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Alkaline phosphatase activity of phytoplankton in East China Sea coastal waters with frequent harmful algal bloom occurrences

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ABSTRACT: Alkaline phosphatase activity (APA) was measured during 3 cruises in spring 2002, 2003 and 2005 using bulk and single-cell assays in coastal waters in the East China Sea which experience frequent harmful algal bloom occurrences. The bulk APA ranged from below the detection limit to 73.53 nmol l^{-1} h^{-1}, with mean values of 15.73 ± 14.12, 23.77 ± 15.20 and 11.48 ± 12.44 nmol l^{-1} h^{-1} for 2002, 2003 and 2005, respectively. The cell-bound fraction (mainly phytoplankton) was the major contributor to APA, with averages of 74 and 80% during 2003 and 2005, respectively. During the spring of 2005, most of the dominant dinoflagellates had high percentages of enzyme-labeled fluorescence (ELF)-labeled cells, while only a few diatoms and chrysophytes were labeled with ELF. Among the dinoflagellates, *Protoperidinium* spp. and *Karenia mikimotoi* had the highest percentages of ELF-labeled cells (84 and 82%, respectively), whereas *Gonyaulax* spp. and *Dinophysis* spp. had the lowest percentages of labeled species (17 and 21%, respectively). An alkaline phosphatase (AP) kinetic experiment was performed during 2005, with a turnover time of 10 h and a maximum potential velocity of 206.1 nmol l^{-1} h^{-1}. The present results showed that severe phosphorus (P) stress occurred during springs in the study area, in particular when *Prorocentrum donghaiense* bloomed during 2003. AP played an important role in hydrolyzing soluble nonreactive phosphorus. The dominant dinoflagellates, which suffered more severe P stress compared to the diatoms, were the major AP producers during the spring of 2005, and differences in physiological P status existed among different dinoflagellate species.

KEY WORDS: Alkaline phosphatase · Phosphorus stress · Phytoplankton · Harmful algal blooms · East China Sea

INTRODUCTION

Phosphorus (P) is an essential macronutrient for the growth of phytoplankton. A growing amount of research has indicated that P might play an important role in controlling primary production in sea areas from open oceans to coastal environments; thus, P has received more and more attention in marine systems (Krom et al. 1991, Cappellen & Ingall 1996, Wu et al. 2000, Thingstad et al. 2005). With the highlighting of P stress or limitation in sea areas, the bioavailability of soluble nonreactive phosphorus (SNP), which was previously ignored, has become one of the focal points of marine ecological research.

Traditionally, orthophosphate was considered to be the sole form of phosphorus that could be utilized by

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microorganisms (Cembella et al. 1984, Björkman & Karl 1994). A number of studies have demonstrated, however, that bacteria and phytoplankton can utilize SNP through phosphatase hydrolysis (Cembella et al. 1984, Ammerman & Azam 1991, Karl & Yanagi 1997, Suzumura et al. 1998, Huang et al. 2005). SNP concentrations can exceed soluble reactive phosphorus (SRP) concentrations in coastal and open oceans (Karl & Yanagi 1997, Benitez-Nelson 2000, Wu et al. 2000), and thus might potentially control P availability and affect production of natural phytoplankton communities (Benitez-Nelson & Buesseler 1999, Kolowith et al. 2001).

Alkaline phosphatase (AP) is one of the enzymes most characterized in studies of the degradation of organic P compounds into inorganic phosphate (Cembella et al. 1984, Björkman & Karl 1994, Karl & Yanagi 1997, Hoppe 2003). AP activity (APA) has been widely used as an indicator of P deficiency (Healey & Henzel 1979, Annis & Cook 2002). P deficiency has been extensively reported for the northern Red Sea (Li et al. 1998), the southern and central Baltic Sea (Nausch 1998, 2004), the central Atlantic Ocean (Vidal et al. 2003) and the NW African upwelling region (Sebastián et al. 2004) by the traditional bulk APA assay. González-Gil et al. (1998) first developed a single-cell APA protocol using enzyme-labeled fluorescence (ELF). The single-cell APA assay can determine P status at the individual taxon level within natural phytoplankton communities (González-Gil et al. 1998, Dyhrman & Palenik 1999, Rengefors et al. 2003). Since then, much research has been done to determine the P status of individual phytoplankton species using the novel single-cell assay (Dyhrman & Palenik 1999, Rengefors et al. 2003, Lomas et al. 2004, Dyhrman & Ruttenberg 2006, Ou et al. 2006).

The Changjiang (Yangtze) River estuary, together with the adjacent coastal waters of the East China Sea (ECS), has been regarded as the most eutrophic area in China in recent years and has suffered frequently from harmful algal bloom (HAB) damage (Zhou et al. 2003). The nutrient input in this area is mainly from the Changjiang River plume and the Taiwan Warm Current (Chung et al. 2001, Shi et al. 2003). Previous studies have indicated that in the area of the most frequent HAB occurrences, the concentration of inorganic phosphate was relatively low and the N/P ratio was higher than the Redfield ratio, which suggested that P might play a potential role in regulating primary production, in particular when HABs occurred (Harrison et al. 1990).

In the present study, we combined the use of bulk and single-cell assays to investigate APA in the study area during 3 spring cruises in 2002, 2003 and 2005. The objectives of this study were to evaluate whether P stress or limitation existed during the spring periods of highly frequent HABs, as well as its variations, and to determine the specific physiological P status of different species, and the environmental factors which influenced APA.

MATERIALS AND METHODS

Description of the study area and sampling. The Changjiang (Yangtze) River estuary and its adjacent ECS coastal waters have become one of the most eutrophic areas in China, and have been noted as a high-frequency HAB occurrence area (Qi et al. 1993). In particular in recent years, a harmful bloom of the dinoflagellate *Prorocentrum donghaiense* covering more than 1000 km^2 occurred annually in this area during spring and early summer (April, May and June), and heavily influenced the ecosystem and economic development of the neighboring Zhoushan Fishing Ground (Zhou et al. 2003, Lu et al. 2005). To make matters worse, toxic *Alexandrium* spp. co-bloomed with *P. donghaiense* in some years and further threatened human health (Zhou et al. 2003). In order to study the ecology and oceanography of HABs in China, the 'China Ecology and Oceanography of Harmful Algal Blooms' (CEOHAB) project was launched in 2001, which was a 5 yr, interdisciplinary program to investigate key scientific questions concerning HABs in China. The major study area of the CEOHAB is located in the Changjiang River estuary and the adjacent coastal waters of the ECS, extending from 27.0 to 32.0° N and 121.0 to 123.5° E. This area is characterized by a high frequency of *P. donghaiense* blooms from spring to summer. Thus, *P. donghaiense* bloomed annually in the study area from the spring to summer of 2002, 2003 and 2005, and a large-scale bloom of *P. donghaiense* co-existed with *Alexandrium* spp. on 3 May 2002 and lasted for 27 d. From 1 to 11 May 2003, an extensive *P. donghaiense* bloom was monitored throughout the 30 m water column at 29.0 to 30.5° N, while a mixed bloom of *P. donghaiense* and *Karenia mikimotoi* occurred in June 2005.

