Limnol. Oceanogr., 51(1), 2006, 299–309

Zinc availability and alkaline phosphatase activity in *Emiliania huxleyi:* Implications for Zn–P co-limitation in the ocean

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Abstract

Zinc (Zn) serves as a cofactor in several extracellular phosphatases, which allow microorganisms to acquire phosphorus from organic P compounds. In oligotrophic ocean water, where both phosphate and Zn concentrations are low, orthophosphate regeneration through enzymatic hydrolysis of organic compounds may be restricted by Zn availability. We examined the possibility of co-limitation by P and Zn in batch cultures of the coccolithophore *Emiliania huxleyi* grown at very low biomass. Both growth rates and extracellular phosphatase activity were inhibited by low Zn. Cultures grown at nanomolar P and subpicomolar, unchelated Zn concentrations had higher phosphatase activity and slower growth rates when grown on organic P than when grown on inorganic P. We calculated that the additional Zn demand for phosphatase activity in the culture with organic P amounted to 16% of the cellular Zn quota. This percentage would be lower at higher organic P concentrations. Extrapolating from our data, we surmise that Zn–P co-limitation may prevail in highly oligotrophic systems such as the Sargasso Sea, but it is not likely to be widespread in the ocean. Nonetheless, the observation of a significant enhancement of extracellular phosphatase activity in Zn-amended water samples from the Bering Sea demonstrates the potential for Zn–P co-limitation during phytoplankton blooms.

In oligotrophic ocean regions, phytoplankton growth may be limited by low ambient inorganic phosphate concentrations (Karl and Bjorkman 2002; Lomas et al. 2004; Wu et al. 2000). To access the phosphorus contained in organic compounds (which is generally not directly available to them), phytoplankton and other microorganisms synthesize extracellular hydrolytic enzymes that cleave phosphate (PO_4^{3-}) from its organic moiety (Kuenzler and Perras 1965; Cambella et al. 1984). Phosphate release from a wide variety of phosphomonoesters is achieved by the activity of broad spectrum phosphatases that are commonly classified as either alkaline or acid according to their pH optima (Cambella et al. 1984). Another ectoenzyme, 5′-nucleotidase, which cleaves phosphate from nucleotides, appears to be expressed in bacteria (Ammerman and Azam 1985) as well as in eukaryotic phytoplankton such as the coccolithophore *Emiliania huxleyi* (Dyhrman and Palenik 2003) and the diatom *Phaeodactylum tricornutum* (Flynn et al. 1986). Alkaline phosphatase and 5′-nucleotidase (which we designate together by the general term ''phosphatases'') are Zn metalloenzymes that also generally require (or are stimulated by) Mg^{2+} or Ca^{2+} (McComb et al. 1979; Cambella et al. 1984). At low Zn concentrations or availability, the synthesis of these enzymes might be limited, leading to Zn–P co-limitation of phytoplankton growth.

The concentrations of phosphate in open ocean surface water (measured as soluble reactive phosphorus [SRP]) range from 2 μmol L^{-1} in the Southern and Indian oceans, to $\leq$50 nmol L^{-1} in the oligotrophic regions of the Atlantic and the Pacific (Benitez-Nelson 2000; Karl and Bjorkman 2002). In many open ocean environments, SRP concentrations are below the detection limit of conventional methods, which ranges from 25 to 50 nmol L^{-1} (Karl and Bjorkman 2001). Application of preconcentration methods such as MAGIC (Karl and Tien 1992) have revealed extremely low SRP concentrations of 0.5 $\pm$ 0.3 nmol L^{-1} in Sargasso Sea surface water (Wu et al. 2000). The concentrations, chemical nature, and distribution of organic P compounds are much less studied. However, organic P concentrations always ex-

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Acknowledgments

We thank M. Lohan for the Bering Sea total and unchelated zinc data and D. Tang for help with the ICP-MS analysis. We thank the crew members and scientists aboard the RV *Kilo Moana* and the chief scientist K. Bruland for a successful Bering Sea cruise. This work was supported by the Center for Environmental Bioinorganic Chemistry funded by NSF; the NSF grant 0351499 to F. Morel; and the NSF grants 0094535, 0328483, and 0327724 to D. Hutchins. Y. S. thanks the ISF Bikura Fellowship for financial support.

ceed those of SRP (Benitez-Nelson 2000; Karl and Bjorkman 2002) with typical values (measured as soluble nonreactive phosphorus [SNP]) in oligotrophic regions ranging between 30 and 300 nmol L^{-1} (e.g., Wu et al. 2000; Bjorkman and Karl 2003; Van Den Broeck et al. 2004). Several attempts to evaluate the bioavailability of marine organic P compounds to microorganisms have been made using physiochemical methods (Suzumura and Ingall 2004); hydrolytic enzyme assays (Suzumura et al. 1998); ^{31}P nuclear magnetic resonance (NMR, Clark et al. 1998); measurements of turnover rates based on naturally occurring or exogenous radioisotope (Benitez-Nelson and Karl 2002); bioassays (Karl and Bjorkman 2001); and γ-P labeling techniques for estimating adenosine triphosphate (ATP) pool turnover (Bjorkman et al. 2000). Despite the differences among methods as well as the heterogeneity and dynamic nature of the dissolved organic P pool, it appears that its bioavailability is generally low with values in the 0–10% range (of SNP) according to some studies, and in the 15–35% range according to others (Karl and Yanagi 1997; Benitez-Nelson 2000; Bjorkman and Karl 2003). Higher estimates can also be found, however: 60–80% of the total dissolved organic P in the northeastern Atlantic and northwestern Mediterranean was characterized as nonrefractory by Aminot and Kerouel (2004).

Dissolved Zn concentrations in the surface waters of the open Pacific or Atlantic oceans are in the subnanomolar range (Bruland 1980; Lohan et al. 2002). Most of this Zn is bound to strong and uncharacterized organic ligands such that the concentration of Zn′, the unchelated Zn, is in the picomolar range (Bruland 1989; Lohan et al. 2002; Ellwood 2004). Picomolar Zn′ concentrations have been found to limit the growth of many marine phytoplankton in culture (Anderson et al. 1978; Sunda and Huntsman 1992; Morel et al. 1994). Zn addition to bottle incubations in the high nutrient low chlorophyll (HNLC) Subarctic Pacific was observed to slightly increase the chlorophyll *a* concentrations (Coale 1991) and to cause subtle changes in phytoplankton composition (Crawford et al. 2003; Leblanc et al. 2005; Lohan et al. 2005)

Emiliania huxleyi (Lohman) Hay et Mohler, the numerically most important coccolitophore in the modern ocean, has a remarkable ability to grow and outcompete other phytoplankton in oligotrophic water. Field observations and diagnostic modeling studies have found that stratified water with depleted N and P and high irradiance favors *E. huxleyi* blooms (e.g., Kristiansen et al. 1994; Townsend et al. 1994; Tyrrell and Taylor 1996). A competitive advantage of *E. huxleyi* for P, which has been observed in competition experiments in continuous cultures (Riegman et al. 1992) and in mesocosms (Egge and Heimdal 1994), appears to stem from its exceptional phosphate assimilation capability as well as its efficient utilization of organic P (Riegman et al. 2000; Dyhrman and Palenik 2003). Particularly high phosphatase activity, probably attributable to two or three different enzymes, has been recorded in P-stressed *E. huxleyi* batch and continuous cultures (Riegman et al. 2000; Dyhrman and Palenik 2003).

In this study, we examine the possibility of co-limitation of phytoplankton growth in the ocean by Zn and P. Using P-limited dilute batch cultures, we attempted to quantify the concentrations of Zn′, phosphate, and organic P that co-limit *E. huxleyi* growth and evaluate their effect on phosphatase activity. The results provide a quantitative basis for extrapolating to oceanic conditions, with emphasis on low-phosphate oligotrophic waters. We also performed a field experiment in the Bering Sea to test the direct effect of Zn on phosphatase activity.

