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Alkaline phosphatase activities and regulation in three harmful *Prorocentrum* species from the coastal waters of the East China Sea

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Abstract

Harmful blooms of *Prorocentrum donghaiense* occur annually in the phosphorus-scarce coastal waters of the East China Sea (ECS). The enzymatic activities of alkaline phosphatase (AP) and its regulation by external phosphorus were studied during a *P. donghaiense* bloom in this area. The AP characteristics of *P. donghaiense* was further compared with *Prorocentrum minimum* and *Prorocentrum micans* in monocultures with both bulk and single-cell enzyme-labeled fluorescence AP assays. Concentrations of dissolved inorganic phosphorus (DIP) varied between 0.04 and 0.73 μmol l^{-1}, with more than half recording stations registering concentrations below 0.10 μmol l^{-1}. Concentrations of dissolved organic phosphorus (DOP) were comparable or even higher than those of DIP. *P. donghaiense* suffered phosphorus stress and expressed abundant AP, especially when DIP was lower than 0.10 μmol l^{-1}. The AP activities showed a negative correlation with DIP but a positive correlation with DOP. The AP activities were also regulated by internal phosphorus pool. The sharp increase in AP activities was observed until cellular phosphorus was exhausted. Most AP of *P. donghaiense* was located on the cell surface and some were released into the water with time. Compared with *P. minimum* and *P. micans*, *P. donghaiense* showed a higher AP affinity for organic phosphorus substrates, a more efficient and energy-saving AP expression quantity as a response to phosphorus deficiency. The unique AP characteristic of *P. donghaiense* suggests that it benefits from the efficient utilization of DOP, and outcompete other species in the phosphorus-scarce ECS.

Keywords Alkaline phosphorus · Organic phosphorus · *Prorocentrum donghaiense* · the East China Sea

Introduction

Prorocentrum donghaiense is one of the most well-known harmful algal bloom species in the Changjiang River Estuary and its coastal waters of the East China Sea (ECS) [1, 2]. *P. donghaiense* blooms have been recorded annually in spring since 2000, at times lasting for more than 1 month and encompassing several thousand square kilometers [1, 3, 4]. The notorious blooms have caused serious damage to the regional ecosystem, marine fisheries, and public health [5, 6]. The estimated economic losses due to *P. donghaiense* blooms range from hundreds of thousands to millions of US dollars every year [7]. Many studies have been carried out to study the mechanism of *P. donghaiense* blooms in this area, among which the importance of phosphorus dynamics has been noted [2, 8–10]. The nutrient structure in the coastal waters of the Changjiang River Estuary has changed greatly in the past 60 years [10]. The concentrations of nitrate and phosphate have been increased significantly with the dissolved inorganic N:P ratios increasing sharply from 40 in the 1960s to 200 in the

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2010s due to a much more abundant nitrate discharge from the Changjiang River compared with phosphate [10, 11]. Phytoplankton suffer from phosphorus stress or limitation during spring to summer times in this area [6, 12, 13]. The dominant species of *Skeletonema costatum* was replaced by *P. donghaiense* during spring when phosphate was scarce in this area [2, 8]. Furthermore, *P. donghaiense* blooms often persist for a long period under phosphorus-deficient conditions and only collapse as nitrate is depleted [2, 12]. All these findings suggest that *P. donghaiense* is adapted to the low-phosphate environment and must possess some advantageous strategies in competition and utilization of phosphorus.

Phytoplankton have developed adaptive strategies to deal with phosphorus deficiency, e.g., inducing high-affinity phosphate transporters, changing phosphorus allocation through polyphosphate production, decreasing cellular phosphorus demand by substituting phospholipids with sulfolipids and/or nitrolipids, and efficiently utilizing organic phosphorus compounds by phosphatases [14–16]. Among the phosphatases, alkaline phosphatase (AP), which hydrolyzes a broad spectrum of organic phosphorus compounds (mostly monophosphoester) into inorganic phosphate, is one of the best studied [17–20]. AP plays a prominent role in the regeneration of organic phosphorus in the marine environment [19, 21]. AP has been widely used to study the phosphorus status of microorganisms or as an indicator of phosphorus deficiency or limitation [13, 17, 19]. Traditionally, bulk AP activity (APA) is quantitatively measured by adding some artificial chromogenic or fluorescent substrate [18, 22]. The origin of AP (heterotrophic bacteria or phytoplankton) can be roughly analyzed through size fractionation of water samples [18]. A qualitative cell-specific assay using enzyme-labeled fluorescence (ELF) made it possible to accurately tag cells that exhibit APA [23]. The combination of bulk AP and single-cell ELF assays are recommended to better explain the phosphorus status of phytoplankton in both natural environments and laboratory studies [24–26]. Transcriptomic and proteomic techniques have also been used to analyze the molecular responses of AP to the variation of phosphorus in recent years [15, 27–29]. Diverse AP characteristics among phytoplankton species have been observed in marine ecosystems. Algal AP might be constitutive or inducible [19, 23]. Some phytoplankton may not even express AP [18]. Algal AP might differ in gene sequence types [27, 30], subcellular localization [27, 31], response threshold value to phosphorus stress [20, 32], and affinity for organic phosphorus substrates [25] etc. These AP differences among microorganisms influence the hydrolysis efficiency in organic phosphorus compounds and thus determine inter-specific competition capability in marine ecosystems, especially when phosphate is scarce.

The regeneration of organic phosphorus compounds cannot be ignored in the coastal waters of ECS, especially during *P. donghaiense* blooms [33, 34]. The dissolved organic phosphorus (DOP) concentrations sometimes are higher than phosphate concentrations and contribute 5–70% of the total dissolved phosphorus (TDP) in these areas [33, 35]. Inducible expression is observed in *P. donghaiense* as it suffers severe phosphorus stress and expresses high APA during blooms [13]. A turnover time of ~ 10 h for organic phosphorus compounds was calculated during *P. donghaiense* blooms in situ by an AP kinetic assay [13]. AP seems to play a prominent role in the regeneration of organic phosphorus during *P. donghaiense* blooms. Previous laboratory studies indicated that *P. donghaiense* was more competitive in growth with organic phosphorus substrates [36, 37]. Shi et al. [9] suggested that *P. donghaiense* enhanced DOP utilization to cope with phosphorus deficiency. *P. donghaiense* showed a higher AP affinity for organic phosphorus substrate compared with *S. costatum* [38]. Thus, we hypothesize that due to their ability to efficiently utilize DOP by AP, *P. donghaiense* is able to outcompete other species and dominate phytoplankton communities. To explain the mechanism of *P. donghaiense* blooms, it is important to comprehensively understand AP characteristics of *P. donghaiense* and its efficiency in utilizing DOP.

