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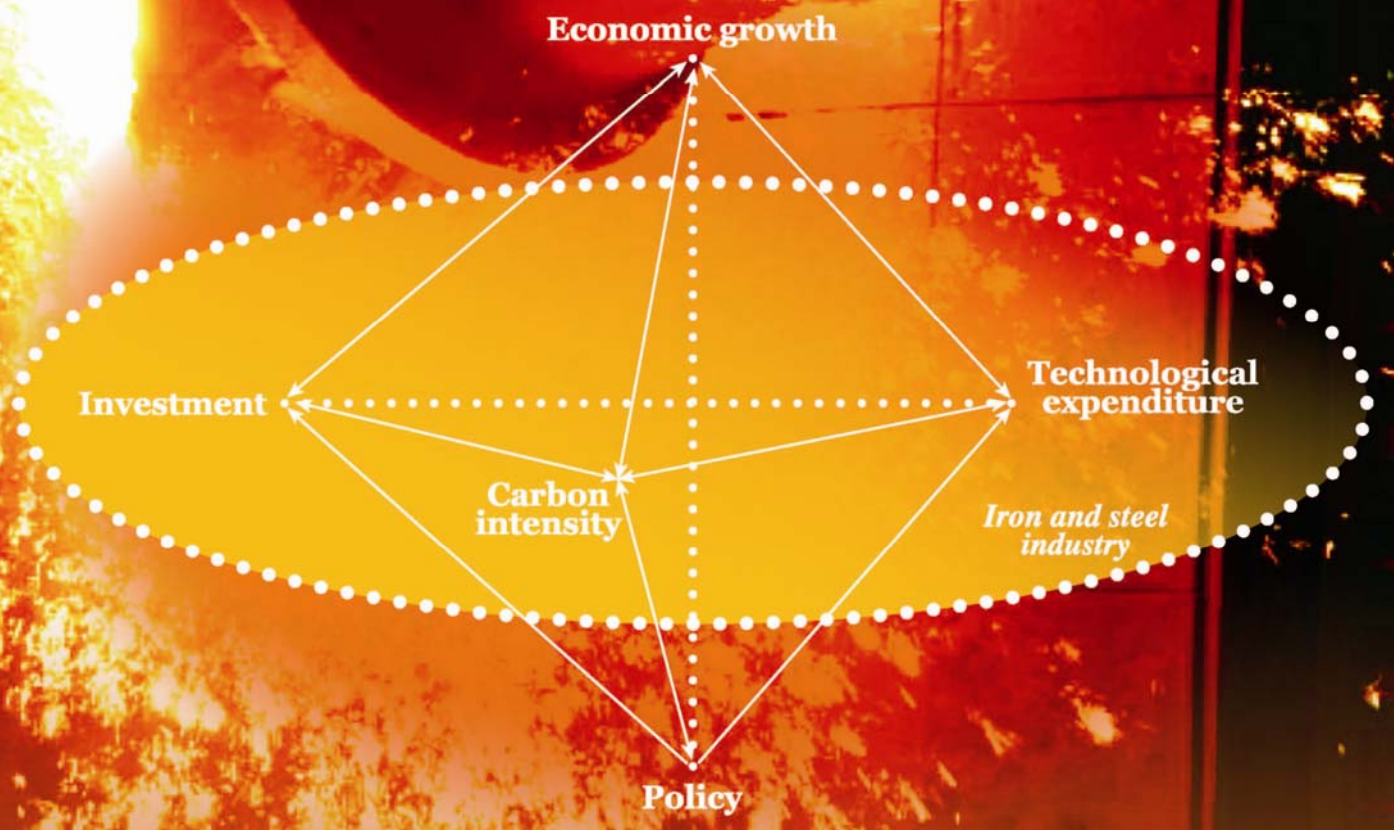

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Growth and alkaline phosphatase activity of *Chattonella marina* and *Heterosigma akashiwo* in response to phosphorus limitation