Materials and Methods

Culture medium—The experimental medium was prepared from microwave-sterilized 0.2 μm filtered Gulf Stream water enriched with chelexed and filter-sterilized nitrate (150 μmol L^{-1}); thiamine (60 nmol L^{-1}); and trace metals (87 nmol L^{-1} Fe, 20 nmol L^{-1} Cu, 50 nmol L^{-1} Mn, 10 nmol L^{-1} Se) buffered with 100 μmol L^{-1} ethylenediaminetetraacetic acid (EDTA). Zn concentrations were adjusted according to the experiment with a maximal Zn addition of 97 nmol L^{-1} (=15 pmol L^{-1} Zn′; Sunda and Huntsman 1995). Background Zn was estimated based on the growth rates with no Zn, Cd, or Co additions at 2–3 nmol L^{-1} (=0.3–0.5 pmol L^{-1} Zn′). No Co or Cd was added to the medium because these trace metals are known to replace Zn in some metabolic pathways in *E. huxleyi*. All organic P compounds were prepared with double-distilled water (Milli-Q, Millipore; 18.2 mΩ) as 0.01 mol L^{-1} stock solutions, which were chelexed, filter sterilized, and kept frozen to minimize hydrolysis. Total P concentration of the stock solutions was measured with a ThermoFinnigan *Element 2* inductively coupled plasma mass spectrometer (ICP-MS). The concentration of PO_4^{3-} in the organic P stock solutions was measured using the molybdenum blue method (Karl and Bjorkman 2001) and was found to be negligible (0.3–0.9% of the organic P). In most experiments the organic P compounds used were α-glycerophosphate (α-gly-P) and guanosine 5′-diphosphate (GDP). Other organic P compounds tested were adenosine 5′-monophosphate (AMP), nicotinamide adenine dinucleotide (NADH), tripolyphosphate (PPP), ribulose bis-P (RUBP), and trimethyl P. Medium of conventional batch cultures contained 1 or 10 μmol L^{-} inorganic P (PO_4^{3-}) or organic P. Medium of dilute batch cultures that contained only 8 and 300 nmol P L^{-1} and sustained low biomass was supplemented with 30 μmol NO_3^{-} (to reduce background levels of P and Zn). Background P level was estimated at 4–6 nmol L^{-1} based on final cell yield obtained in experiments with no added P and minimal measured P quotas (see Results).

Culturing methods—Sterile trace metal clean techniques were applied for culturing and experimental manipulations. Axenic cultures were purchased from the Provasoli Guillard Center of Culture of Marine Phytoplankton (CCMP). It is possible, however, that the experiments had low levels of bacterial contamination. Most experiments were conducted with *E. huxleyi* strain CCMP 374. To expand the applicability of the results, the ability of other *E. huxleyi* strains (CCMP 373, CCMP 1419, CCMP 1516, A1387, 5/90/25j, B11, and DWN53/74/9) to grow on organic P was tested. Cultures were grown in acid-cleaned polycarbonate bottles at 20°C under continuous light (80–100 μmol quanta m^{-2} s^{-1}). Cell numbers and cell volumes were determined using

a Multisizer II Coulter Counter, and specific growth rates were then determined from the linear regressions of the natural log of cell number versus time. The dilute batch cultures were initiated with 40 cells mL^{-1} and were followed by frequent counting (two to three times per day) throughout the exponential phase into the stationary phase. Slowdown of growth was observed above ~2,000 cells mL^{-1} (depending on the added P concentration), and such counts were eliminated from the growth rate calculations. Each sample was counted twice using high enough volume (2 mL) to obtain at most 10% error on the counts (2σ). Significant differences between growth rates were determined using the software SPSS for Windows (v. 13.0) running a one-way analysis of variance (ANOVA) and Tukey's honestly significant difference tests. Before each experiment, the cultures were acclimated to the low P and Zn levels for several generations to ensure that no carryover of P or Zn was introduced with the cells and that the cells did not store these nutrients.

Phosphatase assay—Phosphatase activity was measured in the laboratory using two substrates: 4-nitrophenyl phosphate [pNPP] (Sigma) and 6,8-difluoro-4-methylumbelliferyl phosphate [DiFMUP] (Sigma). Enzymatic cleavage of pNPP was monitored continuously for 2–10 min with a Cary 100 UV/Vis spectrophotometer at a wavelength of 405 nm in 1 cm cuvette. Assays were conducted in the presence of 50 mmol L^{-1} Tris (pH = 8.2) at 20°C using saturating 50 μmol L^{-1} pNPP. An absorbance coefficient of 18,740 ± 1030 (mol $L^{-1})^{-1}$ cm^{-1} was measured using 4-nitrophenyl standard [pNP] (Sigma). Enzymatic cleavage of DiFMUP was measured continuously for 2–10 min in 1 cm cuvette with an LS 55 Luminescence Spectrometer (Perkin Elmer; excitation 385 nm, emission 440 nm). Assays were conducted in the presence of 50 mmol L^{-1} Tris (pH = 8.2) at 20°C using 8 μmol L^{-1} DiFMUP. A conversion factor of 1.4 ± 0.2 (nmol $L^{-1})^{-1}$ cm^{-1} was measured using 6,8-difluoro-4-methylumbelliferyl standard [DiFMU] (Sigma). The enzyme activity was computed from the linear regression of absorbance or fluorescence, respectively, versus time. Detection limits were 20 nmol L^{-1} pNP min^{-1} and 0.5 nmol L^{-1} DiFMU min^{-1}. Blanks were measured in non-inoculated growth medium and were subtracted from all samples. The assays were not conducted under trace metal clean conditions (which were found not be important within the 10–20 min of the assay). Enzymatic hydrolysis of α-gly-P and GDP (0.5 mmol L^{-1}) was measured in P-limited culture filtrate (<0.2 μm) with the molybdenum blue method (Karl and Bjorkman 2001). Phosphatase activity in the Bering Sea was measured continuously for 30–60 min with a Cary 100 UV/Vis spectrophotometer in 10 cm cell. Assays were conducted using 1 mmol L^{-1} pNPP in the presence of 50 mmol L^{-1} Tris (pH = 8.2) at 20°C. Blanks were measured in double-ionized water and were subtracted from all samples.

Other methods—Elemental quotas (P, Ca) were measured with ICP-MS (*Element 2,* ThermoFinnigan) as described by Tang and Morel (in press). Calcification (Ing ^{14}C : Org ^{14}C) in laboratory cultures and field samples was measured according to the microdiffusion technique (Paasche and Brubak 1994) with minor modifications. The nutrient measurements and experimental set-up for the Zn amendment incubations in the Bering Sea are described elsewhere (Leblanc et al. 2005).

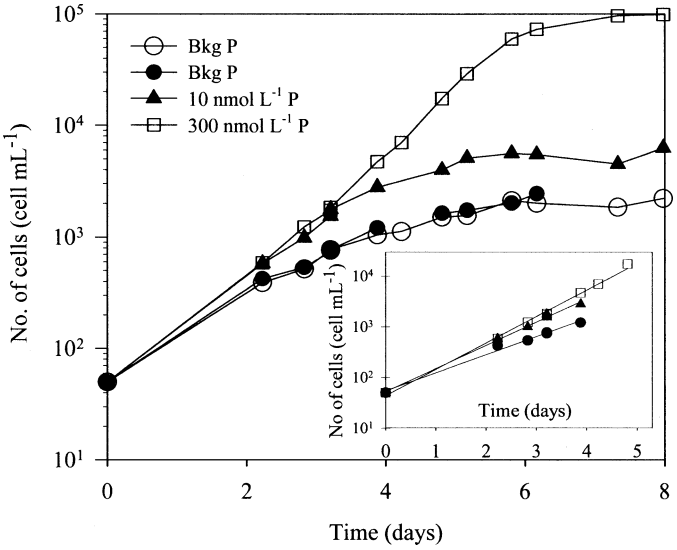

Fig. 1. Growth curves of dilute batch *E. huxleyi* cultures (CCMP 374) in the presence of background P, limiting 10 nmol L^{-1} P, and replete 300 nmol L^{-1} P. Data used to calculate the rate of exponential growth is presented in inset.

