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Growth and photosynthesis limitation of marine red tide alga *Skeletonema costatum* by low concentrations of Zn^{2+}

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Abstract

The specific growth rate, cell final yields and extracellar carbonic anhydrase activity of the red tide alga *Skeletonema costatum* increased with increasing concentrations of Zn^{2+} from 0 to 12 pM, but decreased when Zn^{2+} was over 24 pM. However, cells grown under high concentrations of Zn^{2+} had higher activities of intracellular carbonic anhydrase than those grown under low concentrations of Zn^{2+}. Chlorophyll *a*-specific light-saturated photosynthetic rate ($P_m^{chl\,a}$), dark respiration rate ($R_d^{chl\,a}$) and apparent photosynthetic efficiency ($\alpha^{chl\,a}$) significantly increased with increasing concentrations of Zn^{2+} from 0 to 3 pM, but decreased when increasing concentrations of Zn^{2+} from 3 to 66 pM. Photorespiration is the lowest when cells cultured in 3 pM Zn^{2+}. The results suggest physiological activity of *Skeletonema costatum* is very sensitive to the prevailing concentration of Zn^{2+}.

Introduction

Zinc is widely required in many biological processes and is present in nearly 300 enzymes that perform many different metabolic functions (Vallee & Auld 1990). Recently, extremely low concentrations of Zn^{2+}, determined particularly in oceanic surface waters, have prompted a revaluation of its role with respect to phytoplankton productivity (Morel *et al.* 1994). Uptake of zinc by phytoplankton is related to its free ion concentration rather than organically complexed zinc (Anderson *et al.* 1978, Sunda & Huntsman 1992). More than 98% of zinc is organically complexed in surface seawater of central North Pacific, which results in a concentration of Zn^{2+} as low as 1 pM (Bruland 1989, Bruland *et al.* 1991). Since the optimum Zn^{2+} concentration for oceanic microalgae is often higher than this (Ellwood & Van den Berg 2000), such low zinc availability may limit growth of oceanic phytoplankton and their ability to fix CO_2 from seawater via the enzyme carbonic anhydrase (Sunda & Huntsman 1992, Morel *et al.* 1994).

Some experiments have been run that examine growth limitation of oceanic and coastal species as a function of Zn^{2+} concentrations (Anderson *et al.* 1978, Brand *et al.* 1983). A positive relationship has been demonstrated between growth rate and Zn^{2+} concentrations in a coastal diatom *Thalassiosira weissflogii* culture up to 32 pM Zn^{2+}; the onset of limitation is around 10 pM Zn^{2+} (Anderson *et al.* 1978). The minimum requirement of Zn^{2+} concentration by *Emiliania huxleyi* is 9 pM (Buitenhuis *et al.* 2003). Few studies are conducted corresponding relationships between Zn^{2+} concentrations and photosynthesis of any species (Danilov & Ekelund 2001). And also the evidence for zinc limitation in coastal phytoplankton in general and harmful algal blooms in particular, is lacking (Boyer & Brand 1998). In this study, we carry out comprehensive experiments with *Skeletonema costatum*, one of the major causative organisms of red tide blooms, in order to investigate ef-

fects of various concentrations of Zn^{2+} on the growth, photosynthetic characteristics, photorespiration and carbonic anhydrase activity.

Materials and methods

Organism and growth conditions

Skeletonema costatum was obtained from the Institute of Oceanology, Chinese Academy of Sciences (Qingdao, P.R. China). The artificial seawater was added with sterile f/2 nutrients (Harrison *et al.* 1980) of 880 μM $NaNO_3$, 36 μM NaH_2PO_4, 106 μM $NaSiO_3$, vitamin solution (0.5 μg cyanocobalamin l^{-1}, 0.5 μg biotin l^{-1}, 100 μg thiamine·HCl l^{-1}) and micronutrients (0.9 μM $MnCl_2$, 0.03 μM Na_2MoO_4, 0.04 μM $CuSO_4$, Co^{2+} was not included, Fe^{3+} was substituted by 50 nM $FeCl_2$ and EDTA was at 100 μM) with Zn concentrations as indicated in each individual experiment. Inorganic Zn^{2+} concentrations were calculated from total Zn concentrations using the computer program MINEQL (Westall *et al.* 1976). Five Zn^{2+} were obtained, each of these in duplicate. Stock cultures were grown at without added Zn^{2+} for six generations prior to the inoculation of experimental cultures. Experimental cultures were grown in 200 ml polycarbonate flasks in plant growth chamber at 22 °C, 100 μmol photon m^{-2} s^{-1} and a 12 h light:12 h dark cycle for five or more generations and harvested in the early exponential phase.