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ABSTRACT

The growth and alkaline phosphatase activity (APA) of two raphidophyceae species *Chattonella marina* and *Heterosigma akashiwo* were investigated in response to P-limitation and subsequent addition of dissolved inorganic phosphorus (DIP, NaH_2PO_4) and two dissolved organic phosphorus (DOP) compounds: guanosine 5-monophosphate (GMP) and triethyl phosphate (TEP). APA levels increased greatly after P-starvation as the decrease of the cellular phosphorus quotes (Q_P). *C. marina* responded to P-limitation quickly and strongly, with 10-fold increase in APA within 24 hr after P-starvation. The larger difference between maximal and minimal Q_P values in *C. marina* indicated its high capacity in P storage. APA of *H. akashiwo* was maximally enlarged about 2.5 times at 48 hr of P-starvation. After the addition of nutrients, cell numbers of *C. marina* increased in all treatments including the P-free culture, demonstrating the higher endurance of *C. marina* to P-limitation. However, those of *H. akashiwo* increased only in DIP and GMP cultures. APA increased only after the addition of the monophosphate ester GMP. The results suggest that quick responses of *C. marina* to P-limitation, high capacity in P storage as well as endurance for P-depletion provide this species an ecological advantage in phytoplankton community competition under DIP-limited conditions.

Introduction

Nitrogen (N) and phosphorus (P) are two essential elements for the growth of phytoplankton (Klug, 2006; Mallin et al., 1999). Traditionally, N is considered to be the limiting element in coastal marine habitats (Codispoti, 1989). As the increase in N loading, P limitation has become more evident in many sea areas. P deficiency has been reported for several open-ocean areas and coastal waters (Thingstad et al., 2005; Vidal et al., 2003). However, this determination of P limitation is based on the assumption of dissolved inorganic P (DIP) as the sole source for the growth of phytoplankton, and neglects to consider that dissolved organic P (DOP) may provide an alternative source of P to phytoplankton. Actually, DOP component can comprise a significant portion of the dissolved total P (DTP) pool in a variety of aquatic environments (Hoppe, 2003; Lomas et al., 2010).

Alkaline phosphatase (AP) is an important enzyme for DOP hydrolysis, commonly presented in eukaryotic marine algae (Dyhrman and Ruttenber, 2006). It is typically surface associated and hydrolyzes inorganic P from phosphor-monoester for assimilation by the cell (Yamaguchi and Adachi, 2010). AP activity (APA) is thought to be triggered by low inorganic P availability and has been used as an indicator of P status in a variety of phytoplankton communities (Dyhrman and Ruttenber, 2006; Hoppe, 2003; Lomas et al., 2004; Ou et al., 2010). Quite a few harmful algal bloom (HAB) taxa such as *Alexandrium* (Jauzein et al., 2010), *Karenia mikimotoi* (Huang et al., 2007), *Trichodesmium* (Orchard et al., 2010), and *Chattonella* (Wang et al., 2011; Yamaguchi et al., 2005, 2008) appear

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to have APA and have the competitive advantages in DIP-limited conditions in terms of DOP acquisition.

A lot of studies have shown the differences among the AP characteristics of phytoplankton species (Ou et al., 2008; Rengefors et al., 2003). Our previous study indicated that the abilities in DOP utilization and P-depletion tolerance varied among HAB taxa (Wang et al., 2011). The harmful raphidophyceae species *Chattonella marina* grew well under varieties of DOP compounds, and had high ability to sustain in P-free conditions (Wang et al., 2011). As a HAB species, the first record of *C. marina* bloom in China was in 1991 (Qi et al., 1994), and the blooms occurred again in the Yellow Sea in 1993 and 1995 (Jiao and Guo, 1996). Blooms of this species have occurred more frequently in sea areas along the Chinese coast since 2000 (Li et al., 2005; Wang et al., 2006a). The P strategy of this species may play important roles in its competition in phytoplankton community as the prevalence of its bloom coincides with P-limitation in Chinese coastal waters (Dong et al., 2010; Huang et al., 2007).