Results

Growth limitation by low P—All *E. huxleyi* strains tested (CCMP 374, CCMP 1516, CCMP 1419, Al387, B11, and 5/90/25j) grew at maximal rates when supplemented with 0.3 − 10 μmol L^{-1} inorganic or organic P, and the initial P concentration determined only the final cell yield. In the absence of a convenient buffering system (such as provided by EDTA for trace metals), growth limitation by major nutrients is usually obtained by using continuous cultures. Besides being laborious, this method is problematic for metal-clean procedures. We found, however, that initial P concentrations below 15 nmol L^{-1} yielded stable exponential growth rates in batch cultures when the cell concentration was kept below ~2,000 cells mL^{-1} (Fig. 1). Growth rates of ~50% of μ_{max} were obtained for cultures of *E. huxleyi* CCMP 374 with no P addition (estimated background P = 6.0–6.5 nmol L^{-1}; see below), whereas addition of 10 nmol L^{-1} P yielded growth of ~75% of μ_{max} (Fig. 1). In other experiments, additions of 8 nmol P L^{-1} yielded growth of 70–85% of μ_{max} (data not shown, n = 8). From these experiments we calculated a half saturation constant for growth (K_μ) of 5 ± 2 nmol L^{-1} (depending on background P levels; see below). Other strains of *E. huxleyi* (B11 and 5/90/25j) grew at 85% of μ_{max} in dilute cultures with no P addition (data not shown). The less severe growth limitation by P for these strains may indicate that they have lower K_μ than that of CCMP 374, provided that the background P concentrations in all experiments are comparable.

Effect of P concentrations on P quotas and cell size—We examined the effect of P limitation on the P quota of *E.*

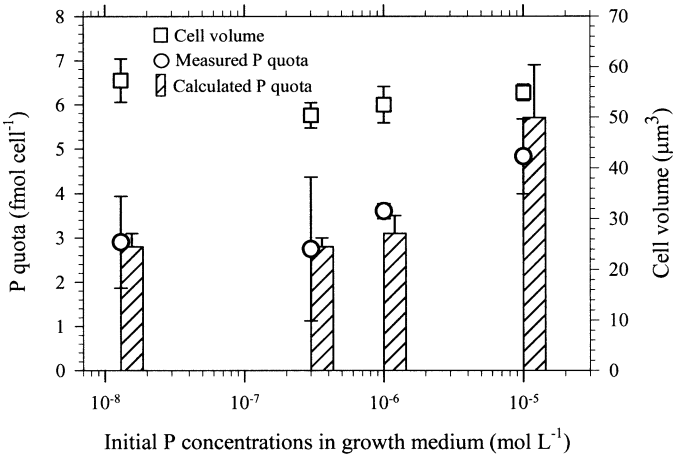

Fig. 2. ICP-MS measured P quotas, calculated cell quotas, and cell volumes of *E. huxleyi* (CCMP 374) grown with ~12 nmol L^{-1}, 300 nmol L^{-1}, 1 μmol L^{-1}, and 10 μmol L^{-1} P. (See text for details of quota calculation.)

huxleyi cultures amended with 12 nmol L^{-1}, 300 nmol L^{-1}, 1 μmol L^{-1}, and 10 μmol L^{-1} inorganic and organic P. P quotas were obtained in two ways: (1) measuring the P content of cells in the late exponential phase (Fig. 2, circles), and (2) dividing the initial P concentration by the final cell yield (Fig. 2, bars). The later calculation is applicable as long as all of the P in the medium is consumed by the cells, as was the case in our experiments (possibly except for the 10 μmol P L^{-1} treatment). Both methods gave similar results, and the P quotas essentially remained constants (2.7–3.6 fmol cell^{-1}) over a range of initial P concentrations from 12 nmol L^{-1} (dilute cultures) to 1 μmol L^{-1} (Fig. 2). A higher P quota (4.8–5.7 fmol cell^{-1}), which probably resulted from P storage, was observed for cultures grown with 10 μmol L^{-1} P (Fig. 2). The low K_μ and the corresponding low P additions make the background P contamination an important variable in these experiments. We were unable to measure directly the background P, and hence it was calculated for each experiment based on the averaged ICP-MS–measured P quota (2.8 ± 0.13 fmol cell^{-1}; see Fig. 2 for the 12 and 300 nmol P L^{-1} treatments) and the measured final cell yield. From this calculation we obtained a background P of 6.0–6.5 nmol L^{-1} for the experiment presented in Fig. 1 and 4.0–4.5 nmol L^{-1} for the Zn–P co-limitation experiment (Table 1; Fig. 3). Cell volumes remained constant for exponentially growing cells in the presence of 0.3–10 μmol L^{-1} P and were independent of the P compound given to the culture (53 ± 2 μm^3 cell^{-1}; Fig. 2, squares). Small increases in cell volume, which corresponded to slower growth rates, were observed in P-limited cultures. A marked increase in cell volume (up to 250%) was observed upon slowdown of growth and the initiation of the stationary phase (data not shown).

Co-limitation of Zn and P on E. huxleyi *phosphatase activity and growth on organic P*—Based on earlier experiments (Fig. 1), we used a series of dilute batch cultures to examine Zn–P co-limitation in *E. huxleyi* CCMP 374. Cultures were inoculated at 40 cells mL^{-1} and supplemented with two levels of Zn (no Zn added and 15 pmol L^{-1} Zn′) and three P treatments: limiting 8 nmol L^{-1} organic P (GDP), limiting 8 nmol L^{-1} inorganic P (PO_4^{3-}), and replete 300 nmol L^{-1} inorganic P (Table 1; Fig. 3). As mentioned before, the experimental medium contained background Zn′ of 0.4 pmol L^{-1} and background P of 4.0–4.5 nmol L^{-1}. Low P treatments were conducted in duplicates and high P treatments in a single replicate. The specific growth rates calculated for the exponential phase of growth (which include at least five data points) were found to remain constant up to the third or fourth day of the cultures (R^2 = 0.992–0.999; Fig. 3). Growth limitation by Zn was obtained in the exper-

Table 1. Experimental conditions, growth rates, cell volumes, and exponential phase phosphatase activities of Zn–P co-limited *E. huxleyi* (CCMP374) dilute batch cultures.

Treatment	[Zn′]* (pmol L^{-1})	[PO_4^{3-}]† (nmol L^{-1})	[GDP]† (nmol L^{-1})	Growth rate (d^{-1})	Replicate (No.)	Cell volume (μm^3)	Phosphatase activity‡ (fmol DiFMUP cell^{-1} min^{-1}) 2.8 d	3.3 d	3.8 d	4.8 d
Low Zn, low Ing P	0(+0.4)	8(+4)		0.99(0.002)	2	60(2.2)	2.8(0.06)	5.2(0.5)	13.5(1.9)	42.3(1.9)
Low Zn, low Org P	0(+0.4)		8(+4)	0.85(0.05)	2	52(1.1)	11.6(4.0)	13.9(2.1)	22.1(0.8)	52.2(0.8)
Low Zn, high Ing P	0(+0.4)	300		1.08	1	50(2.8)	BDL	BDL	ND	ND
High Zn, low Ing P	15	8(+4)		1.15(0.14)	2	59(4.7)	11.5(1.4)	27.3(0.8)	40.0(16)	75.5(16)
High Zn, low Org P	15		8(+4)	1.25(0.04)	2	60(1.8)	14.1(1.3)	29.1(6.7)	40.5(1.5)	65.8(1.5)
High Zn, high Ing P	15	300		1.44	1	51(2.0)	BDL	2.0(0.015)	ND	ND

* Added concentrations of unchelated Zn (plus estimated background unchelated Zn).
† Added concentrations of PO_4^{3-} and GDP (plus estimated background P).
‡ Phosphatase activity of exponentially growing cells, measured at 2.8, 3.3, 3.8, and 4.8 d of the culture initiation.
1 SD of measurements/treatments are presented in parentheses. Ing, inorganic; Org, organic; BDL, below detection limit (=2 fmol DiFMUP cell^{-1} min^{-1}); ND, not determined.