Two other species of *P. minimum* and *P. micans* in the family of Prorocentraceae often coexist with *P. donghaiense* in the ECS [3]. Of special interest is that *P. minimum* is the cause of harmful blooms in many estuarine and coastal waters [11, 39, 40]. We ask why the bloom species in the ECS is *P. donghaiense* instead of *P. minimum* or *P. micans*? Might the difference in the efficiency in utilizing DOP by phosphatase among *Prorocentrum* species help determine the fates in inter-specific competition? In this study, the differences in AP characteristics and the efficiency in hydrolyzing organic phosphorus compounds among three *Prorocentrum* species were compared with the bulk and single-cell ELF assays in both the field and the laboratory. The objectives of this study were to investigate the phosphorus status of phytoplankton and the AP regulation capability during *P. donghaiense* blooms to better understand the adaptive strategies of *P. donghaiense* to phosphorus deficiency and the importance of DOP regeneration through AP hydrolysis during blooms. The inter-specific differences in AP distribution among three *Prorocentrum* species were also investigated.

Materials and Methods

Study of AP Activities During a *P. donghaiense* Bloom in the Coastal Waters of ECS

A field cruise was carried out in the coastal waters of ECS during May 7–24, 2010 using the RV "Science 3." A total of 27 stations were sampled (Fig. 1). Water samples were taken from the surface layer (1-m depth) using 2.5-l Niskin bottles.

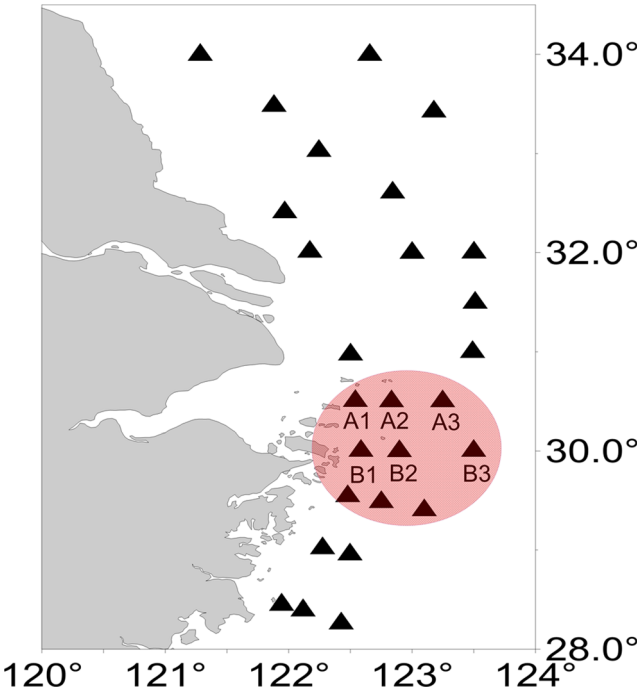

Fig. 1 Sampling stations in the coastal waters of the East China Sea during a bloom of *P. donghaiense* in May 2010. The red oval area with transects A and B indicates the central zone of *P. donghaiense* bloom

During the cruise, a *P. donghaiense* bloom with the density higher than > 10^6 cells l^{-1} were observed in the study area, especially along transects A and B. Cells of *S. costatum*, *P. minimum*, and *P. micans* were observed to coexist with *P. donghaiense* in most stations.

Two replicate seawater samples were filtered through precombusted Whatman GF/F filters (450 °C, 2 h) for measurement of nutrient. The samples were frozen at − 20 °C until analysis in the laboratory in one month. Nitrate + nitrite, ammonium, reactive silicate, and DIP were determined spectrophotometrically using a flow injection analyzer (Technicon AA II autoanalyzer)[41]. Dissolved inorganic nitrogen (DIN) was the sum of nitrate, nitrite, and ammonium. Total dissolved nitrogen (TDN) and TDP were measured after wet oxidation with alkaline $K_2S_2O_8$ [41]. Dissolved organic nitrogen (DON) was calculated by subtracting DIN from TDN whereas DOP was calculated by subtracting DIP from TDP.

Approximately 10 l of seawater was concentrated using a 10 μm mesh and centrifuged for 5 min at 4000 g. The concentrated cell pellets were processed for the analysis of single-cell APA with ELF reagent (2-(5′-chloro-2′-phosphoryloxyphenyl)-6-chloro-4-(3H)-quinazolinone, Molecular Probes, Inc., OR) according to the method of Ou et al. [25]. The pellets were labeled with ELF for 30 min at room temperature in the dark and then was rinsed 3 times using sterile seawater. The samples were stored in 50–100 μl sterile seawater in the dark at 4 °C until analysis in the laboratory in one month. For analysis, the samples were observed under an epifluorescence microscope (OLYMPUS, X61) with a DAPI filter set. The ELF of three *Prorocentrum* species and *S. costatum* were observed. When the density was high, at least 200 cells of the observed species were counted. When the density was relatively low, all cells of the species in the sample were counted. The observed cells were divided into three groups based on the intensity of fluorescence: absence of fluorescence; medium fluorescence, where ELF coverd < 30% of the cell bodies; strong fluorescence, where ELF covered > 30% of the cell bodies. Percentages of ELF for a given species were calculated as the fraction of ELF labeled cells in one group divided by the total number of cells counted.

Study of AP Characteristics of Three *Prorocentrum* Species in the Laboratory

Culture and Growth Conditions

Cultures of *P. donghaiense* (MEL 203), *P. minimum* (MEL 310), and *P. micans* (MEL 56) were maintained in the Algal Collection, Research Center of Harmful Algae and Marine Biology, Jinan University, China. The cultures were inoculated at 21 ± 1 °C in a light: dark cycle of 12: 12 h with irradiation of 100 μmol m^{-2} s^{-1}.

Prior to the experiment, the cultures were re-inoculated 3 times during the exponential phase in the Aquil* artificial seawater medium (enriched with f/2 except for PO_4^{3-})[42] to allow the algae to acclimatize to the subsequent experimental conditions. The initial concentrations of PO_4^{3-} were decreased to ~ 18, 9 and 5 μmol l^{-1} gradually at each inoculation. Antibiotics (penicillin G, 3 g l^{-1}, and streptomycin sulfate, 5 g l^{-1}) were used to eliminate bacterial contamination 48 h before the next inoculation [43]. The cultures were checked for bacterial contamination with 4′,6-diamidino-2-phenylindole (DAPI)(Sigma) stain at regular intervals by microscopic inspection.