In this study, the growth and APA of *C. marina* and *Heterosigma akashiwo* were investigated in response to P-limitation and subsequent addition of DIP and DOP compounds. DTP, DIP concentrations and particulate phosphorus (PP) were measured simultaneously. The purpose of this study is to compare AP characteristics of the two taxa, evaluate the role of AP in DOP utilization, and thus better understand the potential role of the P strategy in phytoplankton competition.

1. Material and methods

1.1. Algal cultures

Chattonella marina (Subrahmanyan) Hara et Chihara and *H. akashiwo* (Hada) Hada were isolated from Daya Bay in 2003. The cultures were maintained in an autoclaved (121°C, 20 min) f/2 media (Guillard, 1973) at 20 ± 1°C, salinity 32, under 80 μmol photon/(m^2·sec) of cool-white fluorescent illumination with a dark:light cycle of 12:12 hr. The stock cultures were maintained by transferring aliquots of exponentially growing culture to new flasks containing fresh f/2 media. In order to eliminate bacterial contamination, antibiotics were added in stock cultures for the experiments.

1.2. Experimental design

The antibiotic-treated axenic algal cells were harvested from the stock culture at late log-phase by centrifugation (4000 ×*g*, 15°C, 10 min), and collected into a 2000 mL Erlenmeyer flask containing 1400 mL of N, P-free f/2 media. APA and PP were measured before P starvation. The collected cells were incubated in conditions as the stock cultures for 24 hr to let the cells recover from the rough treatment of centrifugation. Each of 100 mL above collected cells was distributed into twelve 2000 mL Erlenmeyer flasks with 1100 mL N, P-free f/2 media to make the final volume of 1200 mL. The flasks were incubated in conditions as the stock cultures for another 48 hr. Cell number, DTP and DIP, PP, and APA were measured every 24 hr.

Different forms of P compounds were added into the test flasks after P-starvation for 72 hr. Guanosine 5-monophosphate (GMP) and triethyl phosphate (TEP) were supplied as DOP sources as examples of the highly and lowly valuable DOP compounds, respectively, based on our previous study on DOP utilization of HAB species (Wang et al., 2011), and also the representatives of the monophosphate and non-monophosphate esters. A DIP culture (served by NaH_2PO_4) and P-free culture (P0) were modified. Each treatment was set in triplicate. $NaNO_3$ was served as N source in the experiment. The concentrations of N and P were 36 and 2.4 μmol/L, respectively, approximating maximum nutrient concentrations in Chinese coastal fish farm waters (Wang et al., 2006a). In order to reduce the background N and P concentrations, the media was made with artificial sea salt (Red Coral Sea, nutrient free formula) with salinity 31–32 and pH 7.9 ± 0.1. Cell number, DTP, DIP, PP and APA were assessed at 0.5, 3, 6, 12, 24, 48 and 96 hr after the addition of P.

1.3. Cell counting and growth rate

Cell counts were performed in a cell counting chamber, by placing 0.05–0.1 mL culture into the chamber fixed with a drop of Lugol's fixative, and observed under an inverted microscope (Leica DMIRB, Germany) at a magnification of 200×. Each sample was counted more than three times until differences in cell numbers were less than 10%. Specific growth rate (μ, day^{-1}) was calculated using the following equation:

$$\mu = (\ln N_1 - \ln N_0)/(t_1 - t_0)$$

where, N_0 (cells/mL) and N_1 (cells/mL) are cell density values at times t_0 (day) and t_1 (day).

1.4. Measurement of APA and other parameters

Fifty milliliter algal cultures were sampled periodically and filtered onto Whatman GF/F filters. Twenty-five milliliter filtrate was used for DIP determination, and another 25 mL for DTP measurement. Residues in the filters were for PP measurement. The DIP was determined using the molybdenum blue method described by Murphy and Riley (1962). DTP and PP were measured using the persulfate digestion method (Lampman et al., 2001; Wetzel and Likens, 1995). PP standardized by cell density was represented as the cellular P quota (Q_P).

