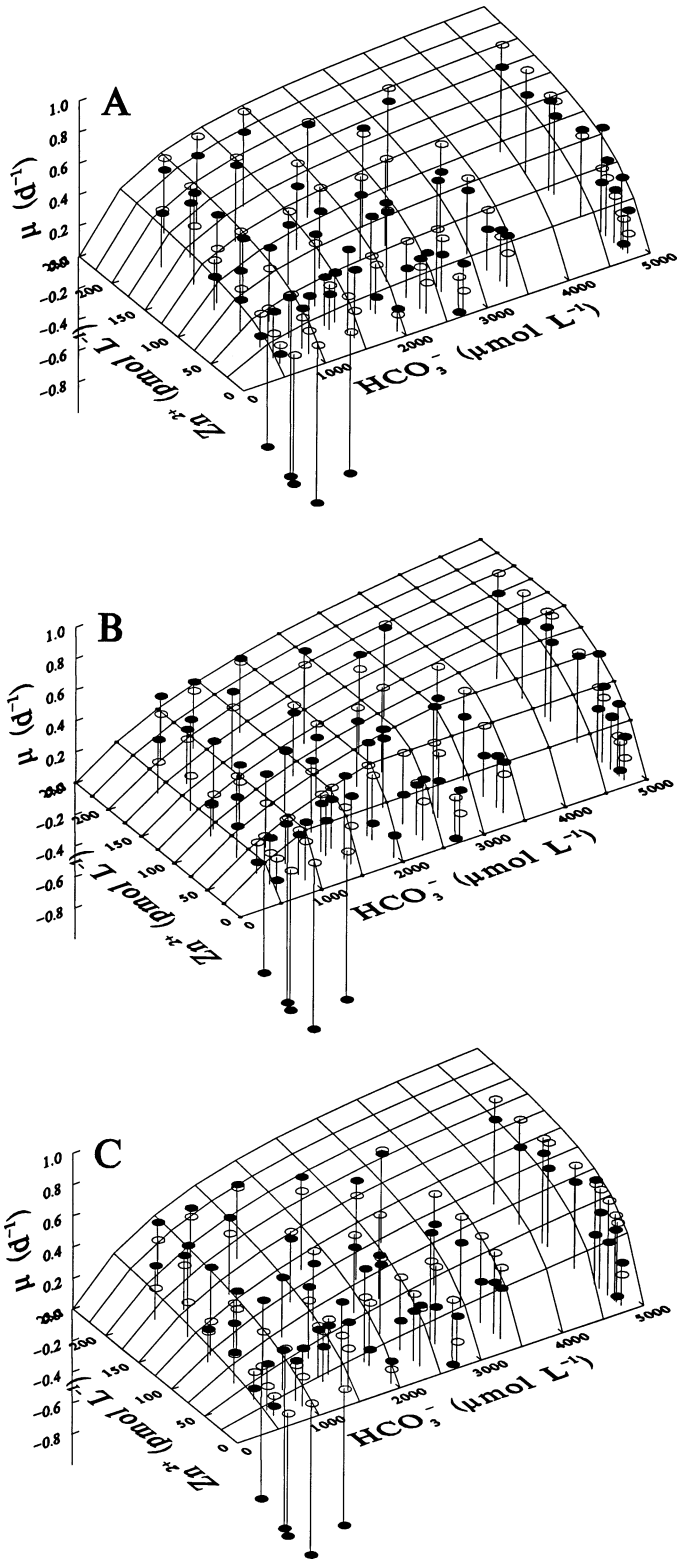

Fig. 4. Growth rate of *Emiliania huxleyi* as a function of HCO_3^- and Zn^{2+} at a constant CO_2 concentration of 16.7 μmol L^{-1}. Filled circles represent experimental data points and are identical in the three panels. The data points of Fig. 3 are included. The data point at 406 pmol L^{-1} Zn^{2+}, 698 μmol L^{-1} HCO_3^-, $\mu = 0.59$ d^{-1} is not shown. The data have been fitted to Eqs. 6–8, and these have been plotted in the form of a mesh. Open circles have been calculated from the equations at the points where growth rates were measured. Thus, the open circles lie on the mesh and indicate whether the measured rates lie above or under the calculated mesh. *See text* for equations and parameter values. (A) Multiplication of limitations, Eq. 6. (B) Law of the minimum, Eq. 7. (C) Affinity for HCO_3^- is a saturating function of Zn^{2+}, Eq. 8.