Given the differences in cell volumes among three *Prorocentrum* species, monocultures of *P. donghaiense*, *P. minimum*, and *P. micans* were inoculated in triplicate with densities as shown in Table 1. The cell volumes of three species were calculated according to the method of Hillebrand et al. [44]. The initial concentrations of PO_4^{3-} were adjusted to ~ 3 μmol l^{-1}

Table 1 The cell volumes and initial inoculate densities of three *Prorocentrum* species

Species	Volumes (μm^3)	Initial densities (cells ml^{-1})
P. donghaiense	580.0 ± 224.2	1.3×10^4
P. minimum	1712.0 ± 274.3	4.4×10^3
P. micans	15,059.2 ± 2116.6	5.0×10^2

Table 2 Variations of nutrients in the coastal waters of the East China Sea in the field study. *DIN*, dissolved inorganic nitrogen; *DIP*, dissolved inorganic phosphorus; *DON*, dissolved organic nitrogen; *DOP*, dissolved organic phosphorus; *TDN*, total dissolved nitrogen; *TDP*, total dissolved phosphorus. The unit of nutrient concentration is μmol l^{-1}

Nutrients	Range	Average ± S.D
DIN	0.93–28.54	12.11 ± 7.72
DIP	0.04–0.73	0.17 ± 0.15
SiO_3^{2-}	0.32–37.57	15.45 ± 8.60
DON	2.97–54.81	14.12 ± 10.27
DOP	0.07–0.58	0.21 ± 0.13
DIN/DIP	13.1–454.9	104.9 ± 104.9
TDN/TDP	34.1–253.0	75.5 ± 42.1

based on the f/2 medium. When the observed DIP concentration in media did not decrease anymore (lower than 0.1–0.2 μmol l^{-1}) and the cells of *Prorocentrum* species stopped growing for 3 days, the cultures were considered as phosphorus-starved cells. ~ 5 μmol l^{-1} of PO_4^{3-} was replenished into each treatment on day 11 (for *P. donghaiense*) or day 12 (for *P. minimum* and *P. micans*) just before sampling. Surprisingly, DIP in each media decrease sharply to lower than 0.5 μmol l^{-1} in the next day; another two times of 5 and 10 μmol l^{-1} of PO_4^{3-} were added separately into each treatment in the next 2 days after the measurement of DIP. Samples for analysis were taken each day at 12:00.

Measurement of Parameters

Cells were fixed in a 2% acid Lugol's solution and the densities were counted using a light microscope (Olympus BX61). The specific growth rates (μ) of each *Prorocentrum* species were calculated according to the equation $\mu = (\ln N_2 - \ln N_1)/(t_2 - t_1)$, where N_2 and N_1 were the cell densities at respective times, t_2 and t_1. For Chl *a* analysis, cells were filtered onto Whatman GF/F filters and were stored at − 20 °C. Chl *a* was extracted with 90% acetone in the dark for 24 h and were analyzed using a spectrofluorometer (Hitachi U-2810, USA) [45].

The seawater was filtered through pre-combusted (450 °C, 2 h) GF/F filters. The filtrate was used for the analysis of DIP with the method of Valderrama [46]. The filters were used for the analysis of particulate phosphorus (PP) according to Solórzano and Sharp [47].

Bulk APA was determined by fluorescence as the release of 4-Methylumbelliferone (MUF, Sigma) from 4-methylumbelliferyl phosphate (MUP, Sigma) according to Hoppe [48]. The seawater was first filtered through a 120-μm filter to remove zooplankton. The assay was run in the dark and at 30 °C for 1 h with the final substrate 4-MUP concentration of 25 μmol l^{-1}. The fluorescence was measured using the spectrofluorometer (Ex = 435 nm, Em = 520 nm). Sterile seawater was used as control blanks. AP in the 0.2-μm filtrate (filtered through 0.22 μm polycarbonate filters under < 100 mmHg pressure) was regarded as free AP. AP in unfractionated samples was regarded as total AP. Bulk APA was expressed as fmol of MUP released $cell^{-1}$ h^{-1}.

Single-cell APA of three *Prorocentrum* species was determined as in the field study. At least 200 cells of each species were counted in one sample. To analyze the location sites of AP, samples labeled with ELF were observed using a confocal epifluorescence microscope (Zeiss, LSM 510 META) to scan in ~ 0.50-μm section through cells.

Table 3 Enzyme labeled fluorescence (ELF) of the dominant species with concentrations of dissolved inorganic phosphorus (DIP) in the coastal waters of the East China Sea during a bloom of *P. donghaiense* in May 2010. The studied station numbers are shown in brackets. The observed cells are divided into three groups based on the intensity of fluorescence: absence of fluorescence; medium fluorescence, where ELF covered < 30% of the cell bodies; strong fluorescence, where ELF covered > 30% of the cell bodies. Percentages of ELF for a given species are calculated as the fraction of ELF labeled cells in one group divided by the total number of cells counted

DIP concentrations (μmol L^{-1})	Species	Cell counted	Percentages of ELF (%)		
			Strong	Medium	None
DIP ≥ 0.30 (3)	*P. donghaiense*	261	1	70	29
	P. minimum	34	9	56	35
	S. costatum	203	1	24	75
0.10 < DIP < 0.30 (11)	*P. donghaiense*	1078	8	66	26
	P. minimum	277	5	81	14
	P. micans	120	18	58	24
	S. costatum	693	6	8	86
DIP ≤ 0.10 (13)	*P. donghaiense*	1638	30	51	19
	P. minimum	471	4	76	20
	P. micans	196	23	48	29
	S. costatum	480	35	21	44

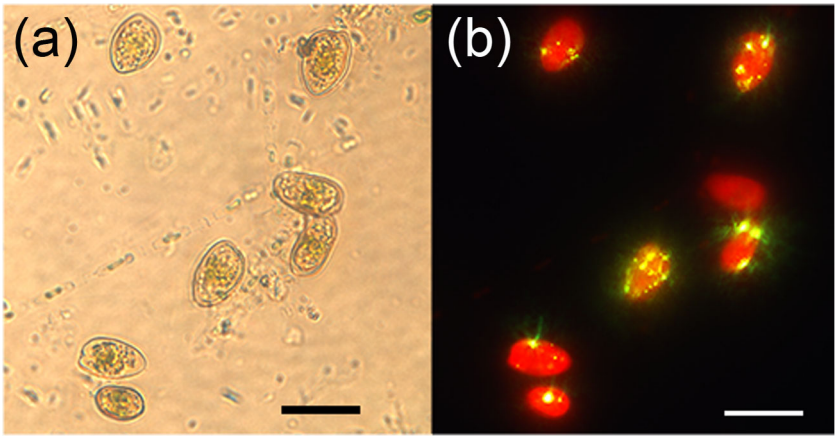

Fig. 2 The enzyme-labeled fluorescence (ELF) of *P. donghaiense* during the *P. donghaiense* bloom in the coastal waters of East China Sea. Samples are observed under **a** bright field and **b** a long-pass DAPI filter set. The bright green fluorescence is indicative of alkaline phosphatase activity. The bar = 10 μm

Study of AP Hydrolytic Kinetics of Three *Prorocentrum* Species in the Laboratory

Monocultures of three *Prorocentrum* species were transferred four times with the initial concentrations of PO_4^{3-} reducing gradually to 18, 9, 4, and 2 μmol l^{-1} at each inoculation. The P-starved cells were used for the study. Substrate MUP was added with the final concentrations of 0, 0.1, 0.2, 0.5, 2, 5, 10, 20, 50 μmol l^{-1} in triplicate. Sample fluorescence was measured in the dark and at 30 °C for 1 h. The hydrolytic kinetic constants (K_s and V_{max}) which followed the Michaelis-Menten equation were calculated using the non-linear least squares method.