APA was determined spectrophotometrically according to Wynne (1977) and Kruskopf and Plessis (2004), using *p*-nitrophenyl phosphate (*p*NPP) as substrate. Algal culture of 10 mL was collected on a 0.2 μm filter and the filters were stored frozen at −20°C until analysis. The filters with algal cells were placed in 3 mL of extraction buffer containing 0.05 mol/L Tris–HCl (pH 9), and immediately disrupted by sonication (Sonopuls Ultrasonic Homogenizer, Bandelin) for 30 min with a repeating duty cycle of 0.3 sec in an ice bath. The cellular homogenate was centrifuged at 35000 ×*g* for 10 min at 4°C, and the supernatant was used for enzyme assay. One hundred microliter *p*NPP (10 mmol/L) was added to 1.5 mL supernatant, and the reaction mixture incubated at 30°C for 2 hr. The reaction was stopped by adding 300 μL NaOH (1 mol/L), and the concentration of the product (*p*-nitrophenol, NP) was measured spectrophotometrically (410 nm) using a Shimadzu UV-2450 spectrophotometer (Japan). Buffer without sample was used as control. The enzyme activity is expressed as fmol of NP released at 1 hr per cells (fmol/(cells·hr)).

1.5. Data analysis

Mean and standard deviation (SD) values were calculated for each treatment from the three independent replicate cultures. Student's *t*-test was performed to compare the test groups with the relative controls, and significant difference from each other was observed using SAS for windows v8 software (SAS Institute lnc., USA).

2. Results

2.1. Growth of *C. marina* and *H. akashiwo*

Cell numbers of *C. marina* increased gradually in P-free culture (P0) (Fig. 1a). After adding the nutrients (36 μmol/L $NaNO_3$-N and 2.4 μmol/L different forms of P), cell numbers increased in all treatments. The maximum specific growth rates between 0.63–1.03 day^{-1} were obtained at the first 24 hr after adding the nutrients (96 hr of the experiment) (Table 1). The growth in DIP and GMP cultures was comparable, while growth in TEP was inferior to that in P0 culture.

H. akashiwo experienced a lag phase after N, P-starvation, and then cell density doubled at 72 hr after adding the nutrients. The growth varied among treatment cultures after nutrient addition (Fig. 1b), displayed by rapid increase in cell number in DIP culture, a 24 hr lag stage and then an increase in GMP culture. Cell numbers in P0 culture increased a bit and maintained low numbers during the experiment. The cell densities in TEP culture decreased and were significantly lower than those in P0 culture and the other treatments ($p < 0.01$), which indicated that *H. akashiwo* could not utilize TEP. The maximum growth rates were 0.51, 0.27, −0.05, 0.14 day^{-1} for DIP, GMP, TEP and P0 cultures, respectively (Table 1).

Table 1 – Maximum specific growth rate (μ, day^{-1}) of *C. marina* and *H. akashiwo* after the addition of different compounds of phosphorus. DIP: dissolved inorganic phosphorus, GMP: guanosine 5-monophosphate, TEP: triethyl phosphate, P0: P-free culture.

	DIP	GMP	TEP	P0
C. marina	0.82	1.03	0.63	0.64
H. akashiwo	0.51	0.27	−0.05	0.14

2.2. Changes in dissolved total phosphorus and dissolved inorganic phosphorus

The DTP concentrations decreased gradually from *ca.* 2 to 1.3–1.5 μmol/L after P-starvation (Fig. 2). DTP concentrations increased shortly after P additions, and decreased sharply within 12 hr (72–84 hr of the experiment) in DIP and GMP cultures of *C. marina*, and then maintained at about 1.5 μmol/L thereafter. However in these two cultures of *H. akashiwo*, DTP decreased all the time during the experiment. DTP was in high levels in TEP cultures, and decreased to a low level in P0 cultures for both species.

The changes in DIP were comparable with those in DTP during the P-starvation (Fig. 2). DIP concentration increased shortly after P addition in DIP cultures, while it reached the maximum in GMP cultures at 3 hr after P addition (75 hr of the experiment). DIP was maintained in low levels in TEP cultures.