Cell density and pigment measurements

Cell density was monitored daily by enumeration with a phytoplankton-counting chamber (0.1 ml). Specific growth rates (μ) were calculated using the equation $\mu = (\ln X_t - \ln X_0)/t$, where X_0 is the initial cell density, X_t is the cell density after t d. Chlorophyll *a* contents were determined according to Jeffrey & Humphrey (1975) with 90% (v/v) acetone extracts.

Photosynthetic activity

Cells were harvested in mid-growth phase and resuspended in artificial seawater enriched with f/2. Their photosynthetic activity was assayed by measuring the rate of O_2 evolution under different irradiances using a Clark-type O_2 electrode (Hansatech Instruments Ltd., UK). The temperature was kept at 22 °C by a circulating water bath. Data were treated by non-linear fitting technique using model $P = P_m \times \tanh(\alpha \times I/P_m) + R_d$ (Henley 1993), where I represents irradiance, and P is photosynthetic rate at certain irradiance. P_m, light-saturated photosynthesis; α, the initial slope at limiting irradiances, was calculated to assess the photosynthetic efficiency; R_d, dark respiration rate. I_c, the light intensity at which net photosynthetic rate is zero, was calculated as R_d/α. I_k, the light intensity at which photosynthesis is initially saturated, was calculated as P_m/α.

Table 1. Maximum cell density and specific growth rate of *Skeletonema costatum* grown under different concentrations of Zn^{2+}.

Zn^{2+} (pM)	Specific growth rate (d^{-1})	Maximum cell density (10^5 cells ml^{-1})
0	0.44	1.9
3	0.55	2.3
12	0.58	4.7
24	0.45	3.4
66	0.52	3.8

Carbonic anhydrase assays

Carbonic anhydrase was measured in homogenized whole cells (total activity) and intact whole cell (extracellular activity) by a pH drift assay (Wilbur & Anderson 1948). Intracellular activity was calculated using extracellular activity subtracted from total activity.

Photorespiration rate

Photorespiration rate (P_R) was determined by the differences in oxygen evolution rate of cells in high O_2 concentration and low O_2 concentration in the air using the Clark-type oxygen electrode (Hansatech Instruments Ltd., UK). The cells harvested in mid-growth phase were placed in the O_2 electrode chamber. After aerated with pure N_2 (net photosynthesis in low O_2, P_{N_2}) or air (net photosynthesis in high O_2, P_{O_2}) for 4–5 min, oxygen evolution rate at saturated light intensity was measured. Finally dark respiration rate (R_d) was measured. Photorespiration was calculated according to the equation: $\frac{P_R}{P_g}\% = \frac{(P_{N_2}+R_d)-(P_{O_2}+R_d)}{P_{O_2}+R_d}$, where P_g is the gross photosynthesis of cells in high O_2 concentration.

Table 2. Photosynthetic parameters of *Skeletonema costatum* grown under different concentrations of Zn^{2+}. These parameters were determined by fitting a three-parameter model, $P = P_m \times \tanh(\alpha \times I/P_m) + R_d$, where I represents irradiance, and P is photosynthetic rate at certain irradiance. These parameters were averaged from three replicate measurements.

Photosynthetic parameters	Zn^{2+} (pM)				
	0	3	12	24	66
$P_m^{chl\,a}$ μmol O_2 (mg chl *a* h)$^{-1}$	356.1 ± 13.8	882 ± 13.9	739 ± 13.9	631.8 ± 13.8	579 ± 13.8
$\alpha^{chl\,a}$ μmol O_2 (mg chl *a* h)$^{-1}$ (μmol photons m^{-2} s^{-1})$^{-1}$	1.6 ± 0.2	3.7 ± 0.1	3.4 ± 0.2	2.9 ± 0.2	2.8 ± 0.2
$R_d^{chl\,a}$ μmol O_2 (mg chl *a* h)$^{-1}$	15.4 ± 12.1	63.5 ± 11.9	43.9 ± 12.1	38.9 ± 12.1	37.9 ± 12.3
I_k μmol photons m^{-2} s^{-1}	218.7	236.8	219.7	217.7	206.2
I_c μmol photons m^{-2} s^{-1}	9.5	17.1	13	13.4	13.5

Chl, chlorophyll; P_m, light-saturated photosynthesis; α, the initial slope at limiting irradiances; R_d, dark respiration rate; I_c, the light intensity at which net photosynthetic rate is zero, was calculated as R_d/α; I_k, the light intensity at which photosynthesis is initially saturated, was calculated as P_m/α.