which results in a 40-fold uncertainty in the intracellular HCO_3^- concentration. The value $k_{cat}^{HCO_3^-}$ would be three times higher if we used the K_1 of Roy et al. (1993), rather than the variable K_1 calculated from the Haldane relationship (Eq. 4). The kinetic parameters were measured on CA from spinach at 25°C (Pocker and Miksch 1978). However, in Table 1 we calculate the rate of uncatalyzed hydration of HCO_3^- to be more than four orders of magnitude smaller than the calcification rate at ambient conditions ($fCO_2 = 444.6$ μatm, pH = 8). Therefore, since calcification was found to be tightly coupled to the photosynthetic use of HCO_3^- (Buitenhuis et al. 1999), it still seems to be justified to conclude that the conversion of HCO_3^- to CO_2 is catalyzed by CA. From the catalytic rate of CA and the conditions in the chloroplast, the amount of CA that is needed for enzyme catalyzed conversion can be calculated to be 2.5×10^{-20} mol $cell^{-1}$ (Table 1). Since we used the $k_{cat}^{HCO_3^-}$ for monomeric CA, which contains one atom of Zn^{2+} as a cofactor, this directly gives the minimum cellular Zn^{2+} requirement in carbonic anhydrase: at a maximum cell density in our experiments of $\sim 10^8$ cells ml^{-1}, a minimum concentration of 2.5 pmol L^{-1} Zn^{2+} is required. This 2.5 pmol L^{-1} was calculated from the cellular Zn^{2+} requirement at a steady state calcification rate and thus constitutes a biomass or yield approach. This minimum yield for Zn^{2+} sufficient growth is of the same order of magnitude as the half-saturation constant of 19 pmol L^{-1} that was measured with the kinetic approach of measuring growth rates as a function of Zn^{2+} concentration.

Zn^{2+}-Co^{2+} interreplacement—As was shown before (e.g., Timmermans et al. 2001) Co^{2+} can replace the Zn^{2+} requirement of *E. huxleyi* in our experiments. Co was not measured, but in medium that was prepared in the same way Co_T was 4 to 7 nmol L^{-1} (A. Daniel, Univ. Liverpool, pers. comm.). Since Co is complexed by EDTA 40 times more efficiently than Zn, there was little Co^{2+} available relative to Zn^{2+}.

Effect of EDTA—In order to test for the possible detrimental effect of EDTA (Muggli and Harrison 1996), the Zn^{2+} concentration was manipulated either by the addition of EDTA or by the addition of $ZnCl_2$ at a high EDTA concentration (200 μmol L^{-1}). In Fig. 2 it can be seen that there is no significant difference between growth rates measured at high EDTA concentrations and high Zn_T concentrations and at low EDTA concentrations and low Zn_T concentrations, corresponding to the same Zn^{2+}. Muggli and Harrison (1996) measured reduced growth rates of *E. huxleyi* at 100 μmol L^{-1} EDTA. However, the Zn^{2+} concentration varied in their experiments. In fact, they measured a growth rate at 16 pmol L^{-1} Zn^{2+}, which was 47% of that at 251 pmol L^{-1} Zn^{2+}, which is almost in perfect agreement with our $K_{1/2}$ of

19 pmol L^{-1} Zn^{2+}. Their interpretation of the detrimental effect of EDTA was possibly suggested by the results of Sunda and Huntsman (1992) that the Zn^{2+} requirement of *E. huxleyi* is extremely low. This was subsequently shown to be due to the presence of Co^{2+} in the medium, which can replace Zn^{2+} (Sunda and Huntsman 1995). Both Muggli and Harrison (1996) and we used low Co^{2+} concentrations (*see materials and methods*). Given our finding that EDTA has no direct poisonous effect on *E. huxleyi*, the advantage of using EDTA is that it buffers the Zn^{2+} concentration so that it remains constant in solution as the algae grow and use Zn^{2+}. This made it possible to simplify the experimental approach to a batch culture and still get cells that were adapted to a specific Zn^{2+} concentration (*see materials and methods*).