2.3. Cellular phosphorus quota in *C. marina* and *H. akashiwo*

Cellular phosphorus quotas (Q_P) of *C. marina* decreased quickly from 134.7 pg P/cell before P-starvation to 43.1 pg P/cell at the 72 hr of P-starvation (Fig. 3). Q_P increased in all treatments within 12 hr of P addition (72–84 hr of the experiment), and then decreased with exposure time as the increase of cell number (Fig. 1) and the decrease of DTP and DIP concentrations (Fig. 2). A minimum Q_P value of 11.7 pg P/cells was obtained in TEP culture after 96 hr of P addition (168 hr of the experiment).

Cellular phosphorus quotas of *H. akashiwo* decreased as well after P-starvation, from 9.47 to 4.52 pg P/cell at the 72 hr of P-starvation (Fig. 3). No significant changes in Q_P were observed after P addition ($p > 0.05$). The quick increase of cell number in the late growth period in DIP and GMP cultures resulted in low Q_P, and a lowest value of 2.35 pg P/cells appeared at 96 hr of P addition in GMP culture. While in TEP and P0 cultures, Q_P values maintained low levels all the time.

2.4. Alkaline phosphatase activity in *C. marina* and *H. akashiwo*

APA of *C. marina* ranged from 4.76 to 46.4 fmol/(cell·hr). APA increased sharply at 24 hr after P-starvation, and decreased as

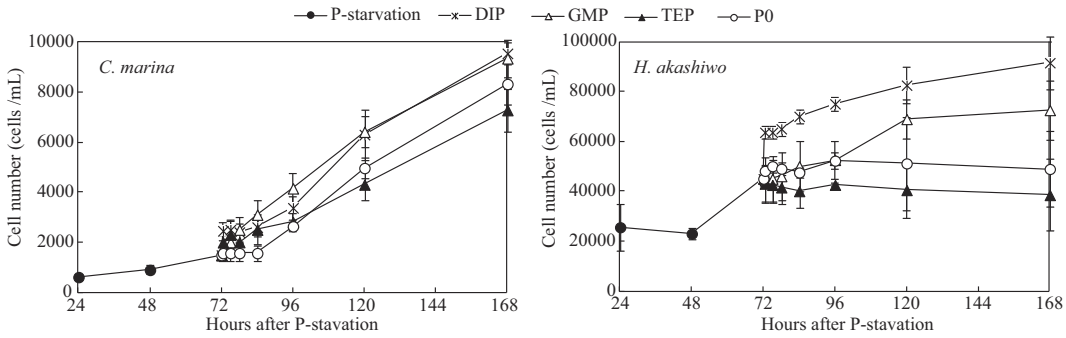

Fig. 1 – Growth of *C. marina* and *H. akashiwo* in different forms of phosphorus (P) compounds. P-starvation started at 0 hr, and different forms of P compounds were added at 72 hr after P-starvation. DIP: dissolved inorganic phosphorus, GMP: guanosine 5-monophosphate, TEP: triethyl phosphate, P0: P-free culture.