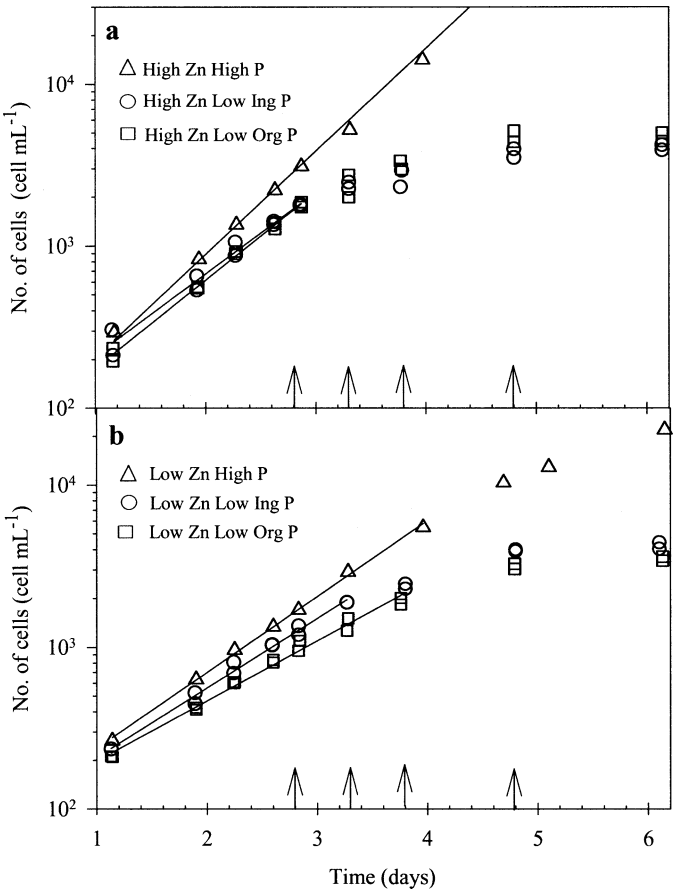

Fig. 3. Growth curves of dilute batch *E. huxleyi* cultures in the Zn–P co-limitation experiment at (a) high Zn (Zn′ = 15 pmol L^{-1}), and (b) low Zn (Zn′ ~ 0.4 pmol L^{-1}). Each Zn level included three treatments: limiting inorganic P (8 nmol L^{-1} PO_4^{3-}, duplicate bottles); limiting organic P (8 nmol L^{-1} GDP, duplicate bottles); and replete inorganic P (300 nmol L^{-1} PO_4^{3-}). Growth rates are calculated from the solid regression lines. Arrows indicate phosphatase activity sampling times.

iment as evidenced by the slower growth rates of the high P–low Zn cultures compared with the high P–high Zn cultures (μ = 1.08 d^{-1} vs. 1.44 d^{-1}, respectively; Fig. 3; Table 1; $t(18) = 5.38$ $p < 0.00002$). Growth limitation by P was observed in both the high Zn (μ = 1.44 d^{-1} for high P vs. average μ = 1.22 d^{-1} for low organic and inorganic P; $t(28) = 5.03$ $p < 0.0001$) and the low Zn cultures (μ = 1.08 d^{-1} for high P vs. average μ = 0.92 d^{-1} for low inorganic and organic P; $t(17) = 2.68$ $p < 0.016$).

Phosphatase activity was measured at four different time points (2.8, 3.3, 3.8, and 4.8 d) in all cultures. In the low P treatments, the activity increased with time, presumably as a result of P depletion in the medium. In contrast, in the high P treatments the activity remained close to or below the detection limit (Table 1; Fig. 4). At all time points, higher phosphatase activity was observed in the high Zn cultures compared with the low Zn cultures (Table 1; Fig. 4), indicating that Zn availability may be limiting the synthesis or activity of phosphatases under these experimental conditions. In the high Zn cultures, growth rates and phosphatase

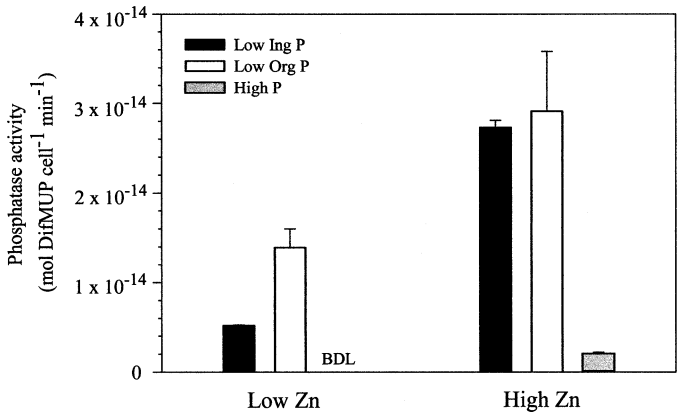

Fig. 4. Cell normalized phosphatase activities of *E. huxleyi* in the Zn–P co-limitation experiment. Phosphatase activity was measured in whole water samples at Day 3.3 of the experiment using DiFMUP (*see Table 1* for more measurements). BDL = below detection limit.

activity of the low organic P and the low inorganic P treatments were not statistically different from each other (Table 1; Figs. 3a and 4). However, in the low Zn cultures, significantly higher phosphatase activity was found at all time points in the low organic P treatments compared with the low inorganic P treatments (Table 1; Fig. 4). Differences in growth rates were also observed for the low P and Zn cultures, as cells grew 16% faster in the presence of inorganic P than in the presence of organic P (Table 1; Fig. 3b; $t(22) = 3.43$, $p < 0.002$). Increases of up to 18% in cell volume were observed between treatments, corresponding in most cases to the decrease in growth rates (Table 1). Since cell volume remained constant in each treatment throughout the exponential phase, cell volume–based growth rates were identical to cell number–based growth rates.

Phosphatase kinetics—We investigated the kinetics of phosphatase activity in whole cultures and 0.2 μm filtered enzyme solutions using two substrates, DiFMUP and pNPP (Fig. 5). Comparable rates of enzymatic P cleavage from the organic P substrate as well as half saturation constants (K_s) were found for whole cultures or filtered enzymes with both substrates (K_s = 2 ± 0.3 μmol L^{-1} for DiFMUP and pNPP; Fig. 5a). The rate of enzymatic P cleavage from GDP and α-gly-P, the actual P substrates on which the cultures grew, was also determined by measuring phosphate formation with time in the presence of filtered enzymes (Fig. 5b). All substrates were cleaved by the enzyme at comparable rates, a behavior that is typical of alkaline phosphatase (McComb et al. 1979). These results imply that our activity measurements with DiFMUP and pNPP, once corrected for the organic P concentration in the growth medium, provide a good measure of the actual enzymatic hydrolysis rate in the culture. An additional enzyme with higher maximal activity, but much lower substrate specificity (K_s = 610 ± 114 μmol L^{-1}), was detected with pNPP (Fig. 5c). This experiment was not performed with DiFMUP because of signal saturation.