Field cruises were carried out using RV 'Haijian 47', from 25 April to 2 May 2002, 1 to 11 May 2003 and 3 to 9 May 2005. A total of 28, 10 and 29 stations were sampled during these 3 years, respectively (Fig. 1). Water samples were taken from the surface layer (1 m in depth) using 20 l Niskin samplers.

Hydrological data. At each station, a YSI 6600 was used to obtain parameters of temperature, salinity and fluorescence. The YSI was calibrated before the cruise.

Nutrient analysis. Water was filtered through Whatman GF/F filters immediately after sampling and stored at –20°C. The concentrations of dissolved inor-

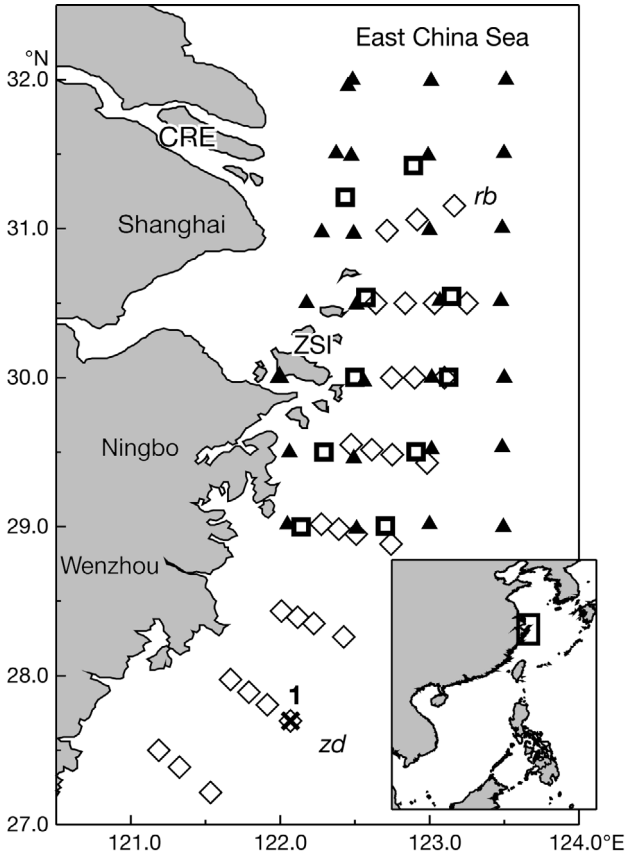

Fig. 1. Sampling stations in the Changjiang (Yangtze) River estuary (CRE) and adjacent coastal waters in the East China Sea. Stations were sampled in 2002 (▲), 2003 (□) and 2005 (◇); 2 transects (*zd* and *rb*) were sampled during 2005. 1: Stn 1 for the kinetic experiment of alkaline phosphatase activity in 2005; ZSI: Zhoushan Islands

ganic nitrogen (DIN, including NO_3^-, NO_2^- and NH_4^+), silicate and SRP were measured spectrophotometrically using a flow injection analyzer (Technicon AA II autoanalyzer) (Fiz et al. 1998). The dissolved organic nitrogen (DON) and SNP (samples were not taken during 2003) were determined after wet oxidation with alkaline $K_2S_2O_8$, followed by analysis of the dissolved nutrients (Grasshoff et al. 1999). TDN (total dissolved nitrogen) was the sum of DIN and DON; TDP (total dissolved phosphorus) was the sum of SRP and SNP.

Chlorophyll *a*. The chl *a* concentration was determined fluorometrically with a Turner Designs (Model 10) spectrofluorometer as described by Parsons et al. (1984). Seawater samples of 500 ml were filtered through Whatman GF/F filters and stored at –20°C. Chl *a* was extracted in cold acetone (90% v/v) in the dark for 24 h in the laboratory on land and then determined.

Bulk alkaline phosphatase activity assay. All seawater samples were first filtered through a 120 µm filter to remove zooplankton. Bulk APA was determined fluorometrically as the release of 3-0-methylfluorescein from 3-0-methylfluorescein phosphate (MFP; Sigma), according to a modified method based on Perry (1972). During the spring of 2002, APA was measured in unfractioned samples, while, during 2003, APA was fractioned into 2 sections: APA in the 0.22 µm filtrate (filtered through 0.22 µm polycarbonate filters under <100 mm Hg pressure), regarded as soluble APA, and cell-bound APA, contributed by the particulate organisms calculated as the difference between APA for the whole sample and that of the 0.22 µm filtrate. Cell-bound APA was further fractioned into APA in the fraction <3 µm (mainly bacteria) and APA in the fraction >3 µm (mainly phytoplankton) during the spring of 2005. The assay was run in duplicate in 1 to 4 h at *in situ* temperature. The fluorescent product was detected using a Shimadzu (RF-5301PC) spectrofluorometer, with the excitation and emission wavelengths set at 435 and 520 nm, respectively. One ml of MFP (1 mg l^{-1}) in the pH 8.72 Tris buffer was added to 9 ml of water sample (the final concentration of MFP substrate was 0.3 µM). Calibration was performed with standard solutions of 3-0-methylfluorescein (Sigma) in the range from 0.01 to 0.74 µM. Control blanks were run using sterilized surface water. Bulk APA was expressed as nmol of MFP released l^{-1} h^{-1}.