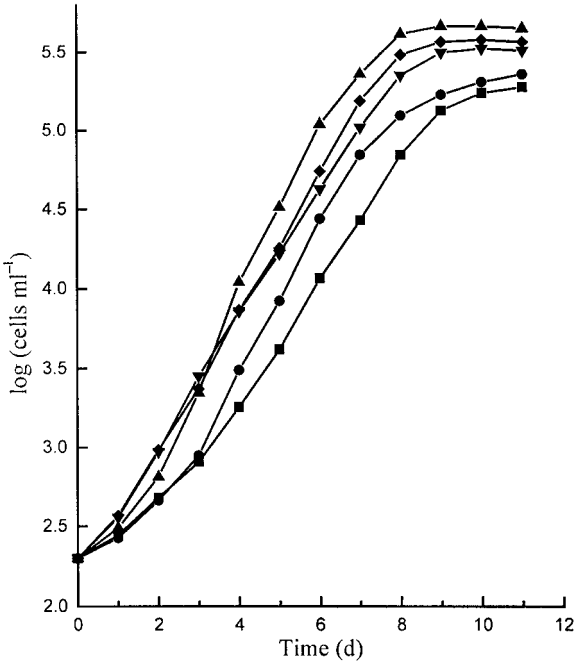

Fig. 1. Growth of *Skeletonema costatum* at different concentrations of Zn^{2+}. Zn^{2+}: ■, 0 pM; ●, 3 pM; ▲, 12 pM; ▼, 24 pM; ♦, 66 pM.

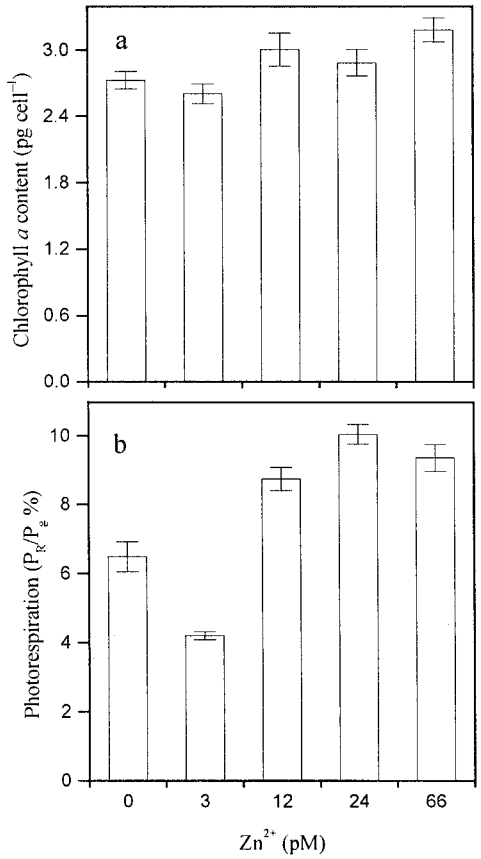

Fig. 2. Chlorophyll *a* contents (a) and photorespiration (b) of *Skeletonema costatum* at different concentrations of Zn^{2+}. Error bars denote the standard deviation of triplicate incubations.

Results and discussion

Zn^{2+}-limited yield and the growth rate

As shown in Figure 1, cell final yields increased with an increase of zinc concentration from 0 to 12 pM, and decreased over 24 pM. The lowest specific growth rate and cell final yields were without addition of zinc to the medium. Cell final yield in 12 pM Zn^{2+} was approx. 2.5-fold that without added Zn^{2+}, and specific growth rate was 1.3-fold of this. Increasing Zn^{2+} to 24 or 66 pM slightly decreased the cell final yields and the specific growth rate (Table 1).

Chlorophyll a *contents and carbonic anhydrase activity*

Increasing concentrations of Zn^{2+} did not change the chlorophyll *a* content of the cells (Figure 2). Zinc limitation had no significant effect on chlorophyll *a* synthesis, which it is different from Fe limitation (Fu & Bell 2003). Zinc is an essential cofactor for carbonic anhydrase and the general effects of zinc limitation on algae are the decrease in carbonic anhydrase activity (Morel *et al.* 1994, Lane & Morel 2000, Reinfelder *et al.* 2000). Positive correlations were detected between total carbonic anhydrase activity, intracellular carbonic anhydrase activity and increasing concentration of Zn^{2+}. The highest level of intracellular carbonic anhydrase activity was recorded in the 66 pM Zn^{2+} culture, which was nearly twice that without added Zn^{2+}. However, the highest activity of extracellular carbonic anhydrase was with 12 pM Zn^{2+} being 1.4 times higher than without Zn^{2+} (Figure 3). The extracelluar carbonic anhydrase activity influenced the uptake of CO_2, while the intracellular carbonic anhydrase activity affected the CO_2 fixation that, in turn, decreased the photosynthetic efficiency. From this, CO_2 fixation during photosynthesis of *Skeletonema costatum* was more sensitive to zinc limitation than was the uptake of inorganic carbon.