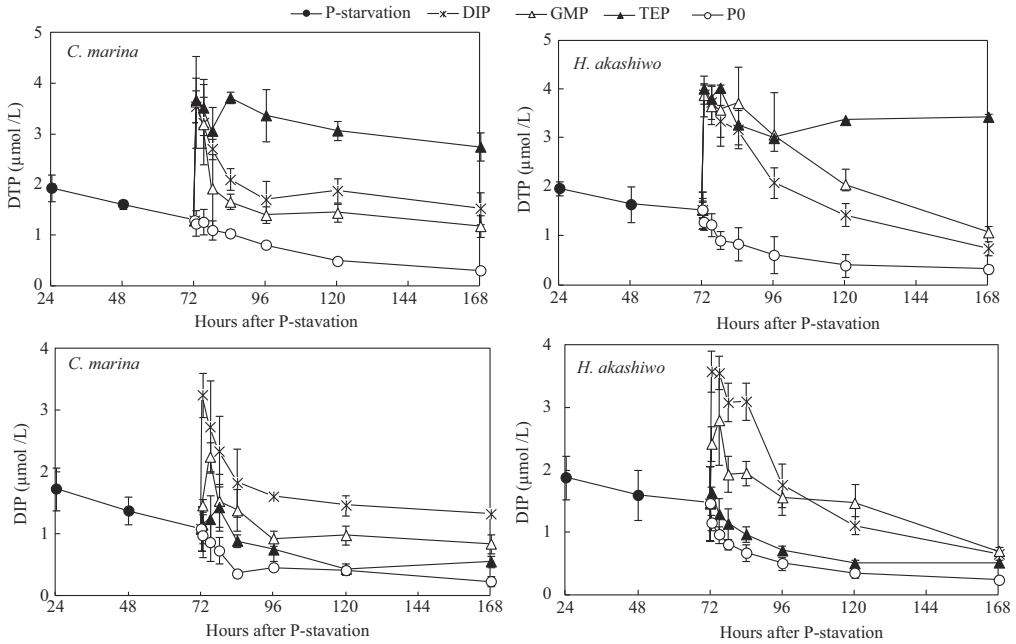

Fig. 2 – Concentrations of dissolved total phosphorus (DTP) and DIP in cultures of *C. marina* and *H. akashiwo* under different forms of P compounds. P-starvation started at 0 hr, and different forms of P compounds were added at 72 hr after P-starvation. DIP: dissolved inorganic phosphorus, GMP: guanosine 5-monophosphate, TEP: triethyl phosphate, P0: P-free culture.

the extension of P-starvation (Fig. 4). APA did not change significantly after P addition, with a trivial increase in GMP and P0 cultures and a little decrease in DIP culture. APA kept almost constant during 24 to 96 hr of P-addition (96–168 hr in the experiment).

The changes in APA of *H. akashiwo* to P-starvation were similar to those of *C. marina*, except for the delayed response. APA was between 1.45 and 2.86 fmol/(cell·hr), and was maximally enlarged about 2.5 times at 48 hr of P-starvation (Fig. 4). APA in GMP culture decreased at the first 12 hr after P addition (84 hr in the experiment) and recovered at 24 hr (96 hr in the experiment), and then decreased. APA decreased in all the other three cultures, and maintained the low levels after 24 hr of P-addition (96 hr in the experiment) though the activity in TEP culture increased at the late period of exposure.

3. Discussion

Cell numbers of *C. marina* kept on increasing slowly after nutrient-starvation, and increased in all treatments including P0 culture after nutrient-addition. The growth in P0 culture was stimulated by the addition of N in the culture. The results indicated that *C. marina* could endure P-limitation, which coincided with our previous study on nutrient utilization of *C. marina* (Wang et al., 2011). On the other hand, *H. akashiwo* grew well only at DIP and GMP cultures, suggesting its less sufferable P limitation than *C. marina*.

Several methods are applied in the measurement of APA in microalgal cells, including cell-specific enzyme-labeled fluorescence measurements (Litchman and Nguyen, 2008; Ou et al., 2010; Ranhofer et al., 2009), using 3-O-methylfluorescein

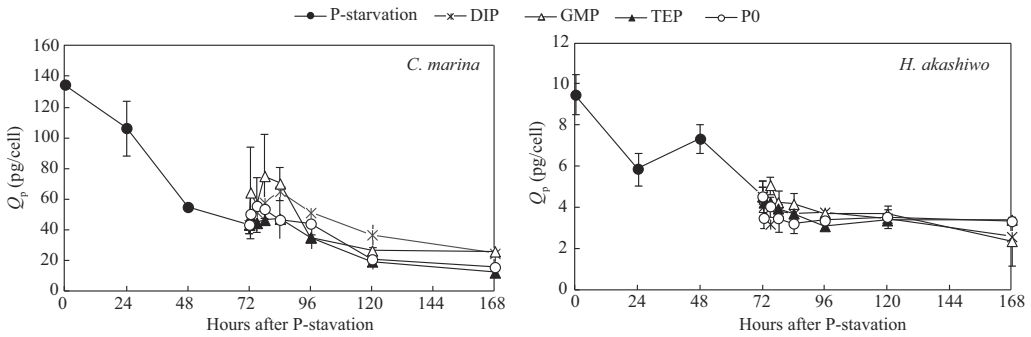

Fig. 3 – Cell phosphorus quotas (Q_P) in *C. marina* and *H. akashiwo* in response to different forms of P compounds. P-starvation started at 0 hr, and different forms of P compounds were added at 72 hr after P-starvation. DIP: dissolved inorganic phosphorus, GMP: guanosine 5-monophosphate, TEP: triethyl phosphate, P0: P-free culture.