Single-cell alkaline phosphatase activity assay. A total of 23 stations were sampled for single-cell APA analysis during 2005. Approximately 10 l of surface seawater was concentrated using a 10 µm mesh and resuspended. The concentrated samples were processed for ELF labeling by centrifuging for 5 min at 4000 × *g*. The supernatant was discarded, and the cell pellets were transferred to a 1.5 ml microfuge tube. After this step, the protocols of Ou et al. (2006) were followed. The pellets were incubated for 30 min with 1 ml of 70% ethanol, the tubes were centrifuged (2 min, at 3000 × *g*), and the supernatant was aspirated off; 5 µl of ELF reagent (Molecular Probes) and 95 µl of 0.22 µm filtered sterile *in situ* seawater were added to the pellets, and the cells were incubated for another 30 min at room temperature in the dark. This incubation was followed by rinsing 3 times using sterile seawater to remove the residual ELF substrate, and then the samples were stored in 100 µl sterile seawater in the dark at 4°C until analysis using epifluorescence microscopy.

For analysis, the sample was placed on a glass slide with the mounting media provided in the ELF Phosphatase Detection Kit (Molecular Probes), and was observed under a Zeiss epifluorescence microscope with a DAPI (4′6′-diamidino-2-phenyl-indole) filter set using a 100 W mercury lamp. All samples were counted using standard light (a Tungsten lamp) alternated with mercury light to check for ELF activity. The

observed cells were divided into 2 groups on the basis of presence or absence of ELF precipitates. The percentage of ELF-labeled cells for a given taxon was determined as the fraction of fluorescently labeled cells relative to the total number of cells counted, and was used as an index of algal sensitivity to P stress.

Kinetics of alkaline phosphatase. During the spring of 2005, an offshore station (Stn 1) located on Transect *zd* was chosen as the site for the AP kinetics study. First, surface seawater subsamples were filtered through a 120 µm filter to remove zooplankton, and then different final concentrations (0 to 15 µM) of MFP substrate were added to the seawater. The estimation of the *in situ* turnover rate $T = (K + S_n)/V_{max}$ was carried out according to the method of Sebastián et al. (2004) based on the model developed by Li (1983), where K is the half-saturation constant, S_n is the ambient concentration of substrates, and V_{max} is the maximum potential velocity.

RESULTS

Environmental parameters in the study area

Temperature increased from the north to the south and from inshore stations to offshore stations (Table 1), so that the highest temperatures existed at the south-east stations. The mean temperatures showed no significant difference among the 3 years ($p > 0.05$). Salinity increased south-east off the Changjiang River estuary, and highest values also occurred at the farthest south-east stations. Salinity showed no significant difference among the 3 years studied ($p > 0.05$).

The distributions of SRP, DIN and silicate concentrations showed a similar trend and decreased from inshore stations to offshore stations (Table 1). Both SRP and silicate concentrations showed significant differences among the 3 years ($p < 0.01$), while DIN did not ($p > 0.05$). The mean SRP concentration during 2002 was significantly higher than those during 2003 and 2005 ($p < 0.01$). SNP comprised 0 to 42.7% of the TDP pool during 2002. During 2005, the mean value of SNP represented 46.3 ± 24.7% of the TDP pool. N/P and TDN/TDP ratios increased from inshore stations to offshore stations, and mean values of N/P and TDN/TDP were all >32 during the 3 years.

Bulk alkaline phosphatase activity

During the spring of 2002, bulk APA ranged from 1.84 to 73.53 nmol l^{-1} h^{-1}, with a mean value of 15.73 ± 14.17 nmol l^{-1} h^{-1}. Maximum APA existed at the inshore station near the Zhoushan Islands. When the APA data set was taken as a whole, no significant relationship was apparent between APA and environmental factors.

During 2003, a large-scale *Prorocentrum donghaiense* bloom occurred in the southern waters of the sampling area (29.0 to 30.5° N). APA ranged from 7.21 to 53.32 nmol l^{-1} h^{-1} in the study area, and the mean value of APA in the HAB zone (the southern waters) (32.82 ± 12.73 nmol l^{-1} h^{-1}) was significantly higher than that in the no-HAB zone (the northern waters) (10.18 ± 3.61 nmol l^{-1} h^{-1}) ($p < 0.01$). Cell-bound APA contributed most to APA, with a mean value of 74 ± 7% (Fig. 2). When compared with environmental factors, APA showed positive correlations with temperature and salinity, while it showed negative correlations with silicate, DIN concentrations and N/P ratio (Table 2). The multiple enter linear regression identified certain variables (temperature + SRP + DIN + N/P) as the significant factors that explained 94% of the APA variation ($p < 0.01$). A stepwise linear regression set to reject any variable that failed to produce an F statistic significant at the $p = 0.05$ level identified temperature as the major significant variable for APA, with r^2 values of 0.84 ($p < 0.01$).

Table 1. Variations of environmental parameters in East China Sea coastal waters during the cruises in 2002, 2003 and 2005. SNP was not measured during 2003. SRP: soluble reactive phosphorus; DIN: dissolved inorganic nitrogen; SNP: soluble nonreactive phosphorus; TDN: total dissolved nitrogen; TDP: total dissolved phosphorus; N/P: ratio of DIN versus SRP; –: parameters not measured

Parameter	2002		2003		2005	
	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD
Temp. (°C)	16.16–20.44	17.46 ± 1.03	15.41–19.61	17.78 ± 1.36	15.83–20.48	17.93 ± 1.23
Salinity (psu)	14.92–34.18	29.58 ± 4.86	21.71–30.88	27.56 ± 2.90	28.44–32.44	30.14 ± 1.16
SRP (µM)	0.31–0.86	0.50 ± 0.16	0.21–0.85	0.34 ± 0.15	0.05–0.70	0.25 ± 0.19
DIN (µM)	2.50–56.74	19.32 ± 18.61	1.83–46.05	17.65 ± 12.45	3.29–21.14	11.52 ± 5.59
Silicate (µM)	1.77–45.16	13.55 ± 12.50	5.60–29.51	16.63 ± 7.16	1.89–19.49	9.04 ± 5.54
SNP (µM)	0–0.47	0.13 ± 0.11	–	–	0–0.35	0.17 ± 0.09
N/P	7–90	32 ± 24	19–84	52 ± 21	21–162	59 ± 32
TDN/TDP	24–90	47 ± 17	–	–	21–48	33 ± 7
Chl *a* (µg l^{-1})	0.25–9.08	1.09 ± 1.63	0.51–11.73	2.71 ± 3.38	0.36–29.07	2.41 ± 5.20