We also found that the phosphatase produced by *E. huxleyi* CCMP 374 is a Ca-dependent enzyme. This observation

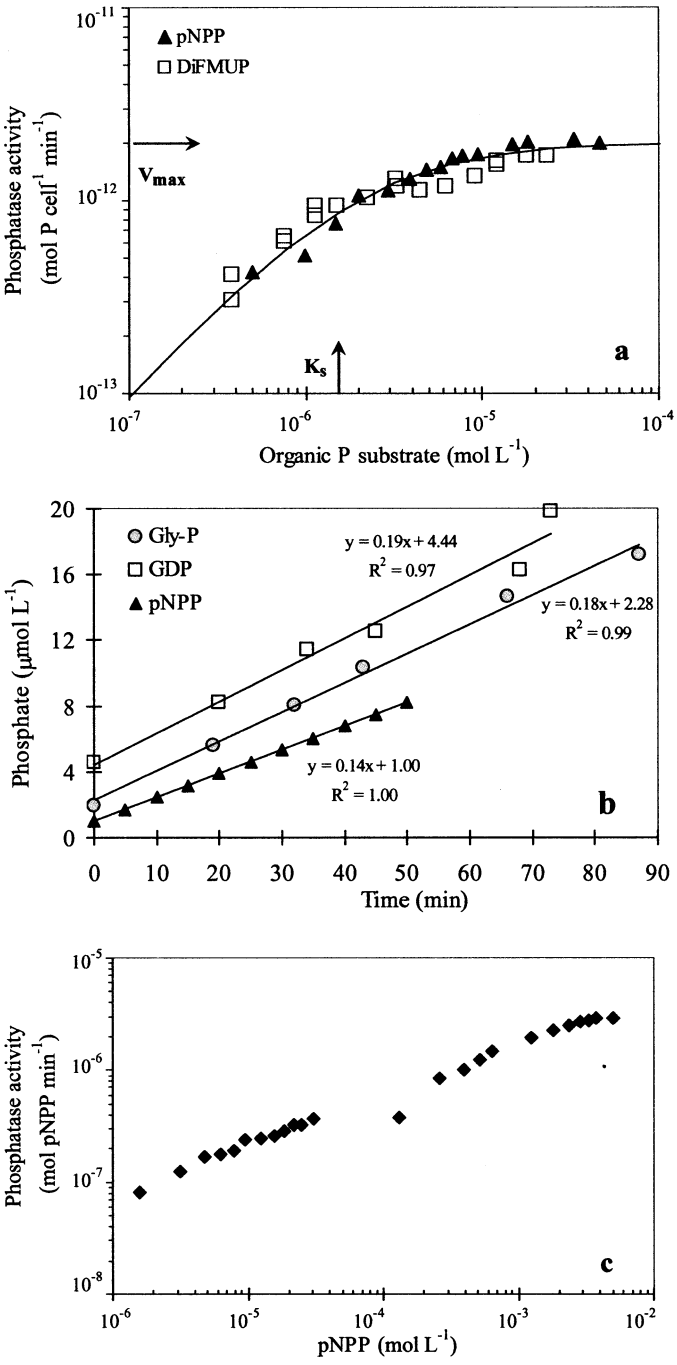

Fig. 5. Phosphatase kinetics and hydrolysis rates of different organic P substrates. (a) Cell normalized phosphatase activity as a function of substrate concentrations. The line through the points corresponds to the Michaelis Menten formula with $V_{max} = 2 \times 10^{-12}$ mol P cell^{-1} min^{-1} and $K_s = 2$ μmol L^{-1}. (b) Rate of enzymatic cleavage of PO_4^{3-} from GDP, α-gly-P, and pNPP by phosphatase in culture filtrate (<0.2 μm). (c) Phosphatase activity of culture filtrate (<0.2 μm) over a wide range of pNPP concentrations.

was made with exponentially growing cultures (1 μmol L^{-1} P) that were transferred into P-free synthetic seawater with increasing Ca concentrations (0, 0.5, 1, and 2 mmol L^{-1}; Table 2). Phosphatase activity was assayed several times within a 2-d period and was found to increase in all cultures. Higher activity was found in the treatments that had higher Ca concentrations (Table 2). To examine whether Ca has a direct or indirect effect on the enzymatic activity, Ca was added as $CaCl_2$ to subsamples of these cultures (Table 2). Immediate enhancement of the enzyme activity upon Ca addition was observed using DiFMUP (Table 2). This pattern was also observed when pNPP was used as a substrate (data not shown). The enzymatic activity peaked around 10 mmol L^{-1} Ca (similar to seawater Ca concentrations of 9.1 mmol L^{-1}), and further Ca addition had only a minor effect on activity (Table 2).

Effect of Zn addition on phosphatase activity in Bering Sea water incubations—We have tested directly the possibility of Zn limitation on phosphatase activity in the open ocean during a research cruise in the Bering Sea by assaying phosphatase activity in whole water samples incubated for 6 d with various trace metal amendments. The incubations were conducted with Bering Sea shelf water (collected at 57.67°N/168.70°W) that contained 4.1 ± 0.2 μg L^{-1} chlorophyll *a* (Chl *a*) and 0.08±0.03, 0.06±0.05, and 0.66±0.47 μmol L^{-1} phosphate, nitrate, and silicic acid, respectively. Total and unchelated Zn concentrations determined at this station were 0.25 nmol L^{-1} and 8 pmol L^{-1}, respectively (M. Lohan pers. comm.). Although large blooms of *E. huxleyi* were observed in the area in previous summers, inorganic ^{14}C assimilation measured in these samples indicated, as confirmed by microscopic observations, that calcifying microorganisms were present at low abundance (Ing ^{14}C : Org ^{14}C = 3 ± 1%). The phosphatase activity determined for water samples from this station varied from 10 to 41 nmol pNPP (μg Chl a^{-1}) h^{-1} during the day (unfortunately no measurements were taken from the incubation bottles at the beginning of the experiment).

The water samples were amended with 0.5 nmol L^{-1} of Zn, 0.25 nmol L^{-1} of Fe, or both. Nutrient drawdown and phytoplankton growth (Chl *a* concentrations) were monitored at Days 3 and 6 of the experiment. At Day 3, Chl *a* concentrations had increased in all treatments (Fig. 6a) accompanied by a decrease in phosphate concentrations to 0.015–0.022 μmol L^{-1}. At Day 6, Chl *a* concentrations had declined in all treatments (Fig. 6a), probably as a result of nutrient depletion [final concentrations: 0.03–0.04 μmol L^{-1} PO_4^{3-}, 0.05–0.06 μmol L^{-1} NO_3^-, and 0.011–0.024 μmol L^{-1} $Si(OH)_4$] and grazing. Phosphatase activity measured in duplicates on Day 6 of the experiment (in only two of the three incubation bottles due to time constrains) was low in the control and the +Fe treatment, whereas +Zn and +Fe+Zn treatments had high phosphatase activity (Fig. 6b). The activity measured initially in the seawater and in the incubations is in agreement with other field studies (0–250 nmol MF-P [μg Chl a^{-1}], h^{-1} [Guildford and Hecky 2000]; 10–300 nmol MF-P [μg Chl a^{-1}] h^{-1} [Vidal et al. 2003]) and with our laboratory culture measurements (~30 nmol DiFMUP [μg Chl a^{-1}] h^{-1}, using 2×10^{-5} μg Chl *a* cell^{-1}).

Discussion

We obtained P limitation in batch cultures of *E. huxleyi* by supplying an initial P concentration of only a few na-

Table 2. Phosphatase activity of *E. huxleyi* grown with different calcium concentrations and short-term effect of calcium addition on phosphatase activity.