Effect of Zn on photosynthesis and photorespiration

Zinc limitation imposed a restriction on the chlorophyll *a*-specific light-saturated photosynthetic rate ($P_m^{chl\ a}$), dark respiration rate (R_d^{chla}) and apparent photosynthetic efficiency (α^{chla}). P_m^{chla}, R_d^{chla} and α^{chla} were, respectively, 2.5-, 4.1- and 2.3-times higher in 3 pM Zn^{2+} culture than without added Zn^{2+}. These values decreased slightly when Zn^{2+} concentrations were further increased (Figure 4 and Table 2).

An increase in photosynthetic efficiency has also been observed when *Chlamydomonas reinhardtii* cells were exposed to Zn^{2+} (Danilov & Ekelund 2001). Our results suggested that 3 pM Zn^{2+} was the critical point for the photosynthesis of *Skeletonema costatum*. The photosynthetic parameters were more sensitive to Zn^{2+} concentration changes than was the cell growth. Moreover, the photosynthetic parameters of *Skeletonema costatum* were more markedly influenced by zinc limitation than by higher Zn^{2+} concentrations (over 12 pM), which might be toxic to the alga. I_c in zinc limitation culture was lower than was in higher

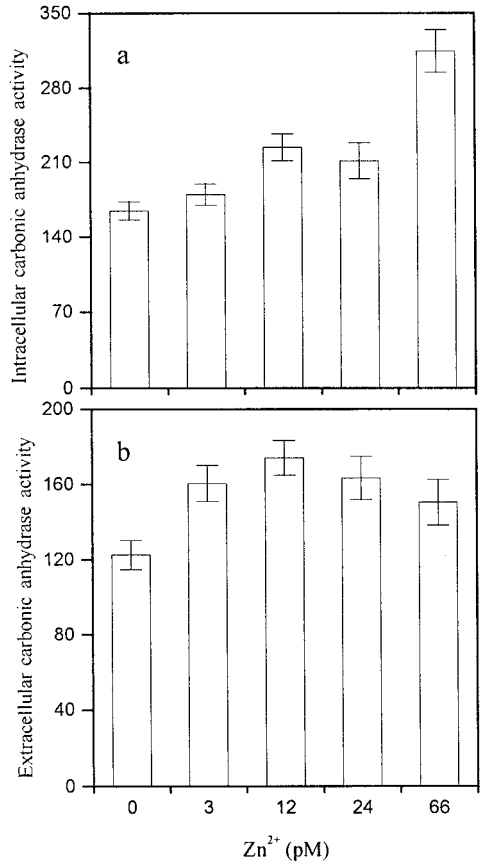

Fig. 3. Relative amounts of intracellular (a) and extracellular (b) carbonic anhydrase activity of *Skeletonema costatum* at different concentrations of Zn^{2+}. Error bars denote the standard deviation of three measurements from each of two independent sets of cultures.

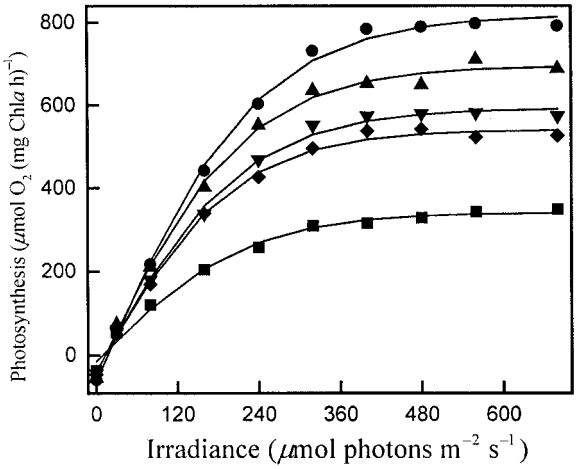

Fig. 4. Representative curves of chlorophyll *a*-specific photosynthesis versus irradiance for 0 Zn^{2+} (■), 3 pM Zn^{2+} (●), 12 pM Zn^{2+} (▲), 24 pM Zn^{2+} (▼), and 66 pM Zn^{2+} (♦) cultures.

Zn^{2+} cultures, and I_k did not show obvious differences at various Zn^{2+} concentration cultures.