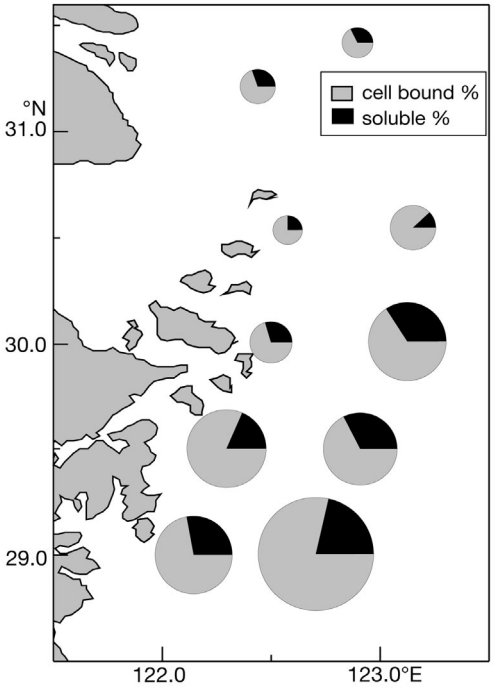

Fig. 2. Distribution of bulk alkaline phosphatase activity (APA) and its size fractions during the spring of 2003. Circles show APA values at various stations. Dark areas represent soluble percent APA; grey areas represent cell-bound percent APA

During the spring of 2005, surface APA ranged from below the detection limit to 56.17 nmol l^{-1} h^{-1}, with a mean value of 11.48 ± 12.44 nmol l^{-1} h^{-1}. APA was undetectable in stations located just outside the Changjiang River estuary (Fig. 3). When we divided bulk APA into different sizes, cell-bound APA contributed 80 ± 20% to the APA, the APA in the fraction >3 µm forming 59 ± 27% and the APA in the fraction <3 µm forming 21 ± 12%. The correlations between APA and the environmental parameters during 2005 were similar with those during 2003 (Table 2). Additionally, APA also showed positive correlations with chl *a* and SNP concentrations, but showed negative correlation with SRP concentration. A multiple linear regression model (APA = temperature + SRP + SNP) showed that these 3 factors explain 32% of the variation of APA ($p < 0.05$).

Comparing the variations of bulk APA in the same sampling area (29.0 to 32.0° N, 121.5 to 123.5° E) during 2002, 2003 and 2005 showed significant differences among the 3 years ($p < 0.05$). The mean APA (23.77 ± 15.21 nmol l^{-1} h^{-1}) in 2003, when a large-scale HAB occurred, was significantly higher than that in 2005 (8.31 ± 9.18 nmol l^{-1} h^{-1}) ($p < 0.05$). If we only considered the APA in the HAB zone during 2003, the mean value was significantly higher than that in either 2002 or 2005 ($p < 0.05$ and $p < 0.01$, respectively).

Table 2. Nonparametric correlations of bulk alkaline phosphatase activity (APA) with different parameters and the significance level (p) during 2003 (n = 10) and 2005 (n = 29). R: Spearman's rank correlation coefficient; +, –: positive and negative correlation between bulk APA and specific parameter, respectively, *$p < 0.05$, **$p < 0.01$; SRP: soluble reactive phosphate; SNP: soluble nonreactive phosphate, not measured during 2003; DIN: dissolved inorganic phosphate; N/P: ratio of DIN versus SRP

Parameter	2003		2005	
	R	p	R	p
Temp. (°C)	0.73(+)	*	0.51(+)	**
Salinity (psu)	0.87(+)	**	0.52(+)	**
Chl *a* (µg l^{-1})	0.36		0.56(+)	**
SRP (µM)	0.56(–)		0.62(–)	**
SNP (µM)			0.54(+)	**
Silicate (µM)	0.76(–)	*	0.79(–)	**
DIN (µM)	0.84(–)	**	0.69(–)	**
N/P	0.70(–)	*	0.28	

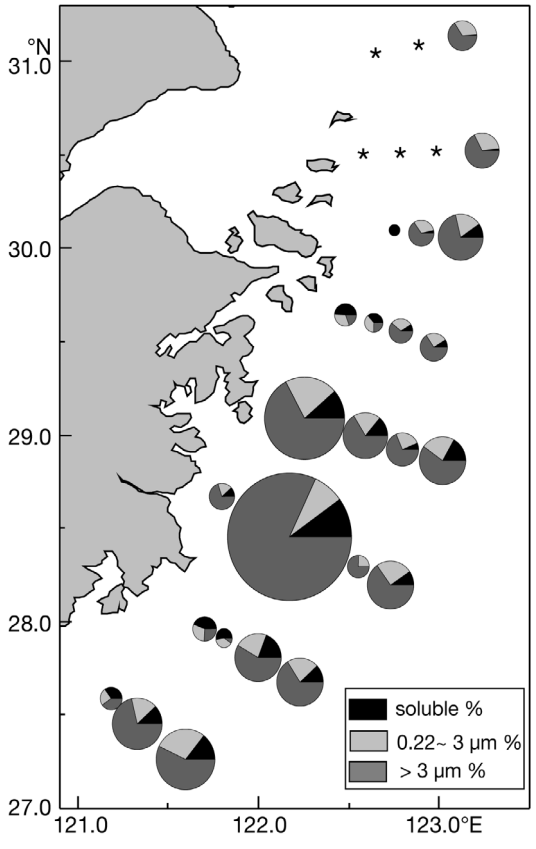

Fig. 3. Distribution of bulk alkaline phosphatase activity (APA) and its size fractions during the spring of 2005. Circles show APA values at various stations. APA values at station locations labeled with asterisks were below the detection limit. Dark areas represent soluble percent APA; light grey areas represent percent APA in the fraction from 0.22 to 3 µm; dark grey areas represent percent APA in the fraction >3 µm

Single-cell alkaline phosphatase activity

Dinoflagellates were the dominant species and showed high species diversity in the phytoplankton community during the 2005 study period (Table 3). In contrast, the abundance and diversity values of diatoms were very low. There was no detectable green ELF product in some samples from stations located outside the Changjiang River estuary, which coincided with the bulk APA results, but other samples were labeled with ELF at least for some species and showed different values of percentages of ELF-labeled cells (Fig. 4, Table 4). *Dictyocha* spp. in the Chrysophyta and the diatom species showed low percentages of ELF-labeled cells. In contrast, the percentage of ELF-labeled cells of most dinoflagellates was higher than that of the chrysophytes or the diatoms, although differences existed among the dinoflagellate species. When we compared the percentages of ELF-labeled cells of species in the same genus (e.g. *Ceratium*) the values of *C. breve* and *C. tripos* were much higher than those of *C. furca* and *C. fusus*.