Medium Ca concentration (mmol L^{-1})	Culture phosphatase activity* (fmol DiFMUP $cell^{-1}$ min^{-1})	Phosphatase activity following Ca addition† (fmol DiFMUP $cell^{-1}$ min^{-1})		
		1.5 mmol L^{-1} Ca	10 mmol L^{-1} Ca	20 mmol L^{-1} Ca
None added	31(5.7)	68(0.78)		
0.1	50(2.3)			
0.5	65(9.0)			
1	52(2.4)	121(5.6)	144	157
2	79(6.6)		159	161

* Late exponential phase P replete cultures were transferred to P-free synthetic seawater with diffeent Ca concentrations. Phosphatase activity and cell number were followed for 2 d.

† Phosphatase activity was measured immediately after addition of $CaCl_2$.

nomolars. In very dilute cultures, the P drawdown was kept small, and steady exponential growth rates were maintained for several doublings in cell numbers. Dilute batch cultures, in which pH was kept nearly constant, have previously been used for studying carbon acquisition and calcification by coccolithophores at different CO_2 levels (Burkhardt et al. 1999; Rost et al. 2002). To our knowledge, this is the first study using P-limited dilute batch cultures, a clean and simple technique that is particularly useful to study co-limitation of major nutrients and trace metals. This method has several advantages: (1) it is straightforward and requires less handling than chemostats; (2) cell-induced changes of the growth medium, such as pH shifts or metabolites excretion, are minimal; and (3) the growth (and uptake) kinetics can be measured directly on exponentially growing cells in the presence of known P concentrations. This method requires the use of clean techniques to minimize background P contamination (although we still suffered from significant P blanks) and a frequent and accurate measurement of cell numbers or other growth-related parameters. Its major drawback is the relatively small phytoplankton biomass, which can be overcome by using large culture volumes.

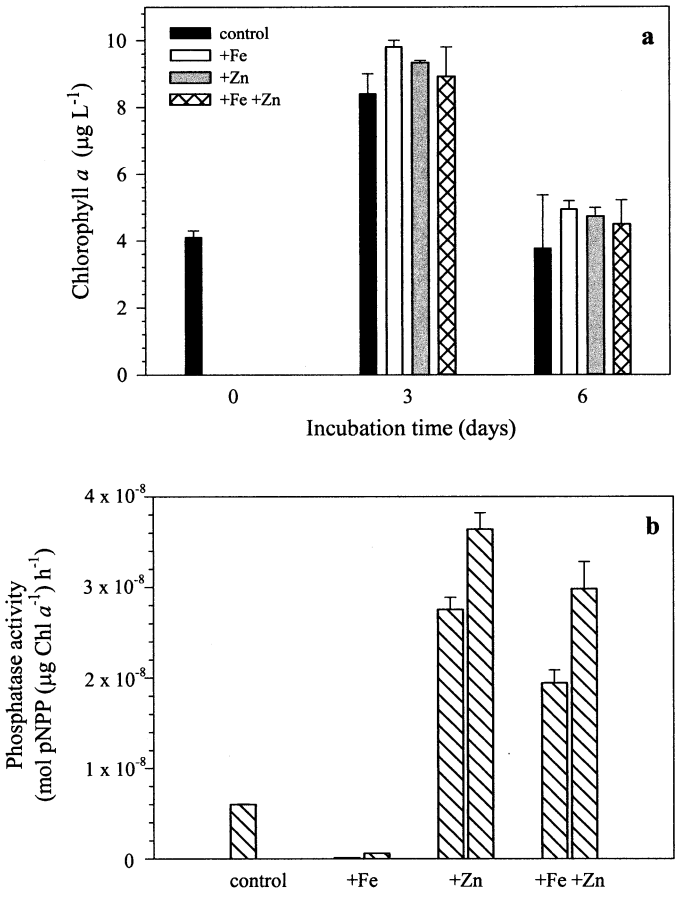

Fig. 6. Chlorophyll *a* (Chl *a*) and phosphatase activity of Bering Sea water incubated in triplicate bottles with 0.5 nmol L^{-1} Zn (+Zn), 0.25 nmol L^{-1} Fe (+Fe), and both (+Fe+Zn) for 6 d. (a) Change in Chl *a* concentrations throughout the experiment. Error bars represent 1 standard deviation of Chl *a* measurements from the different bottles. (b) Chl *a* normalized phosphatase activity of all incubation bottles measured at Day 6 of the experiment. Error bars represent 1 standard deviation of replicate activity measurements from the same bottle.

The measured P quotas (2.7–3.6 fmol $cell^{-1}$ in P-limited cultures and 4.8–5.7 fmol $cell^{-1}$ in P-replete cultures) are in excellent agreement with those of Riegman et al. (2000), who reported values of 2.6–3.7 fmol $cell^{-1}$ for P-limited *E. huxleyi* strain L, and 1.8–6 fmol $cell^{-1}$ for N-limited cells (Riegman et al. 2000). Thus *E. huxleyi* appears to tightly regulate its P quota: a constant minimum cellular P concentration is maintained in all P-limited cultures, and only a modest amount of storage is observed in the presence of excess P.

There are only a few published values of half saturation constants for growth (K_μ) of P-limited phytoplankton. The $K_\mu = 5 \pm 2$ nmol L^{-1} observed here is in the lower range of these values (0.5 nmol L^{-1} for *Monochrysis lutheri* and 18–55 nmol L^{-1} for *Thiocystis* sp., *Ankistrodesmus falcatus, Fragilaria crotonensis, Euglena gracilis,* and *Anabena flos aquea;* Gotham and Rhee 1981). A value of $K_\mu \sim 1$ nmol L^{-1} was calculated indirectly by Riegman et al. (2000) for *E. huxleyi* strain L by making a number of assumptions, including an extremely rapid regulation of P uptake in short-term experiments. Our findings of different growth limitation among the three strains tested without P addition may indicate that the half saturation constant for growth is strain-dependent. The K_μ we observed is between the low phosphate (SRP) concentrations measured in the oligotrophic

Table 3. Evaluation of Zn demand for phosphatase activity, phosphatase-specific activity, and organic P concentrations in which phosphatase activity meet the cells' P requirements.

Parameters	Values	Units	Equations and data sources
R_μ, growth rate ratio (of low Zn cultures grown on Ing P vs. Org P)	1.16		$R_\mu = \frac{\mu_{\text{IngP}}}{\mu_{\text{OrgP}}}$ (Table 1)
R_Q, quota ratio	1.16		$R_Q = \frac{Q_{\text{OrgP}}}{Q_{\text{IngP}}} = \frac{\mu_{\text{IngP}}}{\mu_{\text{OrgP}}}$ (Fig. 3b)
Q_{Zn}, Zn quota	1.0×10^{-18}	mol Zn cell^{-1}	Sunda and Huntsman 1995 Y. Xu pers. comm.
ΔQ_{Zn}, excess Zn requirement of Org P cultures	1.6×10^{-19}	mol Zn cell^{-1}	$\Delta Q_{\text{Zn}} = Q_{\text{Zn}} \times (R_Q - 1)$
$AP_{\max}$, phosphatase activity in the Org P cultures	1.0×10^{-14}	mol DiFMUP cell^{-1} min^{-1}	$AP_{\max}$ = phosphatase activity Org P − Ing P (Fig. 4)
Phosphatase-specific activity	2000	units mg enzyme^{-1}*	$\frac{AP_{\max}}{\Delta Q_{\text{Zn}}} \times 4Zn \times \frac{1}{10^5 \text{ g mol}^{-1}}$
Q_P, P quota	2.8×10^{-15}	mol P cell^{-1}	Fig. 2
ρ_P, steady state P uptake rate	2.4×10^{-15}	mol P cell^{-1} d^{-1}	$\rho_P = Q_P \times \mu$
S, organic P concentration in which phosphatase activity equals ρ_P	0.34	nmol L^{-1}	$S = \frac{\rho_P \times K_m}{AP_{\max} = \rho_P}$

* One unit of alkaline phosphatase is defined as the amount of enzyme that hydrolyzes 1 μmol of pNPP in 1 min (incubation conditions: Tris HCl 0.8 mol L^{-1}, pH 8, $MgCl_2$ 1 mmol L^{-1}, pNPP 2.5 mmol L^{-1}, 20°C).
Ing, inorganic; org, organic.

waters of the Sargasso Sea (0.5 ± 0.3 nmol L^{-1}) and in the Pacific Ocean near Hawaii (9–40 nmol L^{-1}; Wu et al. 2000).