Data Analysis

A one-way ANOVA with Tukey's test was performed to compare the differences among the treatments of each test parameter. A p value < 0.05 was regarded as significant. Prior to analysis, data were tested for normality and homogeneity of variance. A Log 10 or square-root

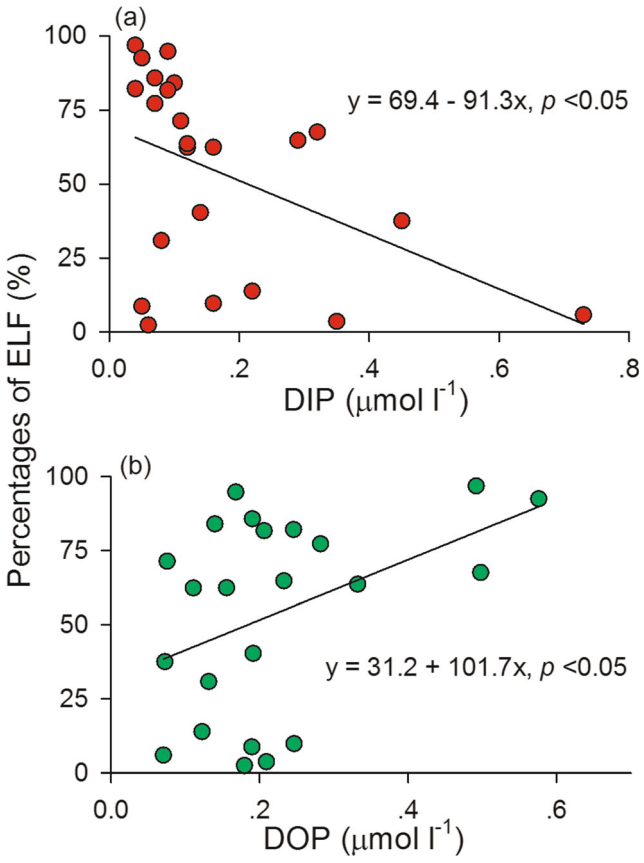

Fig. 3 The relationships between the percentages of enzyme-labeled fluorescence (ELF) of *P. donghaiense* cells and **a** dissolved inorganic phosphorus (DIP) concentrations and **b** dissolved organic phosphorus (DOP) concentrations

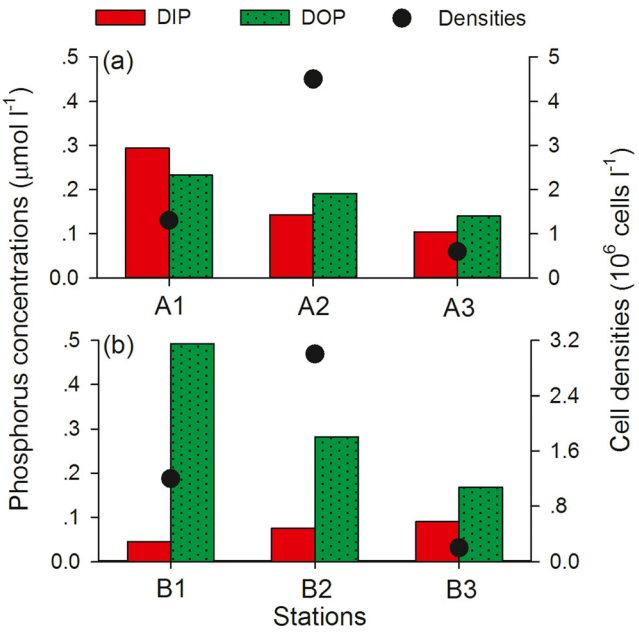

Fig. 4 Variations of dissolved inorganic phosphorus (DIP), dissolved organic phosphorus (DOP) concentrations, and densities of *P. donghaiense* along **a** transect A and **b** transect B

transformation of the data was performed prior to any statistical test when necessary. All tests were performed using the SPSS 19.0 software (SPSS Inc., USA).

Results

Nutrients and APA in the Coastal Waters of ECS

DIN, DON, DIP, and SiO_3^{2-} decreased gradually from inshore to offshore. DIN and DON varied between 0.93–28.54 and 2.97–54.81 μmol l^{-1}, with the averages of 12.31 ± 7.39 and 13.65 ± 9.76 μmol l^{-1}, respectively (Table 2). DIP changed from 0.04 to 0.73 μmol l^{-1}, with most stations in the southern part of the Changjiang River Estuary (latitude < 30°) lower than 0.10 μmol l^{-1}. DOP varied between 0.07–0.58 μmol l^{-1} and the high concentrations were observed close to the estuary. The high values of DIN/DIP and TDN/TDP suggested that most stations suffered P stress.

Phytoplankton in the studied stations were divided into three groups according to the DIP concentrations in situ. Measured cellular fluorescences showed that single-cell APA strengthened with the decrease of DIP, particularly at levels lower than 0.10 μmol l^{-1} (Table 3). When DIP was higher than 0.10 μmol l^{-1}, most *S. costatum* cells were not labeled with fluorescence whereas 65–86 % of *Prorocentrum* cells were labeled with medium or even strong fluorescence (Fig. 2). When DIP decreased to lower than 0.10 μmol l^{-1}, higher percentages of strong ELF were found in the sampled species, especially *S. costatum*. The percentages of ELF of *P. donghaiense* decreased with DIP but increased with DOP ($p < 0.05$ for both) (Fig. 3). No obvious difference was found in single-cell APA of three *Prorocentrum* species.

Transects A and B were in the central zone of *P. donghaiense* bloom. The densities of *P. donghaiense* in the nearshore stations were all higher than 10^6 cells l^{-1}, with the maximum of 4.5 × 10^6 cells l^{-1} in Stn A2 (Fig. 4). The morphology of *P. donghaiense*, abundant observed green fluorescent debris in ELF samples, together with decomposing odors in the air in situ, suggested that the bloom along transect B, in particular in Stns B1 and B2, was during the declining phase. DIP was all higher than 0.10 μmol l^{-1} and was similar with DOP along transect A. However, DIP decreased to lower than 0.10 μmol l^{-1} and DOP contributed most to TDP pool along transect B. Most *Prorocentrum* cells were labeled with strong or medium fluorescence whereas only 0–30% of *S. costatum* cells were labeled with fluorescence along transect A (Fig. 5). The percentages of strong and medium fluorescing cells increased greatly along transect B, especially in Stn B1. When compared among three *Prorocentrum* species, it seemed that the fluorescences of *P. donghaiense* and *P. micans* were stronger than that of *P. minimum*.