When analyzing percentages of ELF-labeled cells of the dominant species against environmental parameters, it could be seen that phytoplankton single-cell APA was controlled by different factors. The percentages of ELF-labeled cells of *Prorocentrum* spp. and *Alexandrium* spp. were negatively correlated with salinity ($p < 0.05$, $n = 15$ and $p < 0.01$, $n = 20$, respectively), while the percentage of *Gonyaulax* spp. increased with temperature ($p < 0.05$, $n = 16$). The percentage of ELF-labeled cells of *Dinophysis* spp. showed positive correlation with SRP ($p < 0.05$, $n = 15$) and negative correlation with SNP and N/P ($p < 0.05$, $n = 15$).

Table 3. Dinoflagellate species detected by microscope in samples for the enzyme-labeled fluorescence assay during the spring of 2005

Genus	Species
Alexandrium	*A. catenella*, *A. tamarense*
Ceratium	*C. breve*, *C. furca*, *C. fusus*, *C. trichoceros*, *C. tripos*
Dinophysis	*D. acuminata*, *D. caudata*, *D. fortii*
Gonyaulax	*G. polyedra*, *G. polygramma*, *G. spinifera*, *Gonyaulax* sp.
Gymnodinium	*G. abbreviatum*, *Gymnodinium* spp.
Karenia	*K. brevis*, *K. mikimotoi*
Prorocentrum	*P. balticum*, *P. compressum*, *P. donghaiense*, *P. gracile*, *P. micans*, *P. minimum*, *P. sigmoides*
Protoperidinium	*P. bipes*, *P. conicum*, *P. depressum*, *P. divergens*, *P. oceanicum*, *P. pellucidum*, *P. pentagonum*
Scrippsiella	*S. trochoidea*

Table 4. Percentages (%) of enzyme-labeled fluorescence (ELF)-labeled cells of difference species of phytoplankton during the spring of 2005. –: without ELF labeling on cell; +: ELF labeling covered <50% of the cell; ++: ELF labeling covered >50% of the cell

Taxon	Cell count (n)	–	+	++
Chrysophyta				
Dictyocha fibula & *Disterphanus speculum*	113	99	0	1
Bacillariophyta				
Actinoptychus undulatus	103	77	10	13
Chaetoceros spp.	173	94	1	5
Coscinodiscus spp.	447	89	5	6
Cyclotella striata	160	98	0	2
Paralia sulcata	150	88	4	8
Pyrrophyta				
Alexandrium spp. (*A. tamarense*, *A. catenella*)	825	31	30	38
Ceratium spp.	544	33	9	58
C. breve	208	5	3	92
C. furca	118	72	19	9
C. fusus	95	85	7	7
C. tripos	123	3	11	85
Gonyaulax spp.	596	83	4	13
G. polygramma	493	89	4	7
G. spinifera	47	57	2	40
G. polyedra	52	54	8	38
Dinophysis spp.	680	79	9	12
D. acuminata	46	82	7	11
D. caudata	299	80	11	9
D. fortii	335	77	8	15
Karenia mikimotoi	650	18	18	64
Prorocentrum spp.	444	33	29	37
P. balticum	193	37	36	37
P. micans	204	32	32	36
Protoperidinium spp.	1156	16	29	56
P. pellucidum	989	13	31	55
P. bipes	76	34	36	29
Scrippsiella trochoidea	3526	32	31	36

Kinetics of alkaline phosphatase

At Stn 1, SRP concentration was 0.12 µM and SNP concentration was 0.14 µM, which contributed ca. 54% to the TDP pool. Bulk APA at the station was 20.40 nmol l^{-1} h^{-1}, with APA in the >3 µm fraction contributing 66%. From the nonlinear regression obtained by the least-squares method, the constants V_{max} (206.1 nmol l^{-1} h^{-1}) and K (2.22 µM) could be calculated ($r^2 = 0.98$). The turnover rate (r) was 0.09 h^{-1}, and the turnover time (T) was 10.8 h.

DISCUSSION

Phosphorus stress on the natural phytoplankton community

Many previous studies on APA induced under P stress conditions have been carried out in natural marine environments. Some studies were done in marine systems with a specific character, such as in upwelling regions (Sebastián et al. 2004, Ruttenberg & Dyhrman 2005), but few focused on seawater where eutrophication or high-frequency HABs had occurred. The study area of the Changjiang River estuary and the adjacent coastal ECS is one of the most eutrophic