The half saturation constant we found for the high affinity phosphatase enzyme produced by *E. huxleyi* CCMP 374 (K_s of 2 ± 0.3 μmol L^{-1}) is in good agreement with the value of 2.2–2.7 μmol L^{-1} found by Dyhrman and Palenik (2003) for the strains CCMP 373 and 374 and the value of 1.9 μmol L^{-1} found by Riegman et al. (2000) for strain L. These previous studies also reported the presence of additional enzyme(s) with higher K_s, although their half saturation constants of 10–12 μmol L^{-1} are significantly lower than ours. Kinetic studies in different ocean regions have reported half saturation constants of phosphatases in whole and fractionated water samples that are lower than those found for *E. huxleyi* cultures (K_s = 0.017–1.8 μmol L^{-1}; Sebastian et al. 2004). Unlike most phosphatases, the *E. huxleyi* phosphatase was found to be one or more calcium-dependent enzyme(s) (Table 2). It is currently unclear what is the significance of this finding, which is in agreement with several calcium-dependent extracellular phosphatases that were identified in *Haloarcula marismortui*, *Chlamydomonas reinhardtii*, and *Volvox carteri* (Hallmann 1999).

As expected from other studies (Sunda and Huntsman 1992, 1995), we observed Zn limitation of growth in *E. huxleyi* cultures at concentrations of unchelated Zn in the subpicomolar range (Zn′ ~ 0.4 pmol L^{-1}). Under these conditions, lower phosphatase activities were also observed (Fig. 4; Table 1), indicating that the synthesis of a fully active phosphatase by *E. huxleyi* became limited by Zn availability. Thus, the more severe inhibition of growth observed for cells grown on an organic P substrate, under Zn-limited conditions, than for cells grown on inorganic P (Fig. 3) must have resulted from the higher Zn demand associated with higher phosphatase activity. If we assume that the Zn uptake rate in these two cultures (which were grown at the same limiting Zn′ concentration and had the same P quota) are about the same, then the Zn quota in the organic P culture, which is growing 16% slower, should be about 16% higher (Table 3). Although the precision of our quota measurements did not allow us to obtain direct verification of this assumption, the result is unlikely to be far off, and so about 16% of the cellular Zn must be used in alkaline phosphatase in the low Zn, low organic P treatment.

We can verify that this result is reasonable by calculating the corresponding specific phosphatase activity. The Zn quota of *E. huxleyi* grown at Zn′ = 0.4 pmol L^{-1} is about 1×10^{-18} mol Zn cell^{-1} (9×10^{-19} mol Zn cell^{-1} for clone A1387 [Sunda and Huntsman 1995] and 1.4×10^{-18} mol Zn cell^{-1} for clone CCMP 374 [Y. Xu pers. comm.]). Thus the cellular Zn involved in phosphatase in the culture grown on organic P is about 1.6×10^{-19} mol Zn cell^{-1} (Table 3), and it supports a maximum phosphatase activity of $\sim 1 \times 10^{-14}$ mol DiFMUP cell^{-1} min^{-1} (based on the difference in phosphatase activity between the organic and inorganic P low Zn cultures; Fig. 4). Assuming that the enzyme's molecular weight is 100 KD and that it contains four Zn atoms (the enzyme is commonly active as a dimer; McComb et al. 1979), we obtain a specific activity of ~2,000 units (mg protein)$^{-1}$ (Table 3). This value is in good agreement with the value of reported alkaline phosphatase and 5′-nucleotidase–specific activities (5–2,000 units (mg protein)$^{-1}$; McComb et al. 1979; Cambella et al. 1984). Although this calculation is necessarily approximate, it provides a first estimate for the Zn requirement associated with phosphatase activity in *E. huxleyi.*

If the cells are co-limited by Zn and P, the phosphatase activity in the low Zn, low organic P treatment should be just sufficient to satisfy the cell's demand for P. At steady state the P uptake rate is given by the product of the P quota (Fig. 1) and the growth rate (Table 1): 2.8×10^{-15} mol P

Table 4. Growth rates and maximal stationary phase phosphatase activities of *E. huxleyi* (CCMP 374) batch cultures amended with 1 μmol L^{-1} of inorganic P or organic P and different Zn concentrations.

[Zn′]* (pmol L^{-1})	Inorganic P (PO_4^{3-}) growth rates (d^{-1})	Organic P growth rates (d^{-1})	P compounds	Max phosphatase activity† (pmol pNP $cell^{-1}$ min^{-1})
(1)‡				
0	0.40(0.03, n = 3)	0.25 (0.10, n = 4)	α-Gly-P	BDL
0.5	0.35	0.33	α-Gly-P	0.39(0.12, n = 3)
0.7	0.52(0.02, n = 2)	0.46(0.10, n = 2)	α-Gly-P	1.6
1	0.52(0.01, n = 2)	0.51(0.00, n = 2)	α-Gly-P	2.0
1.5	0.67(0.05, n = 2)	0.76(0.05, n = 2)	α-Gly-P	18(4.9, n = 5)
15	1.03(0.20, n = 7)	0.96(0.16, n = 15)	α-Gly-P	31(5.2, n = 5)
(2)§				
0.75	0.70	0.74	AMP	34(4.0, n = 3)#
0.75		No growth	Trimethyl P	
0.75		0.78	NADH	
15	1.16(0.00, n = 2)	1.09	AMP	41(4.0, n = 3)#
15		No growth	Trimethyl P	
15		1.15	RUBP	
15		1.11	PPP	
15		1.17	GDP	
15		1.24	NADH	

1 SD and number of repetitions are presented in parentheses. BDL, below detection limit of 0.1 pmol pNPP $cell^{-1}$ min^{-1}.
* Unchelated concentrations of added Zn (not including background).
† Maximum activity obtained at late stationary phase from various experiments with Org and Ing P.
‡ Data compiled from five experiments (conducted using chelaxed P compounds).
§ Data compiled from two experiments (conducted using nonchelaxed P compounds).
# Maximum activity obtained at late stationary phase form cultures grown in Ing P, AMP, and NADH.

$cell^{-1} \times 0.85\ d^{-1} = 2.4 \times 10^{-15}$ mol P $cell^{-1}$ $d^{-1} = 1.7 \times 10^{-18}$ mol P $cell^{-1}$ min^{-1} (Table 3). Given a maximum phosphatase activity of $\sim 1 \times 10^{-14}$ mol $cell^{-1}$ min^{-1} (the difference between the organic and inorganic P low Zn cultures; Fig. 4) and a half saturation constant of 2 μmol L^{-1}, a matching rate of phosphatase activity would be obtained at an ambient organic P concentration of 0.34 nmol L^{-1} (Table 3). Thus the high phosphatase activity measured after 2.8 d of growth should correspond to an almost complete exhaustion of the organic substrate. We can calculate that at that time the cells have already taken up about 5.6 nmol L^{-1} of the initial P in the culture. Thus the bulk of the remaining P (8–5.6 = 2.4 nmol L^{-1} + background) must be presumably in inorganic form to maintain the growth rate of the culture. A tighter verification of the match between phosphatase activity and P uptake would necessitate a (difficult) measurement of phosphatase activity earlier in the experiment when the cell concentration, and hence the total rate of P uptake, are lower and the organic P concentration in the medium is higher.