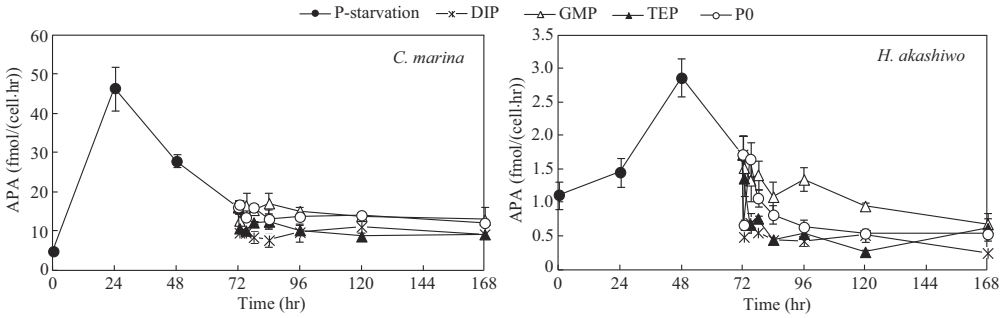

Fig. 4 – Alkaline phosphatase activity (APA) of *C. marina* and *H. akashiwo* in response to different forms of P compounds. P-starvation started at 0 hr, and different forms of P compounds were added at 72 hr after P-starvation. DIP: dissolved inorganic phosphorus, GMP: guanosine 5-monophosphate, TEP: triethyl phosphate, P0: P-free culture.

phosphate (MFP) (Ou et al., 2008), methylumbelliferyl phosphate (MUF-P) (Jauzein et al., 2010), *p*NPP (Kruskopf and Plessis, 2004), or disodium phenylphosphate (Oh et al., 2010) as substrate. APA varied greatly among microalgal species, such as 10–13.4 fmol/(cell·min) (600–804 fmol/(cell·hr)) for toxic dinoflagellate *Alexandrium catenella* using MUF-P as substrate (Jauzein et al., 2010), 1–3 fmol/(cell·hr) for *Prorocentrum donghaiense*, and maximum to 150 fmol/(cell·hr) for *Alexandrium catenella*, less than 2 fmol/(cell·hr) for the diatom *Skeletonema costatum* using MFP as substrate (Ou et al., 2008), and 0.02–0.7 pmol/(cell·hr) (20–700 fmol/(cell·hr)) for *Gymnodinium impudicum* using disodium phenylphosphate as substrate (Oh et al., 2010). The APA levels of 4.76–46.4 fmol/(cell·hr) for *C. marina* in this study were far less than those reported for dinoflagellates species *A. catenella* (Jauzein et al., 2010; Ou et al., 2008) and *G. impudicum* (Oh et al., 2010), however much high than those reported for *P. donghaiense* and *S. costatum* (Ou et al., 2008). APA levels for *H. akashiwo* were comparable to those of *P. donghaiense* and *S. costatum* (Ou et al., 2008).