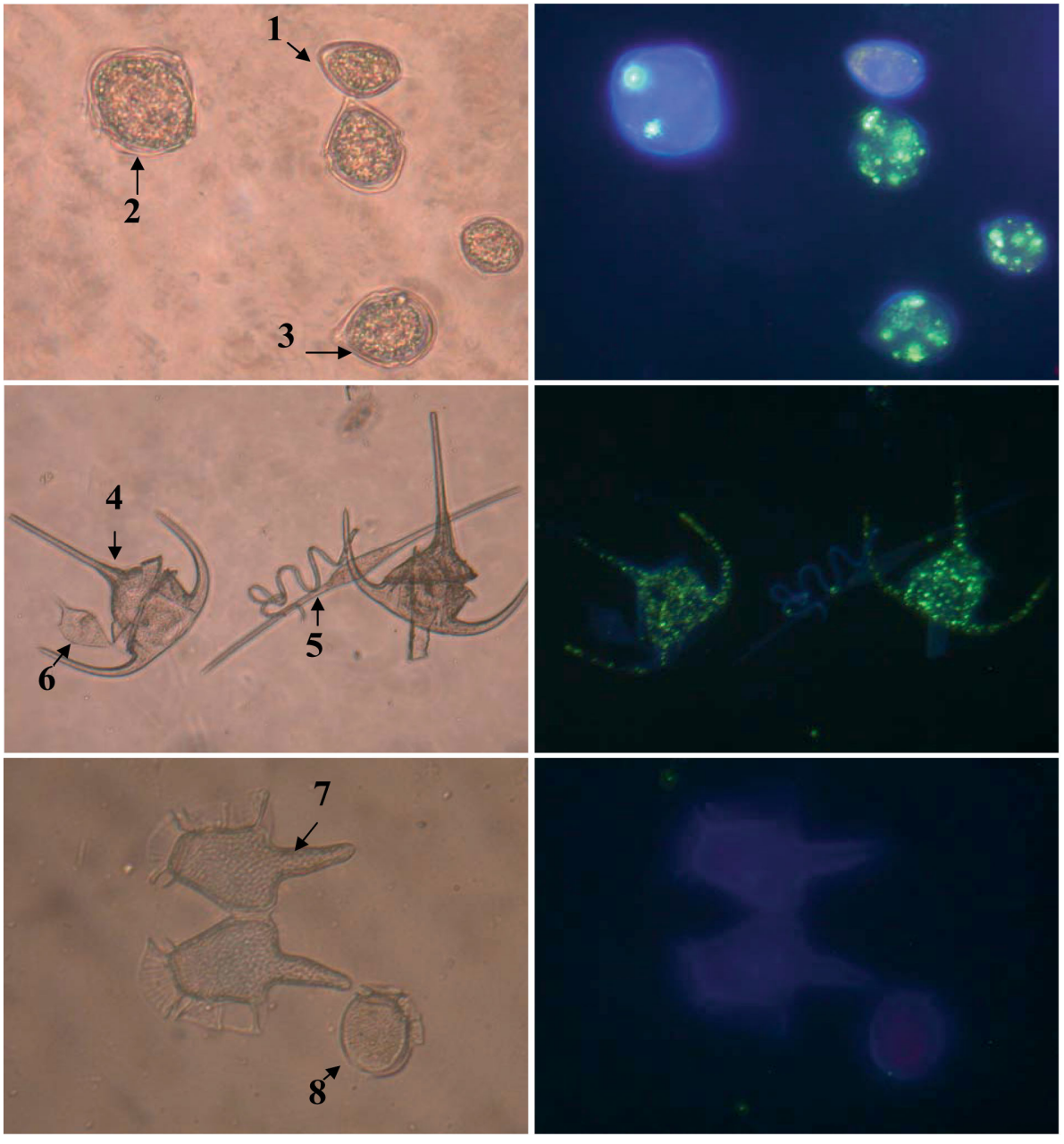

Fig. 4. Microscopic observations of enzyme-labeled fluorescence in phytoplankton during the spring of 2005. Dinoflagellates — 1: *Prorocentrum micans*; 2: *Alexandrium* sp.; 3: *Scrippsiella trochoidea*; 4: *Ceratium tripos*; 5: *Ceratium fusus*; 6: *Ceratium furca*; 7: *Dinophysis caudate*; 8: *Dinophysis acuminata*. Left panels show samples observed under bright field; right panels show samples observed under a long-pass DAPI filter set. Bright green fluorescence in right panels was indicative of alkaline phosphatase activity

bodies of water in China; *Prorocentrum donghaiense* blooms have occurred here annually from spring to summer since 2000 (Zhou et al. 2003). Since relatively high SRP concentrations were detected in the study area during the 3 years surveyed (in particular the mean concentration of SRP was as high as 0.50 ± 0.16 µM during the spring of 2005), it might be thought that APA would not be detectable in such a eutrophic area. However, this was not the case in the study. High APA values were detected during the spring of 2002, 2003 and 2005 whether or not a bloom occurred. The APA values obtained in our study ranged from below the detection limit to 73.53 nmol l^{-1} h^{-1}. In general, the APA in the study area showed an increasing trend from north to south, which meant that different degrees of P stress existed, and indeed showed that the trend of P stress strengthened towards the south in the study area. This phenomenon might be attributed to the location of the study area, since the Changjiang River input contains a large amount of nutrients, including phosphate, which would somewhat alleviate P stress in the study area (Chung et al. 2001, Shi et al. 2003). Low (even undetectable) APA values were found only near the Changjiang River estuary, which meant that phytoplankton was not P stressed in this area.

Due to different experimental conditions, it is difficult to compare APA values in our study with other references (Ou et al. 2006). However, when compared with APA data we had taken from other coastal areas in China under the same experimental conditions, APA in this study area was even higher than that with lower SRP concentrations (data not shown). Therefore, it might be wrong to conclude that seawater was or was not P stressed based directly on environmental SRP concentrations.

During the spring of 2003, a large-scale *Prorocentrum donghaiense* bloom occurred in the southern part of the study area. Although such a bloom did not occur during the study periods 2002 and 2005, it was reported that massive blooms took place soon after our cruises. In particular, in 2002, an algal bloom occurred just 1 d after our cruise. Comparing the APA values among the 3 years showed that the natural phytoplankton community suffered much more severe P stress under algal bloom conditions during 2003. The sea area adjacent to the Changjiang River estuary is well known for its eutrophication and for highly frequent HAB occurrences (Zhou et al. 2003). Some previous studies have shown that in HAB zones of the coastal ECS, the concentration of SRP is lower than that of DIN, and the N/P ratio is significantly higher than 16 (Harrison et al. 1990, Shi et al. 2003). In the present study, we found that N/P and TDN/TDP ratios were >32 in most of the stations. It might be concluded that P was the potentially limiting nutrient in this study area, a conclusion also confirmed by the enrichment mesocosm experiments done on deck during the cruises of 2002 and 2003 (data not shown). Phytoplankton numbers with phosphate addition were significantly higher than those without any addition during the mesocosm experiments, which meant that phosphate might be the potentially limiting nutrient for phytoplankton growth. When phytoplankton bloomed, P, compared to N, would be exhausted first and so the phytoplankton would suffer P stress and induce the expression of AP.

Li et al. (1998) suggested that the P history of a sea area based on the relative levels of soluble and cell-bound APA should be considered. When cell-bound APA was high and soluble APA was low, short-term P limitation (hours to days) happened. During the spring of 2003 and 2005, cell-bound APA was much greater than soluble APA. The mean values of cell-bound APA during 2003 and 2005 contributed 74 ± 7 and 80 ± 23% of the APA, respectively. These results showed that phytoplankton and bacteria were P stressed in the study area during 2003 and 2005. Comparing APA in the fraction >3 µm and APA in the fraction <3 µm during the spring of 2005, it might be concluded that phytoplankton was the main contributor to the APA.