Based on the cell's P quota (Fig. 1), the phosphatase half saturation constant (Fig. 5a), the uniform enzymatic P hydrolysis rates from different organic P compounds (Fig. 5b), and our estimate of the phosphatase specific activity (Table 3), we can calculate the enzymatic activity and the associated Zn demand required to supply phosphate for cells growing under various laboratory and field conditions. The steady state P uptake rate of *E. huxleyi* 374 cells growing at maximal rates with 1 μmol L^{-1} organic P is $\sim 5 \times 10^{-12}$ mol P $cell^{-1}$ d^{-1} (3.7×10^{-15} mol P $cell^{-1} \times 1.4\ d^{-1}$), only 2.5-fold higher than for the P-limited cells in the experiment of Fig. 3. In the presence of 1 μmol L^{-1} organic P, the phosphatase activity necessary to hydrolyze the substrate at a given rate is 100 times lower than that of the dilute cultures grown with $\sim$10 nmol L^{-1} P (since we are below the K_s of the enzyme). Thus the phosphatase activity and its associated Zn demand should be approximately 40 times lower in such cultures than in our P-limited cultures. We would therefore expect that the phosphatase activity of the exponential phase batch cultures with 1 μmol L^{-1} organic P (except in late exponential phase when the organic P concentration drops to nanomolar level) would be $\sim 2.5 \times 10^{-16}$ mol P $cell^{-1}$ min^{-1}, which is below the detection limit of most current methods. The 40-fold lower enzyme concentration should correspondingly require only 2.5×10^{-20} mol Zn $cell^{-1}$, which is a negligible fraction of the Zn quota, even in Zn-limited cultures. Thus in the presence of 1 μmol L^{-1} P, *E. huxleyi* should grow on organic and inorganic P substrates at comparable rates, regardless of the Zn concentration in the medium. Moreover, because of the comparable substrate hydrolysis rates, the cultures are expected to grow at similar rates on different organic P substrates. These predictions have been confirmed in experiments conducted by us (Table 4) and by others (Dyhrman and Palenik 2003). In batch cultures grown with 1 μmol L^{-1} organic P, phosphatase activity could not be detected during the exponential phase, but it became measurable in the early stationary phase when the P concentration dropped to very low levels (Dyhrman and Palenik 2003; Y. Xu pers. comm.). To our initial surprise, we observed no difference in growth rate between Zn-limited cultures grown on organic or inorganic P (Table 4).

Extrapolating these laboratory data to oceanic conditions is necessarily more tenuous. Particularly so, since phosphatases in ocean waters could originate from grazers, bacteria

or phytoplankton may have long residence times and are not exclusively associated with P stress (Hoppe 2003). Nonetheless, phosphatase activities have been measured in numerous oceanic regions and shown in many cases to be correlated to low phosphate and Chl *a* concentrations, suggesting some relationship between low PO_4^{3-} availability and phytoplankton-produced phosphatase activity (e.g., Guildford and Hecky 2000; Vidal et al. 2003; Sebastian et al. 2004). Further, *E. huxleyi* appears to possess the most efficient phosphatases among eukaryotic oceanic phytoplankters (Riegman et al. 2000). According to our laboratory studies with *E. huxleyi,* Zn–P co-limitation may prevail in waters that have below ~20 nmol L^{-1} inorganic phosphate and 1 pmol L^{-1} Zn′. In addition, the concentration of available organic P should be in the nanomolar range, necessitating high phosphatase activity and a corresponding requirement for Zn that is a significant fraction of the Zn quota. This last condition is hard to assess, since the natural organic P pool is heterogeneous and its availability for enzymatic hydrolysis may vary substantially in time and space (Benitez-Nelson 2000; Karl and Bjorkman 2002). Another complication in extrapolating the laboratory data to the field is the ability of organisms such as *E. huxleyi* to substitute Zn with other trace metals such as cobalt (Sunda and Huntsman 1995, 2000).

Bearing all these complications in mind, we speculate that Zn–P co-limitation may occur in the Sargasso Sea, which has low phosphate concentrations (~0.5 nmol L^{-1}; Wu et al. 2000); low total and unchelated Zn concentrations (~ 0.06 nmol L^{-1} and ~1 pmol L^{-1}; Bruland and Franks 1983); and low total dissolved organic P (75 ± 42 nmol L^{-1}; Wu et al. 2000) that is mostly unavailable for enzymatic hydrolysis (see introduction paragraphs). Indeed, the high alkaline phosphatase activities of 90 ± 80 nmol MF-P (μg Chl *a*) $^{-1}$ h^{-1}, which have been measured in the Sargasso Sea (Guildford and Hecky 2000), would require, according to our calculation of specific activity, that a large fraction of the cellular Zn serves as a metal center in the enzymes. In addition, a new cell-labeling technique has shown that 30–70% of the eukaryotic cells in the Sargasso Sea express cell surface phosphatase activity (Lomas et al. 2004). The oligotrophic Pacific gyre near Hawaii has higher phosphate (9–100 nmol L^{-1}; Bjorkman et al. 2000; Wu et al. 2000) and dissolved organic P (100–300 nmol L^{-1}; Bjorkman and Karl 2003; Van Den Broeck et al. 2004), but low Zn concentrations (0.08 nmol L^{-1}; Bruland 1980). These concentrations are slightly higher than those found to cause Zn–P co-limitation in our cultures, although it is conceivable that it would take place locally under episodic phosphate depletion. Other low P environments such as the Mediterranean Sea have high Zn concentrations (0.7–17 nmol L^{-1}; Zeri 2004), whereas low Zn environments such as the HNLC regions (e.g., Lohan et al. 2002; Ellwood 2004), have high phosphate concentrations.

The incubations we conducted with Bering Sea water (Fig. 6) should be interpreted with caution in view of the decrease in Chl *a* concentration at the end of the experiment. Nonetheless, since parameters known to affect phosphatase expression like phosphate and Chl *a* were similar in all treatments (Fig. 6a), the low phosphatase activity measured in the control and the +Fe treatments was most likely the result of limited Zn availability (Fig. 6b). The pronounced difference in phosphatase activity between the Zn-amended treatments and the other incubations may be viewed as a demonstration for the potential of Zn limitation of phosphatase activity, rather than a demonstration of limitation in situ. Zinc concentrations or availability seemed to have no effect on phytoplankton growth during the experiment and probably not on the phosphatase activity of the ambient water. Only toward the end of the incubation, when phosphate became limiting, did the Zn limitation of phosphatase activity become evident. Similar trends (although not as clear cut) were obtained in another incubation experiment from the same research cruise (data not shown). These results possibly suggest that Zn–P co-limitation may develop throughout the spring or summer bloom, when the increasing demand for P and Zn is not replenished by mixing or atmospheric deposition.

Based on our study of Zn–P co-limitation in *E. huxleyi*, we conclude that this phenomenon is not likely to be widespread in the oceans. Zn–P co-limitation of this organism may occur in oligotrophic waters containing exceedingly low phosphate, low or unavailable organic P, and low or strongly organically bound Zn. These conditions are found only in a few areas or may prevail episodically during blooms. The possibility remains that other phytoplankton species, which may have less efficient phosphatases than *E. huxleyi*, will be subjected to Zn–P co-limitation. We note that since organic P compounds are found everywhere in surface waters, the conjectured absence of Zn–P co-limitation implies the absence of P limitation *tout court* (assuming that the organic P compounds are phosphatase labile to some extent). The high efficiency of the phosphatases may be a reason why ocean waters are usually limited by N rather than P.

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Received: 26 March 2005
Accepted: 24 August 2005
Amended: 9 September 2005