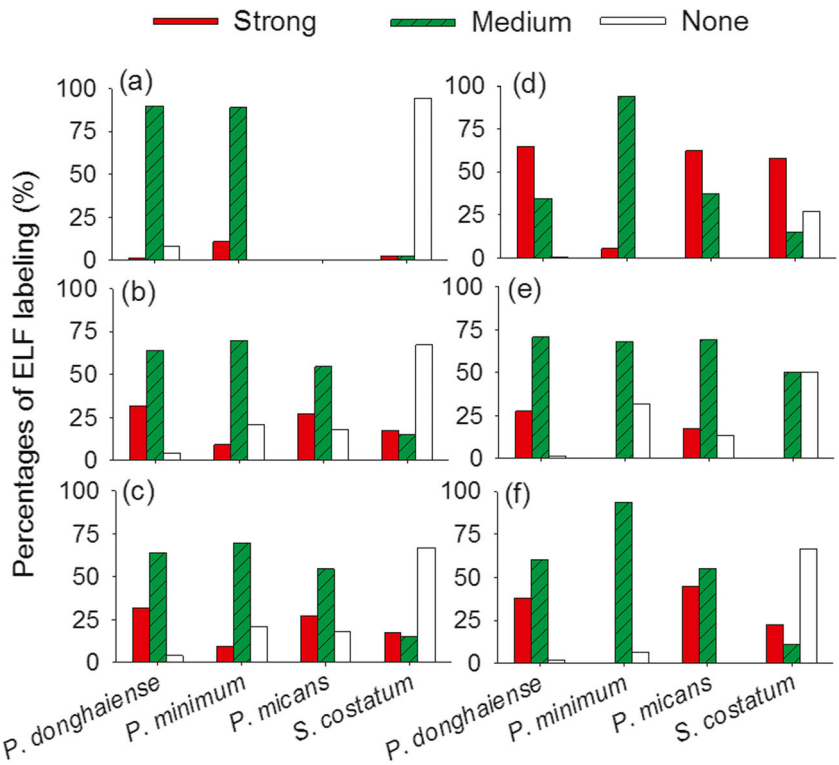

Fig. 5 Percentages of enzyme-labeled fluorescence (ELF) of *P. donghaiense*, *P. minimum*, and *P. micans* in stations **a** A1, **b** A2, **c** A3, **d** B1, **e** B2, and **f** B3

Physiological and Growth Response of three *Prorocentrum* to the Variation of Phosphorus in the Laboratory

Variations of DIP, PP, and Cell Densities

DIP in the treatment of *P. donghaiense* decreased quickly to ~0.2 μmol l^{-1} compared with that in *P. minimum* or *P. micans* (Fig. 6a). DIP decreased sharply to lower than 0.5 μmol l^{-1} in all treatments on the other day after the first two doses of 5 and 10 μmol l^{-1} of PO_4^{3-} were re-added separately. 3–6 μmol l^{-1} of DIP were observed in all treatments at the end of the study.

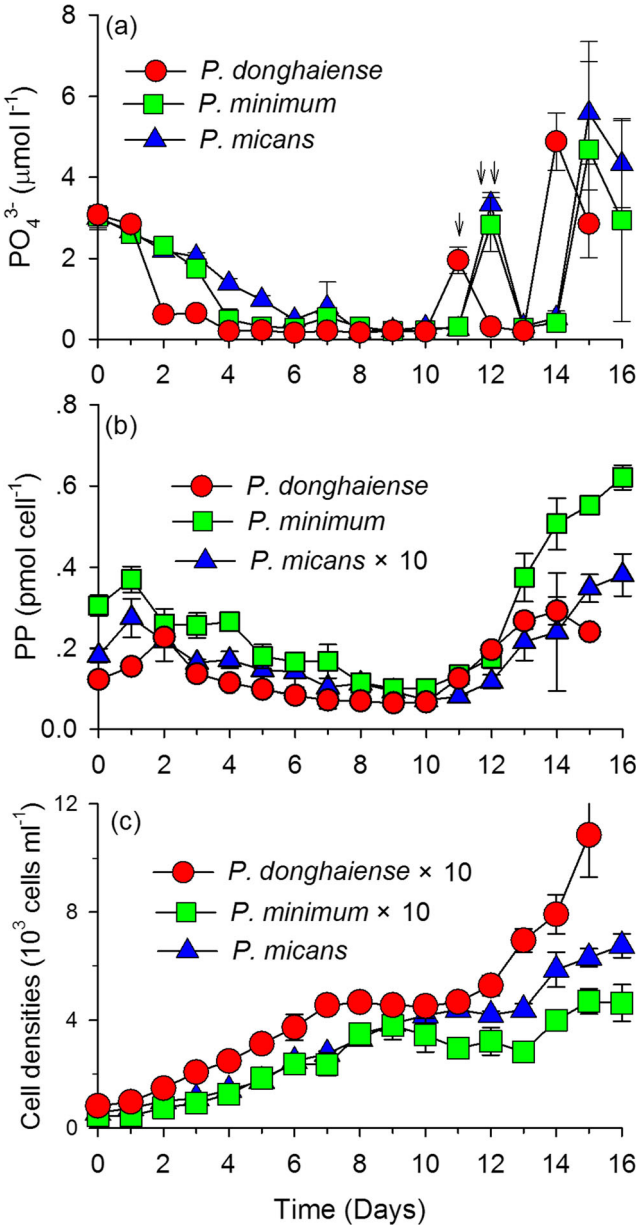

Fig. 6 Variations of **a** dissolved inorganic phosphorus (DIP) concentrations, **b** particulate phosphorus (PP) concentrations, and **c** cell numbers in the treatments of *P. donghaiense*, *P. minimum*, and *P. micans*. The arrows show the initial PO_4^{3-} replenishment in the media

PP decreased gradually after the first days (Fig. 6b). The lowest PP of *P. donghaiense*, *P. minimum*, and *P. micans* were 0.07, 0.10, and 0.72 pmol $cell^{-1}$ respectively. PP increased sharply when PO_4^{3-} were replenished in all treatments.

The cell densities of all three *Prorocentrum* species stopped increasing when PP became the lowest in all treatment (Fig. 6c). The maximum growth rate of *P. donghaiense*, *P. minimum*, and *P. micans* were 0.51, 0.38, and 0.35 d^{-1} respectively. All three *Prorocentrum* species continued to grow after PO_4^{3-} were replenished and *P. donghaiense* grew the fastest.

Variation of APA

The bulk APA in all treatments were undetectable at the beginning. APA of *P. minimum* and *P. micans* increased sharply after day 9 when PP was depleted and decreased gradually when PO_4^{3-} was replenished in the media (Fig. 7). The maximum APA of *P. minimum* and *P. micans* were 43.28 ± 6.44 and 485.84 ± 46.09 fmol $cell^{-1}$ h^{-1}, respectively. Variation of APA in *P. donghaiense* was quite different. The APA of *P. donghaiense* increased sharply when phosphate decreased to ~ 0.2 μmol l^{-1} and the maximum value of 3.40 ± 0.33 fmol $cell^{-1}$ h^{-1} was one to two orders of magnitude lower than those of *P. minimum* and *P. micans* ($p < 0.01$). Some AP of all three species was released into the media and the free AP showed similar variation trends with total AP. Free APA contributed 33–60% to total bulk APA at the peak.

Most sites with APA of *P. donghaiense* and *P. minimum* were located on or near cell surface whereas those of *P. micans* were located evenly inside the whole cell body (Fig. 8). The ELF was not observed for all three species on day 0 but became apparent with time (Fig. 9). Single-cell APA did not show an obvious immediate decrease after PO_4^{3-} were replenished in media. Higher percentages of medium and Strong ELF cells were found in *P. donghaiense* and *P. micans* compared with those in *P. minimum*. However, the percentages of ELF cells didn't increase with the bulk APA in all cultures.