APA is inducible by low extracellular DIP concentrations for many marine phytoplankton species (Ivancic et al., 2009; Jauzein et al., 2010; Oh et al., 2010), and increased with decreasing DIP concentrations in both natural sea waters and batch cultures (Duhamel et al., 2010; Dyhrman and Ruttenber, 2006; Hoppe, 2003; Huang et al., 2007; Ou et al., 2010). However the threshold DIP concentrations for AP induction varied greatly among species, *e.g.*, 0.83 μmol/L for *G. impudicum* (Oh et al., 2010); 0.4–1 μmol/L for *A. catenella* (Jauzein et al., 2010), 3.3 μmol/L for *Gymnodinium catenatum* (Oh et al., 2002), 1 μmol/L for the natural phytoplankton community in the southern Baltic Sea (Nausch, 1998), and 0.1 μmol/L in the transition zone of the North-western African upwelling system (Sebastián et al., 2004). Meanwhile the intracellular P pool and the internal N:P ratio have often been reported as the potential controlling factor that may regulate AP synthesis (Hoppe, 2003; Lomas et al., 2004; Vidal et al., 2003). In this study, APA of both species increased quickly as the decline of DIP levels. The DIP concentrations were about 2 μmol/L at the maximum enzyme activity. Meanwhile, the synthesis of AP by *C. marina* and *H. akashiwo* appears to be induced and maximized as soon as P limitation sets in. The responses of *C. marina* to P limitation were quicker and stronger, and APA increased about 10-folds within 24 hr after P-starvation.

After P addition, APA increased only in the monophosphate ester GMP cultures, and decreased or showed no significant differences in other treatments. The results suggest that enzyme is required for the utilization of monophosphate substrates such as GMP, in agreement with that AP is one of the most common and important enzymes for phosphate monoester utilization (Yamaguchi and Adachi, 2010). Furthermore the production of AP was not completely inhibited but only decreased after the relief of P-limitation (DIP culture) as the same with the results conducted by Štrojsova et al. (2008).

The range of Q_P values gives an indication of potential P-storage capacities of algal cells as the ratio between the maximum and the minimum quotas (Droop, 1974). Large ranges of minimal and maximal Q_P values have been reported for marine phytoplankton species (Jauzein et al., 2010; Ou et al., 2008; Sakshaug et al., 1984), and suggest different P requirements and P-storage capacities between species (Jauzein et al., 2010). In this study, the minimal (11.7 pg P/cell) and maximal (134.7 pg P/cell) Q_P values were recorded for *C. marina* compared to 2.35 and 9.47 pg P/cell for *H. akashiwo*, suggesting high P-storage capacity of *C. marina*. Previous studies also showed high P-storage in *Chattonella* species (Kimura et al., 1999). The result explained why the cell number of *C. marina* kept increasing in P-free culture in this study and our previous studies (Wang et al., 2011). Another possibility to overcome P limitation could be the ability of algal cells to lower their physiological P demand (Bertilsson et al., 2003; Geider and La Roche, 2002; Krauk et al., 2006). In regions of oligotrophic oceans where DIP is scarce, phytoplankton reduces their cellular P requirements by substituting phospholipids with non-phosphorus membrane lipids (Van Mooy et al., 2009).

Chattonella marina is a HAB species whose massive presence in Chinese sea waters has been documented since the 1990s, with recurrent blooms in some coastal areas (Jiao and Guo, 1996; Li et al., 2005; Qi et al., 1994; Wang et al., 2006a), pointing to the possibility that these massive proliferations have been favored by some changes in environmental conditions. The high occurrence of *C. marina* bloom in the Chinese coastal waters followed a long-term decrease in DIP concentrations and increase of N:P ratios (Wang et al., 2006a). For example, DIP concentrations in Daya Bay, a sea area with frequent *Chattonella* blooms, were in an average of 30–40 μg/L at the end of 1980s, and decreased to about 10 μg/L averagely since middle 1990s (Wang et al., 2006b). However, dissolved inorganic nitrogen (DIN) concentrations increased about four to five-folds during the same time period and resulted in about

40-fold increase of N:P ratio (Wang et al., 2006b). DOP contributes significantly to DTP in coastal waters (Sebastián et al., 2004), such as 70%–95% in Daya Bay, a frequent *C. marina* bloom sea area (Sun et al., 2002). Therefore, the dominance of *C. marina* may be explained by its competitive capacity for P-storage and by its ability to use DOP resources. DIP limitation and rich DOP compounds in the Chinese coastal waters may have led to the outbreaks of its blooms in recent years.

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