The lowest photorespiration level was obtained in 3 pM Zn^{2+} culture (Figure 2). In zinc limitation cultures, not only the photosynthetic efficiency was restricted, but also the photorespiration was promoted, accordingly CO_2 fixation in photosynthesis decreased. In high Zn^{2+} cultures the result was the same as zinc limitation culture.

Conclusions

Zinc limitation resulted in decrease in cell yield, specific growth rate, carbonic anhydrase activity and photosynthetic rate, and increase in photorespiration. The photosynthetic parameters were suggested to be far more sensitive measures of zinc limitation of *Skeletonema costatum* than was the cell growth. In zinc limitation cultures, both the ability of cells to uptake inorganic carbon and the inorganic carbon fixation in photosynthesis were affected; therefore the efficiency of carbon utilization decreased and finally resulted in decrease in the cell yield. In high Zn^{2+} cultures the reduction in photosynthetic efficiency and promotion in photorespiration resulted in slight decrease in the cell yield.

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References

Anderson MA, Morel FMM, Guillard RRL (1978) Growth limitation of a coastal diatom by low zinc ion activity. *Nature* **276**: 70–71.

Boyer GL, Brand LE (1998) Trace elements and harmful algal blooms. In: Anderson DM, Cembella AD, Hallegraeff GM, eds. *Physiological Ecology of Harmful Algal Blooms*. Berlin: Springer Verlag, pp. 489–508.

Brand LE, Sunda WG, Guillard RRL (1983) Limitation of marine phytoplankton reproductive rates by zinc, manganese and iron. *Limnol. Oceanogr*. **28**: 1182–1198.

Bruland KW (1989) Oceanic zinc speciation: complexation of zinc by natural organic ligands in the central North Pacific. *Limnol. Oceanogr*. **34**: 269–285.

Bruland KW, Donat JR, Hutchins DA (1991) Interactive influences of bioactive trace metals on biological production in oceanic waters. *Limnol. Oceanogr*. **36**: 1555–1577.

Buitenhuis ET, Timmermans KR, de Baar HJW (2003) Zinc-bicarbonate colimitation of *Emiliania huxleyi*. *Limnol. Oceanogr*. **48**: 1575–1582.

Danilov RA, Ekelund NG (2001) Effects of Cu^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+} and pentachlorophenol on photosynthesis and motility in *Chlamydomonas reinhardtii* in short-term exposure experiments. *BMC Ecol*. **1**: 1.

Ellwood MJ, Van den Berg CMG (2000) Zinc speciation in the Northeastern Atlantic Ocean. *Mar. Chem*. **68**: 295–306.

Fu F, Bell PRF (2003) Growth, N_2 fixation and photosynthesis in a cyanobacterium, *Trichodesmium* sp., under Fe stress. *Biotechnol. Lett*. **25**: 645–649.

Harrison PJ, Waters RE, Taylor FJR (1980) A broad spectrum artificial seawater medium for coastal and open ocean phytoplankton. *J. Phycol.* **16**: 28–35.

Henley WJ (1993) Measurement and interpretation of photosynthetic light-response curves in alga in the context of photoinhibition and diel changes. *J. Phycol*. **29**: 729–729.

Jeffrey SW, Humphrey GF (1975) New spectrophotometric equations for determining chlorophylls *a*, *b*, c_1 and c_2 in higher plants, algae and natural phytoplankton. *Biochem. Physiol. Pflanz*. **167**: 191–194.

Lane TW, Morel FMM (2000) Regulation of carbonic anhydrase expression by zinc, cobalt, and carbon dioxide in the marine diatom *Thalassiosira weissflogii*. *Plant Physiol*. **123**: 345–352.

Morel FMM, Reinfelder JR, Roberts SB, Roberts SB, Chamberlain CP, Lee JG, Yee D (1994) Zinc and carbon co-limitation of marine phytoplankton. *Nature* **369**: 740–742.

Reinfelder JR, Kraepiel AML, Morel FMM (2000) Unicellular C_4 photosynthesis in a marine diatom. *Nature* **407**: 996–999.

Sunda WG, Huntsman SA (1992) Feedback interaction between zinc and phytoplankton in seawater. *Limnol. Oceanogr*. **37**: 25–40.

Vallee BL, Auld DS (1990) Zinc coordination, function and structure of zinc enzymes and other proteins. *Biochemistry* **29**: 5647–5659.

Westall JC, Zachary JL, Morel FMM (1976) *MINEQL*. R.M. Parsons Laboratory, Massachusetts Institute of Technology, Cambridge, MA.

Wilbur KM, Anderson NG (1948) Electrometric and colorimetric determination of carbonic anhydrase. *J. Biol. Chem*. **176**: 147–154.