Zn^{2+} limitation—Zn^{2+} plays a role in a great number of cellular processes (Fraústo da Silva and Williams 1991); thus there is a conceivable risk of ascribing effects of Zn^{2+} limitation to the lack of carbonic anhydrase while in fact another cellular process is responsible for this effect. We consider that the risk of such a misinterpretation is limited, first because it was found that in a diatom most of the cellular Zn^{2+} was contained in carbonic anhydrase (Morel et al. 1994), and second, and more importantly, we have specifically addressed the role of carbonic anhydrase by growing *E. huxleyi* under Zn^{2+}-HCO_3^- colimitation in order to distinguish between general effects of Zn^{2+} limitation and those effects that are linked to inorganic carbon use. The fact that at intermediate Zn^{2+} concentrations of ~150 pmol L^{-1} a reduction of growth rate is only observed at low HCO_3^- concentrations (Fig. 4) shows that Zn^{2+}-HCO_3^- colimitation does indeed occur.

Zn^{2+}-HCO_3^- colimitation—Previous investigations of colimitations have shown that applying the law of the minimum is in better agreement with the data than multiplying limitations of independent nutrients (Droop 1983; Zonneveld 1996). There is compelling circumstantial evidence that for dependent nutrients (where one nutrient is involved in the use of the second nutrient) it would be more appropriate to describe colimitation as the influence of the first nutrient on the affinity for the second (e.g., Fe-NO_3^- colimitation, Timmermans et al. 1994). According to the conceptual model, Zn^{2+} is involved in the C metabolism of *E. huxleyi* as a cofactor in carbonic anhydrase. This would enable the cells to use HCO_3^- in photosynthesis (Fig. 1). From this it is expected that the efficiency of HCO_3^- use in photosynthesis declines with decreasing Zn^{2+}, whereas the use of CO_2 in photosynthesis should be unaffected.

We suggest three possible explanations for the decline in efficiency of HCO_3^- use with decreasing Zn^{2+} as seen in Figs. 3 and 4.

a. The simplest explanation is that Zn^{2+} is involved in the use of HCO_3^-. This is consistent with the conceptual model in Fig. 1, in which Zn^{2+} acts as the cofactor of carbonic anhydrase. In this case Zn^{2+} increases the efficiency with which HCO_3^- is converted to CO_2, which is the substrate for Rubisco.
b. Zn^{2+} and HCO_3^- can partly replace each other in maintaining intracellular pH within the range needed for cell survival. It has been shown that HCO_3^- affects the cytoplasmatic pH, from which it was concluded that the intracellular speciation of DIC acts as a pH buffer (Nimer et al. 1994*a*). Carbonic anhydrase would be needed to keep this pH buffer functioning at low HCO_3^- concentrations if the flux of HCO_3^- into the cell is much higher than the uncatalyzed equilibration of intracellular DIC. The rate of uncatalyzed equilibration would be 10 times faster in the more acidic cytoplasm (pH 6.9, Anning et al. 1996) than in the chloroplast. This would still mean that this rate is slow compared to the rate of HCO_3^- use (cf. Table 1). Although no carbonic anhydrase was found in the cytoplasm (Nimer et al. 1994*b*), it has been found that a carbon concentrating mechanism can have carbonic anhydrase-like properties, and a putative new type of carbonic anhydrase in *Synechococcus* that is directly linked to a HCO_3^- transporter has been identified (Price et al. 2002). Since the presence of either of these functions is still uncertain in *E. huxleyi*, this question should be considered open.
c. The cells produce more carbonic anhydrase at low concentrations of HCO_3^-, and the Zn^{2+} in this enzyme competes with the other sites where Zn^{2+} is used in the cell and thus leads to an indirect Zn^{2+} limitation at low HCO_3^-. This explanation seems to be directed against survival of the cell when they could grow on CO_2 only, but since HCO_3^- never becomes low enough in the sea for this problem to occur it might still be functional.