Phytoplankton species-specific phosphorus stress

Our results showed that during the spring of 2005, extensive phytoplankton was labeled with ELF, and, thus, P stress occurred in the study area, in particular among the dominant dinoflagellates. Low percentages of ELF-labeled cells existed among the diatoms (such as *Coscinodiscus* spp., *Chaetoceros* spp. and *Cyclotella striata*) and the chrysophytes (such as *Dictyocha fibula* and *Disterphanus speculum*), which suggested that these species did not suffer P stress or were only slightly P stressed. It might be considered that, in the same environment, dinoflagellates were more easily P stressed and thus expressed abundant AP, and the high APA value in the fraction >3 µm detected in the study areas might have been contributed by the dinoflagellates. Previous studies have shown that, related to high DNA content, dinoflagellates might have a higher P demand compared to diatoms; therefore, diatoms might bloom more easily in P-stressed or P-limited sea areas (Berdalet et al. 1996). It is not clear what mechanism determined the blooms of dinoflagellates (which suffered more P stress), such as the *Prorocentrum donghaiense* blooms that occurred annually in our study area. Some researchers have suggested

that dinoflagellates might develop some unique mechanisms to compete for nutrients, such as storing surplus nutrients in the internal nutrient pool, switching to utilize dissolved organic nutrients, nutrient retrieval migrations, or mixotrophic nutrition tendencies (Nygaard & Tobiesen 1993, Stoecker 1999, Anderson et al. 2002, Smayda & Reynolds 2003).

Significant differences in percentages of ELF-labeled cells existed among the dinoflagellates, indicating that species of dinoflagellates could be differentiated in terms of their physiological requirement for P. From the results of percentages of ELF-labeled cells, it might be concluded that, in the same environment, *Protoperidinium* spp. and *Karenia mikimotoi* suffered the most severe P stress, while only a few *Gonyaulax* spp. and *Dinophysis* spp. suffered P stress. Therefore, *Protoperidinium* spp. and *K. mikimotoi* might require more P for growth than any other species of dinoflagellates. In the genus *Ceratium*, the percentages of ELF-labeled cells of *C. breve* and *C. tripos* were much higher than those of *C. furca* and *C. fusus*, showing that the P requirements of *C. breve* and *C. tripos* were much higher than those of *C. furca* and *C. fusus*. Much previous research has shown that most dinoflagellates have the ability of mixotrophy (Stoecker 1999, Smayda 2002). Under nutrient-limited conditions, endocytosis of particulate N or P has been extensively reported among dinoflagellates (Nygaard & Tobiesen 1993, Smayda 2002). Stoecker (1999) performed some field experiments and found that nutrient limitation, in particular P limitation, would stimulate dinoflagellates to 'catch' particulate nutrients. Nutritional strategies are diverse among dinoflagellates: some are mainly autotrophic, some are mainly heterotrophic and others are mixotrophic. Due to the difficulties involved in performing mixotrophy experiments, the nutritional strategies of dinoflagellates are still unclear. Our results of single-cell APA showed that dinoflagellates differed in their need for P and therefore their response to P stress. This might be relevant to their diverse nutrient strategies.

Factors regulating alkaline phosphatase activity

APA has been widely used to indicate P stress or limitation in the sea. Many studies have examined the negative correlation between APA and SRP concentrations (Nausch 1998, Annis & Cook 2002). In our study, such a relationship was only found during the spring of 2005. Surprisingly, under most conditions, we also found that APA increased significantly with a decrease in DIN and silicate concentrations. These negative correlations have not been reported in previous studies. Through laboratory experiments, we considered that when phosphate was ample, saturation or absence of nitrate would not influence algal AP expression and its activity (data not shown). Since SRP, DIN and silicate concentrations showed similar trends in the coastal waters of the Changjiang River estuary, whether these negative correlations between APA and DIN or silicate concentrations indicated a relationship between APA and SRP concentration, or whether DIN and silicate concentrations might indeed be important but previously ignored factors for APA, still needs further study.

Some work has pointed out that the control mechanism of APA is complicated in natural ecosystems and that the affecting factors might include temperature, salinity, UV-B, nutrient availability and so on (Hoppe 2003). No significant correlation between APA and environmental factors was found during the spring of 2002. During the spring of 2003, the variables temperature + SRP + DIN + N/P could explain 94% of the APA. During the spring of 2005, the variables SRP + SNP + temperature significantly affected APA with an r^2 of 0.32. We could conclude that temperature and nutrients play more important roles on APA in the study area.

APA regulation was different among different species of phytoplankton, and, since the composition of the phytoplankton community varied between years, this result also indicated why the control mechanism of APA was complicated in natural ecosystems. In our study, the percentages of ELF-labeled cells of *Prorocentrum* spp. and *Alexandrium* spp. decreased significantly with the increase in salinity; the percentage of ELF-labeled cells of *Dinophysis* spp. increased significantly with temperature; and the percentage of ELF-labeled cells of *Gonyaulax* spp. increased with temperature but decreased with SNP concentration and N/P ratio. Smayda (2002) divided dinoflagellate bloom taxa into 10 types according to the characteristics of their habitats. Among these types, *Alexandrium tamarense* and *Karenia mikimotoi* were notable examples of Type IV 'frontal zone taxa' adapted for entrainment and dispersal within coastal currents. We wondered whether these different types of habitat-adapted taxa might develop diverse mechanisms to accommodate the variation of habitats, such as, for example, the frontal zone taxa *A. tamarense* and *K. mikimotoi* being more sensitive to salinity so that variation in salinity might regulate their expression of AP.

In summary, factors regulating APA differed with conditions such as the physico-chemical characteristics of environments, the composition of the natural phytoplankton community, as well as algal-specific physiological requirements and status.