AP Hydrolytic Kinetics of Three *Prorocentrum* Species in the Laboratory

AP hydrolytic kinetics of three *Prorocentrum* species followed the Michaelis-Menten equation (Fig. 10). The V_{max} of *P. donghaiense* (2.24 fmol $cell^{-1}$ h^{-1}) was much lower than those of *P. minimum* (17.21 fmol $cell^{-1}$ h^{-1}) and *P. micans* (738.83 fmol $cell^{-1}$ h^{-1}). The K_s of *P. donghaiense* (0.28 μmol l^{-1}) was comparable with that of *P. minimum* (0.35 μmol l^{-1}) but was much lower than that of *P. micans* (1.86 μmol l^{-1}).

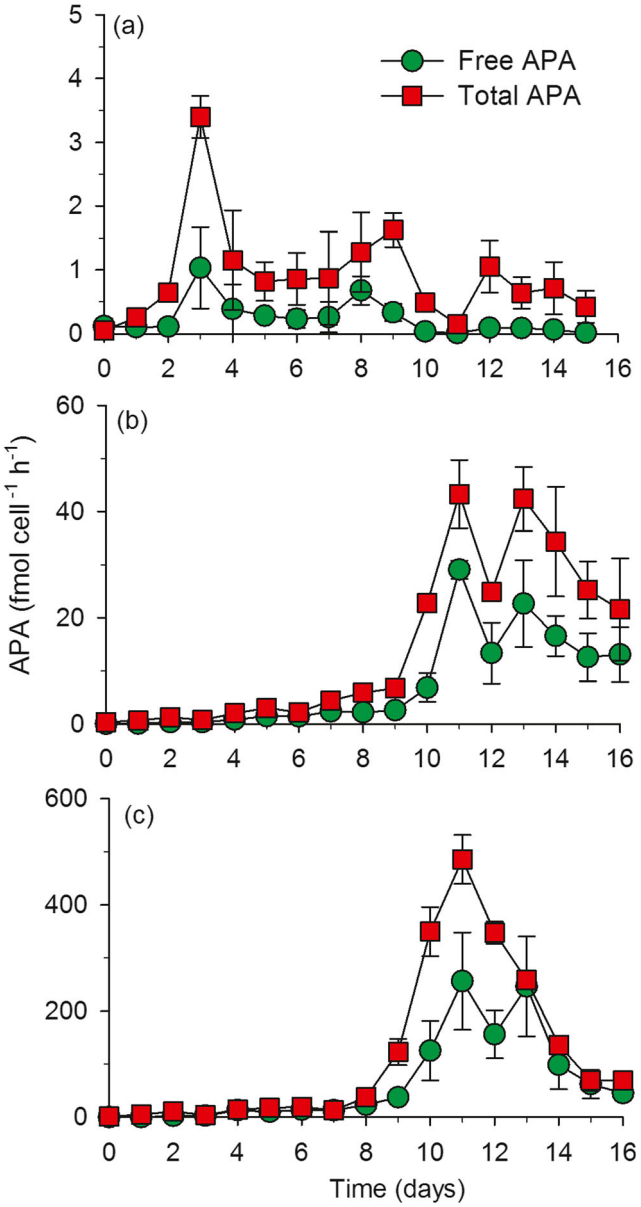

Fig. 7 Variation of free and total bulk alkaline phosphatase activities (APA) in the treatments of **a** *P. donghaiense*, **b** *P. minimum*, and **c** *P. micans*

Discussions

Phytoplankton in the coastal waters of ECS suffer from phosphorus stress or limitation, especially during spring and summer when phytoplankton bloom occurs [6, 7, 10]. During the *P. donghaiense* bloom in the spring of 2010, phosphate concentrations in more than half of the studied stations were lower than 0.10 μmol l^{-1} which is considered as the phosphorus threshold limit value of primary production [13, 35]. DOP concentrations were comparable or even higher than phosphate concentrations in this area. AP, which is one of the most important phosphatases, plays a prominent role in the regeneration of organic phosphorus compounds. The phosphorus status of *P. donghaiense* and the AP regulation by external phosphorus during the bloom were analyzed by the single-cell ELF assay. Because three *Prorocentrum* species coexisted in the water, it was possible to compare the different AP characteristics of phytoplankton that belonged to the same genus but different species in natural environments. Moreover, both the bulk and single-cell AP assays were used in the laboratory to further clarify the regulation mechanisms of AP by phosphorus in monocultures of three *Prorocentrum* species.

The observed three *Prorocentrum* species and *S. costatum* all expressed AP *in situ* which suggested phytoplankton suffered phosphorus stress during the bloom period. AP has been widely used as an indicator of phosphorus stress or limitation as studies have proven APA are highly correlated with external phosphate concentrations [17, 20, 49]. Our findings were similar in the field study. The phosphorus stress status of three *Prorocentrum* species strengthened with the decrease of phosphate. The AP intensity of *P. donghaiense* was inversely correlated with phosphate concentration. Furthermore, our results suggested the APA of *P. donghaiense* was positively correlated with DOP concentrations *in situ*. The existence of available DOP might induce phytoplankton to express more AP [50, 51]. In contrast, a negative correlation between APA and DOP were found by Bogé et al. [52, 53] and refractory organic phosphorus compounds interfering with phosphate for the

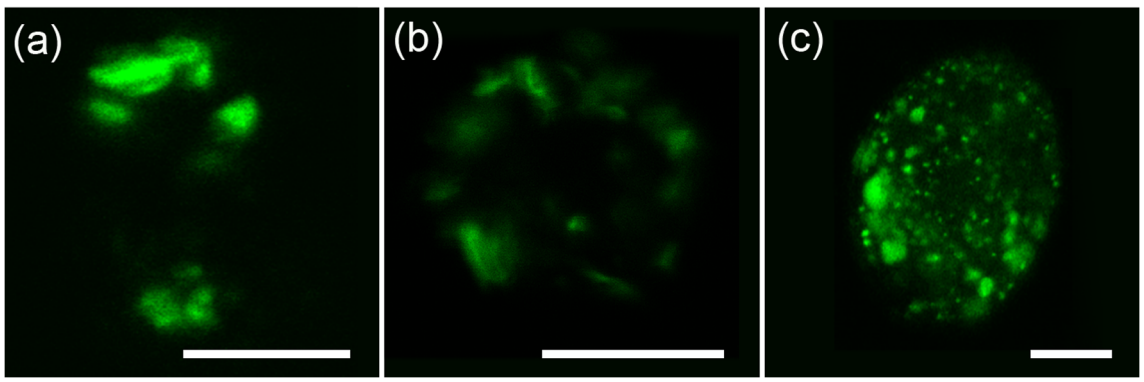

Fig. 8 Enzyme-labeled fluorescence of alkaline phosphatase activities in **a** *P. donghaiense*, **b** *P. minimum*, **c** and *P. micans*. The pictures are taken in the midsection of the fluorescent cell under a confocal fluorescence microscope. The bar = 10 μm