In addition to the effect of Zn^{2+} on HCO_3^- use, Zn^{2+} was seen to have an effect on CO_2 use, since at low Zn^{2+} and low HCO_3^- *E. huxleyi* died (negative μ), even though the CO_2 concentration was constantly high at 17 μmol L^{-1}. This is in contradiction with our conceptual model, in which CO_2 is the direct substrate for Rubisco (Fig. 1). It is important to note that the negative growth rates cannot be explained by just Zn^{2+} limitation, since they only become apparent at low HCO_3^- concentrations. Neither is it just a lack of HCO_3^-, since cells can perform photosynthesis using CO_2 at HCO_3^- concentrations where calcification stops (Buitenhuis et al. 1999). It is also not due to a decreasing CO_2 in the medium. In the cultures with active growth the CO_2 concentration will have decreased due to algal use of inorganic carbon. The maximum influence would be to reduce the CO_2 concentration by 22% to 13.1 μmol L^{-1}. The maximum influence will have occurred at the second HCO_3^- concentration of 958 μmol L^{-1}, at which growth rates were appreciable but not maximum, the buffering effect of the seawater was low, and calcification was reduced (in the calculation we assumed that calcification rate was zero to calculate the maximum effect, although under Zn^{2+} sufficient growth calcification still occurred at this HCO_3^- concentration [Buitenhuis et al. 1999] and the Zn^{2+}-limited cells were still calcified). While this 22% reduction of CO_2 concentration is by no means negligible, this would still be saturating under Zn^{2+} sufficient conditions (Buitenhuis et al. 1999). Thus we can conclude that there is at least some support for explanations (b) and (c) above, in which there are other effects of Zn^{2+} limitation in addition to the decline in efficiency of HCO_3^- use. A mech-

anism that would account for the Zn^{2+}-CO_2 colimitation, independent of the mechanisms of Zn^{2+}-HCO_3^- colimitation suggested above, would be conversion of CO_2 into HCO_3 at the cell membrane, transportation of HCO_3^- to the chloroplast, and conversion of HCO_3^- into CO_2 at the site of fixation by Rubisco. This relatively convoluted mechanism could possibly function to prevent diffusive loss of inorganic carbon, since the charged HCO_3^- ion can be retained in the cell, while CO_2 diffuses through the cell membrane (cf. Price et al. 2002). As mentioned under explanation (b) above, carbonic anhydrase has been measured only in the chloroplast under normal conditions, but alternate mechanisms have been identified that could have the same effects, such as CO_2 recycling (Price et al. 2002, and references therein). While these recent advances in detecting alternate mechanisms of inorganic carbon use have complicated matters, it would still have been useful to know the changes of carbonic anhydrase in our experiments. Unfortunately, our measurement of carbonic anhydrase enzyme by chemoluminescent detection on Western blots was not sensitive enough to detect carbonic anhydrase in *E. huxleyi* (data not shown). Therefore we can conclude from our results that while the current conceptual model of inorganic carbon use by *E. huxleyi* (Fig. 1, explanation a) can account for Zn^{2+}-HCO_3^- colimitation (Fig. 4), it does not account for the Zn^{2+}-CO_2 colimitation. We cannot distinguish between a direct effect of carbonic anhydrase deficiency to account for Zn^{2+}-CO_2 colimitation (explanation b, as suggested by the fact that this colimitation is only measured when both Zn^{2+} and HCO_3^- are low, and by the close correspondence between the calculated cellular Zn requirement in carbonic anhydrase and the half-saturation constant for Zn^{2+} use) or a much more indirect effect of Zn on the efficiency of CO_2 use (explanation c).