Combination of bulk and single-cell APA results as a better indicator of P stress

Table 5 shows the variations in bulk and single-cell APA of the dominant dinoflagellates with varying environmental factors at the 2 transects (*zd* and *rb*) during the spring of 2005. Temperature, salinity and SNP concentration increased, while SRP concentration decreased along these 2 transects with the distance from the shore. The bulk APA of the 2 transects increased with the decrease in SRP concentrations offshore. However, the dominant dinoflagellate taxa along these transects contrasted in terms of single-cell APA. Although bulk APA at the inshore station was below the detection limit along Transect *rb*, some dinoflagellates, in particular *Protoperidinium* spp., were labeled with ELF. With distance offshore, SRP concentrations decreased and bulk APA increased significantly. The percentages of ELF-labeled cells of *Alexandrium* spp., *Gonyaulax* spp. and *Scrippsiella trochoidea* also increased significantly offshore. The results showed that offshore along Transect *rb*, phytoplankton suffered more severe P stress and induced abundant AP; thus, phytoplankton might utilize SNP via the hydrolysis of APA. However, results along Transect *zd* showed some difference. Although bulk APA increased with the decrease in SRP concentration offshore, the percentages of ELF-labeled cell values of *Alexandrium* spp., *Prorocentrum* spp. and *Protoperidinium* spp. decreased, while the percentage values of *Dinophysis* spp. and *Scrippsiella trochoidea* did not change significantly with the variation in SRP concentrations. The explanation of these results might be due to the different P histories at these stations. The percentages of soluble APA at the 2 inshore stations were much higher than those of the 2 offshore stations along Transect *zd*, which indicated that phytoplankton was P stressed at an earlier time at the inshore stations, but that this status was diminishing. Some research has shown that AP induced by phytoplankton would not lose its activity in a short time and that the lifetime of AP might even exceed the average generation time of most phytoplankton species (Li et al. 1998, Vidal et al. 2003). Although enzyme activity decreased, AP could still be detected for a period using the method of single-cell ELF (Dyhrman & Palenik 1999). Therefore, single-cell APA data by the ELF assay might sometimes lead to the incorrect conclusion that phytoplankton suffered severe P stress during the study time. On the other hand, the bulk APA method also had its disadvantages, such as the relatively insensitive detection limit and the sparse information concerning species-specific APA contribution. To better understand the P status of natural phytoplankton communities, a combination of bulk APA and single-cell ELF methods is a promising protocol.

Importance of SNP as a potential P source for the growth of phytoplankton

In the past, marine ecologists paid much more attention to the influence of dissolved inorganic nutrients on primary production, and the significance of organic nutrients was ignored for a long time. Concomitant with phosphate being shown to be the limiting nutrient in many coastal and open oceans (Krom et al. 1991, Wu et al. 2000, Thingstad et al. 2005), the availability of SNP might finally affect the primary

Table 5. Variations of environmental factors, bulk alkaline phosphatase activity (APA) and percentages of enzyme-labeled fluorescence (ELF)-labeled cells of dominant dinoflagellates from coastal to offshore stations along Transects *zd* and *rb* during the spring of 2005. Stns *zd* 1 to 4 and Stns *rb* 1 to 3 are located progressively away from the coast. SRP: soluble reactive phosphorus; SNP: soluble nonreactive phosphorus; U: data at some stations undetectable or lack of the significance of statistics

Parameter	Transect *zd*				Transect *rb*		
	1	2	3	4	1	2	3
Temp. (°C)	18.15	18.44	19.51	20.48	16.34	16.54	17.37
Salinity (psu)	28.77	29.41	31.12	31.92	29.96	30.54	31.13
SRP (µM)	0.44	0.31	0.12	0.12	0.47	0.14	0.13
SNP (µM)	0.08	0.12	0.14	0.14	0.07	0.22	0.27
Chl *a* (µg l^{-1})	1.34	1.60	1.28	0.99	1.35	0.59	1.69
Bulk APA (nmol l^{-1} h^{-1})	4.48	1.65	18.65	20.39	U	5.03	18.01
Soluble bulk APA (%)	44	53	19	12	U	3	10
Alexandrium ELF (%)	U	95	37	7	22	59	U
Dinophysis ELF (%)	26	11	U	30	U	9	U
Prorocentrum ELF (%)	85	92	57	43	17	U	U
Gonyaulax ELF (%)	U	U	24	33	U	14	33
Protoperidinium ELF (%)	98	88	24	51	59	91	92
Scrippsiella ELF (%)	100	96	63	97	26	74	87

production of natural phytoplankton communities (Karl & Yanagi 1997, Benitez-Nelson 2000). In the coastal areas of oceans, the SNP pool generally represents 0 to 50% of the TDP pool, while in the open ocean this could be as high as 75% (Karl & Yanagi 1997, Benitez-Nelson 2000). Most of the SNP has been proved to be available to microorganisms (Benitez-Nelson 2000, Kolowith et al. 2001), and AP might play an important role in the recycling of SNP. AP is one of the best known phosphatases in marine environments, and it could hydrolyze a series of monophosphoesters, including sugar-P, linear polyphosphates and nucleotides (Benitez-Nelson 2000). Therefore, AP could hydrolyze 10 to 50% of the SNP pool (Karl & Yanagi 1997). By hydrolyzation of phosphatases such as AP, the cycling of the TDP pool might control the availability of P and finally determine the biomass of phytoplankton and the bacterial community (Björkman & Karl 1994).

In 2002 and 2005, SNP concentrations contributed >40% of the TDP pool in the study area. In the location where we carried out the AP kinetics experiment in 2005, SNP even represented 46% of the TDP pool. Since we proved that P stress existed in the natural phytoplankton community in the study area, in particular when algal blooms occurred, SNP could play an important role in controlling primary production with the hydrolyzation of AP. The hydrolytic V_{max} (206.1 nmol l^{-1} h^{-1}) was comparable to that observed on the Louisiana Gulf Coast (ca. 500 nmol l^{-1} h^{-1}) (Ammerman & Glover 2000), but it was 100s of times higher than the values reported by Sebastián et al. (2004) in a northwest African upwelling system (up to 2.1 nmol l^{-1} h^{-1}) and by Dyhrman & Ruttenberg (2006) in the Oregon coastal system (up to 3.3 nmol l^{-1} h^{-1}). This result had 2 implications: (1) the natural plankton community in the study area, in particular the phytoplankton, which contributed most to APA, suffered severe P stress and thus induced high APA; and (2) AP had a high capacity to hydrolyze SNP to SRP and could supply a P source for phytoplankton growth under P-scarce conditions. Thus, AP might play an important role in the recycling of P in coastal waters of the ECS.

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