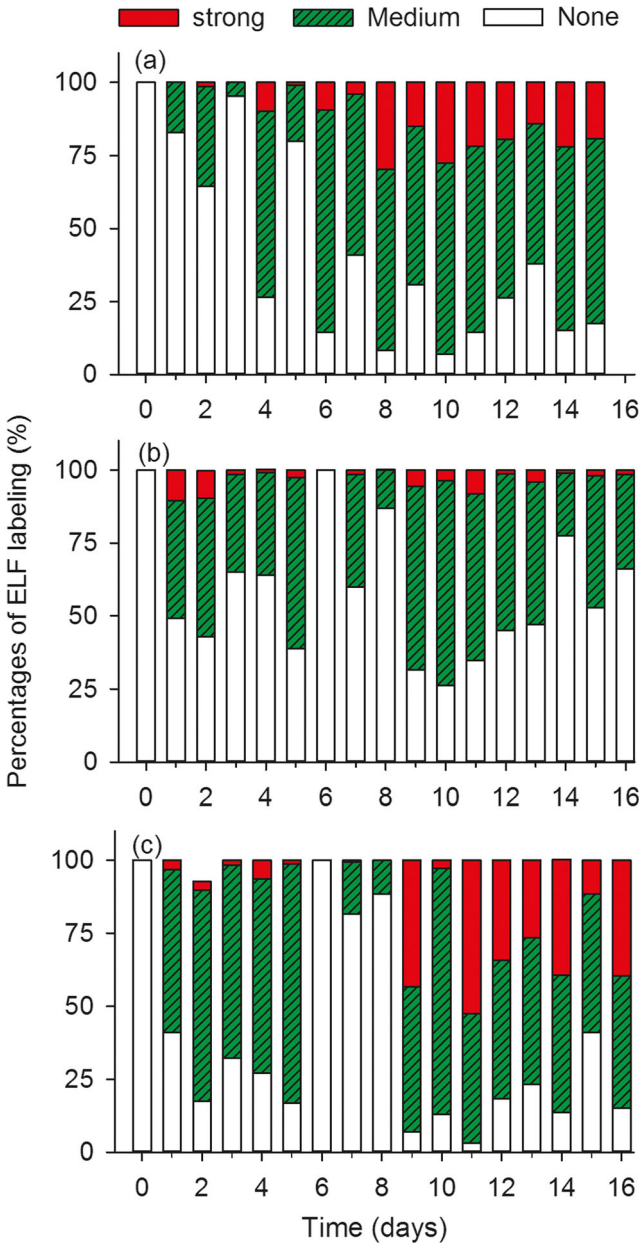

Fig. 9 Variations in the percentages of enzyme-labeled fluorescence (ELF) of **a** *P. donghaiense*, **b** *P. minimum*, **c** and *P. micans*. The observed cells are divided into three groups based on the intensity of fluorescence: absence of fluorescence; medium fluorescence, where ELF covered < 30% of the cell bodies; strong fluorescence, where ELF covered > 30% of the cell bodies. Percentages of ELF for a given species are calculated as the fraction of ELF labeled cells in one group divided by the total number of cells counted.

control of AP synthesis was suggested. Thus, we consider the bioavailability of DOP in seawater determines AP regulation. Following initial phosphate depletion, phytoplankton turn to utilize DOP, as the abundantly available DOP induce phytoplankton to upregulate AP expression. However, after the easily hydrolyzable organic phosphorus compounds have been utilized, the rest stable DOP might inhibit AP expression in return. Furthermore, phytoplankton APA is also regulated by the internal phosphorus pool [18, 54, 55]. Our laboratory results showed that it was not until cellular phosphorus pool was exhausted that APA of two *Prorocentrum* species rapidly increased by two to three orders of magnitude when external phosphate initially depleted. In summary, AP expression and its activities are determined by both the external available phosphorus and the internal phosphorus pool, and the internal phosphorus pool which determines the phosphorus status of phytoplankton might play a more important role.

Although AP expression might be constitutive in some species [19, 23], most agree that AP is an inducible enzyme and is upregulated when phosphate is lower than a threshold limit value [17, 18]. However, the phosphate threshold limit value we found was quite different from previous studies, varying from 0.05 to 1 μmol l^{-1} [50, 56–58]. Nausch [59] found an inverse correlation between APA and phosphate at the range of 0.2–1 μmol l^{-1} and an obvious increase in APA at phosphate concentrations lower than 0.2 μmol l^{-1}. Furthermore, certain amounts of APA were observed in nutrient-repleted conditions, thus some studies suggest AP is not a good indicator of phosphorus limitation [20, 32, 60]. The phosphate concentrations in our field studies varied from below the detection limit (0.02 μmol l^{-1}) to 0.73 μmol l^{-1}, mostly lower than 0.2 μmol l^{-1}. The studied species expressed low levels of APA when phosphate was higher than 0.2 μmol l^{-1}. The APA expression did not change much in the phosphate range of 0.1–0.2 μmol l^{-1}. However, the APA expression of the studied species increased significantly when phosphate was lower than 0.1 μmol l^{-1}. Our laboratory findings were consistent with those in the field study. Small amounts of APA were found in all three *Prorocentrum* species when external phosphate was in the range of 0.2~3 μmol l^{-1} and the APA increased significantly until cellular P pool was exhausted. Meseck et al. [32] observed ten species of phytoplankton express AP in response to high phosphate concentrations (10.1–16.4 μmol l^{-1}), especially *P. minimum*. Fuentes et al. [61] suggested that AP could be stimulated by stressors other than phosphate deficiency, such as silicon deficiency. The physiological status other than phosphorus stress might also induce phytoplankton to express AP. Thus, from our results, we still considered AP a good indicator of phosphorus limitation and the phosphorus threshold limit value is 0.10 μmol l^{-1} in situ. However, this indicator should be more cautiously used, in particular when only detecting low levels of APA.

Compared with diatoms, dinoflagellates are more sensitive to phosphorus stress and express AP in the same environment [50, 62, 63], as was observed in our field study. Even when phosphate was lower than 0.10 μmol l^{-1}, the diatom *S. costatum* ELF was less intense compared with the dinoflagellates represented by three *Prorocentrum* species. No obvious difference in the AP expression of three *Prorocentrum* species could be observed in the field with the single-cell ELF assay. However, with the combination of both bulk and