It is noteworthy that the abilities of the cells to use HCO_3^- and CO_2 from the medium seem to be independent. In Fig. 3 it was shown that *E. huxleyi* was unable to use the high concentration of CO_2, even at a Zn^{2+} concentration of 50 pmol L^{-1}, but that they were able to use HCO_3^-, albeit with a reduced efficiency. Thus, the supply of CO_2^{ex} and CO_2^{in} to Rubisco in Fig. 1 is apparently independent with respect to the effect of Zn^{2+} limitation.

Since we have insufficient data to decide about the mechanisms involved in the Zn^{2+}-HCO_3^- colimitation, we will apply a mathematical criterion to compare the three equations that express growth rate as a function of two nutrient concentrations. We have applied Akaike's information criterion ($AIC = n \log(\hat{\sigma}^2) + 2K$, where K is the total number of estimated parameters, Burnham and Anderson 1998) to calculate the significance in the differences in mean residuals for Eqs. 6–8. The AIC for Eq. 6 is lowest, while for Eq. 7 $\Delta_{AIC} = 4.1$ and for Eq. 8 $\Delta_{AIC} = 5.8$. This indicates that there is considerably more support for Eq. 6 (Burnham and Anderson 1998). This would indicate that Zn^{2+} and HCO_3^- are independent nutrients. However, there are two caveats to this result. First, the results of the second experiment gave rise to the opposite conclusion: that Zn^{2+} and HCO_3^- are dependent nutrients. Second, the inability to express negative growth rates in any of the three equations and the exclusion of these data in the comparison using the AIC indicates that the real conclusion should be that we have reason to say that the current model of inorganic carbon use by *E. huxleyi* (Fig. 1) is incomplete but that we cannot propose a better one at present.

Significance in the ocean—There are very few assessments of Zn^{2+} concentrations in the open ocean (data compilation of Town and Filella, cf. Town and Filella 2000). These data are in agreement with the trend found for other metal ions, with low values in the open ocean where the concentration of organic ligands can get higher than the total Zn concentration. Zn^{2+} concentrations can get as low as 0.4 pmol L^{-1} in the Northeast Pacific Ocean (Donat and Bruland 1990), while in the coastal region, where total Zn concentrations are higher than the ligand concentration, the Zn^{2+} concentrations can get into the nmol L^{-1} range. The open ocean values are much lower than our measured half-saturation constant of 19 ± 8 pmol L^{-1} Zn^{2+} for *E. huxleyi*. Thus, Zn^{2+} may limit carbon fixation by *E. huxleyi* in the open ocean and thus affect the efficiency of the biological carbon pump, as proposed by Morel et al. (1994). In contrast to Zn^{2+}, the concentration of HCO_3^- varies only over the range of about 1.7 to 2.3 mmol L^{-1} in surface waters. Thus, little effect of HCO_3^- is to be expected under Zn^{2+} limitation.

We have shown that there is a variable cellular requirement for Zn^{2+} that is a function of the HCO_3^- concentration. While the Zn^{2+}-HCO_3^- colimitation is in agreement with our present understanding of the inorganic carbon use of *E. huxleyi*, the inability of Zn^{2+}-limited cells to grow on an abundant supply of CO_2 indicates that our understanding is incomplete. The ability of cells to grow on high HCO_3^- concentrations at low Zn^{2+} indicates that any explanation that is put forward concerning the Zn requirement must focus on the carbon physiology, including pH regulation. The finding that carbonic anhydrase forms the major cellular pool of Zn in the diatom *Thalassiosira weissflogii* (Morel et al. 1994) suggests that carbonic anhydrase is the factor mediating Zn^{2+}-HCO_3^- colimitation. However, since we were unable to detect carbonic anhydrase even with the sensitive chemoluminescent technique, the mechanism behind the Zn^{2+}-CO_2 colimitation should be considered unresolved and could be an indirect effect on other cellular processes. More experimental evidence, such as Zn^{2+}-limited rates of photosynthesis and calcification, Zn uptake rates, carbonic anhydrase content, and discrimination against ^{13}C in the organic and inorganic fractions (at constant CO_2 and at constant HCO_3^-) will be needed to resolve the problem we have presented here.

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