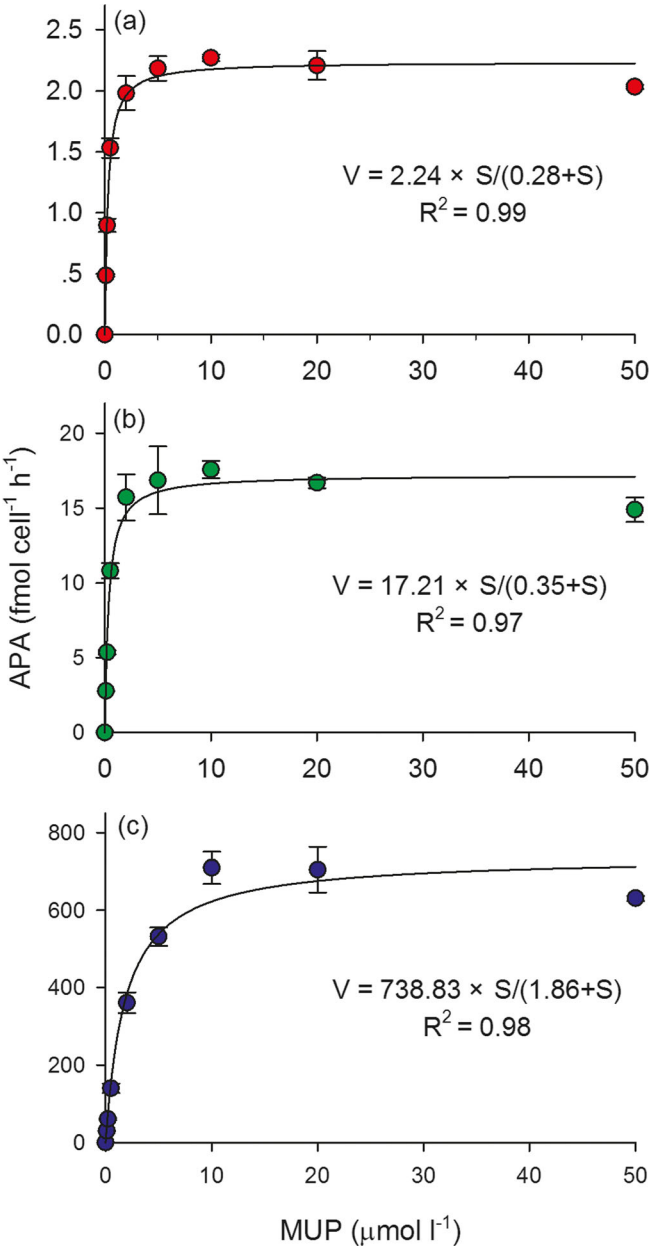

Fig. 10 Alkaline phosphatase activity (APA) of **a** *P. donghaiense*, **b** *P. minimum*, and **c** *P. micans* as a function of substrate addition. The artificial substrate used here is 4-methylumbelliferyl phosphate (MUP, Sigma)

single-cell ELF assays in the laboratory, the similarities and differences in AP characteristics of three *Prorocentrum* species were observed. All three *Prorocentrum* species were inducible, and increased with the decrease of phosphate. Considerable amounts of AP were released into the water as the soluble state. The repletion of phosphate could not completely inhibit APA in several days. Obvious differences existed in the AP of three *Prorocentrum* species. First of all, the *Prorocentrum* species differed in AP location sites. Most AP of *P. donghaiense* and *P. minimum* is localized on or close to the cell surface, which might be more efficient in the coupling effect of hydrolysis and uptake of DOP, as suggested by some studies [64, 65]. In contrast, most AP expression was intracellular in *P. micans*. It requires more energy and time to translocate the AP on the cell surface to directly interact with exteral orgainc phosphorus substrates [23]. Alternatively, the intracelular AP is an isoform that plays a distinct role, such as hydrolyzing the internal organic phosphorus [27, 30, 31]. Second, a higher AP affinity for DOP substrate was observed in *P. donghaiense* and *P. minimum* whereas a higher maximum hydrolysis rate for DOP in *P. micans* was observed. *P. donghaiense* and *P. minimum* seemed to be more efficient in hydrolyzing the relatively low amount of organic phosphorus compounds in natural seawaters. Finally, and most importantly, the APA expression levels and response time profiles are quite different among the three *Prorocentrum* species. APA of *P. donghaiense* rapidly increased to the peak value of 3.40 fmol cell^{-1} h^{-1} when phosphate was lower than 0.2 μmol l^{-1} in the early stage, and then decreased; APA reached the second peak value of 1.63 fmol cell^{-1} h^{-1} when PP was exhausted. As for *P. minimum* or *P. micans*, a sharp increase in APA was observed until the celluar phosphorus pool was exhausted and the maximum APA was two to three orders of magnitude higher than that of *P. donghaiense*. The maximum growth rates of *Prorocentrum* were 0.35~0.51 day^{-1} in this study. Suppose *Prorocentrum* cells could growth at a very high growth rate of 1 day^{-1}, the phosphorus requirement of *Prorocentrum* species in the unit of fmol cell^{-1} h^{-1} could be roughly calculated as the minimum PP (PP_{min}) plus a growth rate of 1 day^{-1} and then divided by 24 h. The AP of *P. donghaiense* is sufficient in hydrolyzing organic phosphorus compounds once they are available in seawater (Table 4). In contrast, the over-expression of AP in the other two *Prorocentrum* species, especially *P. micans*, might be a waste of energy. Young et al. [66] also observed an over-expression of APA in phytoplankton and they suggested this characteristic might not benefit the algae itself. In summary, the AP characteristic of *P. donghaiense* suggests that it benfits from its efficiency in utilizing DOP when compared with the other two *Prorocentrum* species.

Table 4 Comparison between phosphorus (P) requirement and the maximum alkaline phosphatase activities (APA_{max}) of three *Prorocentrum* species. Suppose cells of *Prorocentrum* could double a day that was a very high value, the P requirement of species is roughly calculated as the minimum particulate phosphorus (PP_{min}) plus a double time of 1 day^{-1} and then divided by 24 h. The PP_{min} data are observed in the laboratory study

Species	PP_{min} (pmol cell^{-1})	P requirement (fmol cell^{-1} h^{-1})	APA_{max} (fmol cell^{-1} h^{-1})
P. donghaiense	0.07	2.9	3.4
P. minimum	0.10	4.2	43.3
P. micans	0.72	30.0	485.8

Results from the bulk AP and single cell AP assays in the laboratory were compared. Considering that phytoplankton AP would be released into seawater as the soluble state that could not be measured by the single-cell ELF assay, as shown in our results, high bulk APA did not necessarily accompany with high ELF results at some time nodes. Cells with low ELF or without ELF might still suffer phosphorus stress or limitation. Furthermore, although three *Prorocentrum* species show significant differences in AP quantity, that could not be differentiated with the single-cell ELF assay no matter in the field or the laboratory study. The bulk AP assay failed to show the detailed information of phosphatase producers whereas the single-cell ELF assay was merely a qualitative estimate [26, 50]. Thus, the combination of the bulk and single-cell AP assays are strongly recommended to give an accurate analysis in future studies.

Conclusion

The species of *P. donghaiense*, *P. minimum*, and *P. micans* all suffered from P stress and expressed abundant AP, in particular when DIP was lower than 0.10 μmol l^{-1} during the *P. donghaiense* bloom. The APA expression was regulated by both external DIP and DOP, and internal phosphorus pool. Compared with *P. minimum* and *P. micans*, *P. donghaiense* showed some unique AP characteristics that might help it efficiently utilize organic phosphorus substrates and outcompete other concurrent species to outburst in the P-deficient ECS.

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