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Utilization of organic phosphorus and production of alkaline phosphatase by the marine phytoplankton, *Heterocapsa circularisquama*, *Fibrocapsa japonica* and *Chaetoceros ceratosporum*

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Abstract: Production of alkaline phosphatase (AP) and organic phosphorus utilization of *Heterocapsa circularisquama*, *Fibrocapsa japonica* and *Chaetoceros ceratosporum* were examined to evaluate the contribution of organic phosphorus to the outbreaks of red tides in coastal environments. *H. circularisquama* and *C. ceratosporum* were able to use inorganic phosphate, monophosphate monoester compounds, adenosine di- (ADP) and tri-phosphate (ATP) as a sole phosphorus source. AP activity of *C. ceratosporum* appeared to be induced when orthophosphate concentration decreased to less than 0.41 μM, and its maximum AP activity was 1.04 fmol cell^{-1} min^{-1}. *H. circularisquama* also produced AP under the phosphate-starved condition but it required the presence of organic phosphorus. Its maximum AP activity (3.44 fmol cell^{-1} min^{-1}) was potentially sufficient to maintain saturated growth rate under the phosphorus-limited condition. In contrast, *F. japonica* was able to use inorganic phosphate, ADP and ATP, but not monophosphate monoester compounds as a sole phosphorus source and its AP production was not found even under the phosphate-starved condition. The present results indicate that AP production for organic phosphorus utilization of marine phytoplankton is different between phytoplankton species. We concluded that organic phosphorus can contribute to not only the growth of red tide phytoplankton but also the competitive interaction between phytoplankton species in coastal environments.

Key words: *Heterocapsa circularisquama*, *Chaetoceros ceratosporum*, *Fibrocapsa japonica*, organic phosphorus, alkaline phosphatase, phosphate monoester, orthophosphate

Introduction

Red tides have caused mass mortality of cultured and wild fish in eutrophic coastal environments and have been a serious problem around the world (Hallegraeff 1993). Effects of some environmental factors, such as temperature, salinity (Nakamura & Watanabe 1983; Yamaguchi & Honjo 1989; Yamaguchi et al. 1991; Yamaguchi et al. 1997), nutrients (Watanabe et al. 1982; Nakamura 1985; Matsuda et al. 1999; Yamaguchi & Itakura 1999; Yamaguchi et al. 2001; Anderson et al. 2002), vitamins and trace metals (Nishijima 1985; Nishijima & Hata 1989; Imai et al. 1996) on the growth of red tide phytoplankton, have been studied to elucidate the mechanism of the outbreaks of red tides.

Phosphorus (P), especially dissolved inorganic phosphate (orthophosphate: PO_4^{3-}), is generally known to be one of the major nutrients controlling the occurrence of red tides. In coastal waters, however, the existence not only of inorganic phosphate but also of organic phosphorus has been reported (Miyata & Hattori 1986; Suzumura et al. 1998;

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Monaghan & Ruttenberg 1999). The concentration of dissolved organic phosphorus in surface coastal waters often exceeds that of dissolved inorganic phosphate, sometimes by an order of magnitude (Kobori & Taga 1979; Hernández et al. 2000; Yamamoto et al. 2002; Yamaguchi et al. 2004b).

Furthermore, culture experiments have shown that some of red tide phytoplanktons produce alkaline phosphatase (AP) (Kuenzler & Perras 1965; Rivkin & Swift 1980; Cembella et al. 1984a; Uchida 1992; Riegman et al. 2000; Yamaguchi et al. 2004a). AP cleaves the phosphate moiety from the alkaline phosphatase-hydrolyzable phosphorus (APHP) which consists of phosphate monoester compounds such as sugar phosphates, nucleotide phosphates, and phospholipids (e.g., glycerophosphate) (Price & Morel 1990). Once released from phosphate monoesters, free orthophosphate is taken up by the phytoplankton. Our previous report indicated that some AP-producing red tide phytoplanktons have a sufficient potential AP activity to maintain saturated growth rate under the phosphorus-limited condition (Yamaguchi et al. 2004a).

Recently, distribution and seasonal variation of AP activity in coastal water have been investigated. Most of AP activities in coastal surface waters consist of planktonic AP (Yamaguchi et al. 2004b). Moreover, APHP was widely distributed in coastal environments and represents a significant part of the algal-available phosphorus in the spring–autumn periods (Taga & Kobori 1978; Kobori & Taga 1979; Hernández et al. 2000; Yamaguchi et al. 2004b). Thus, APHP plays an important role as a phosphorus source for AP-producing phytoplankton in coastal environments.

Production and its mechanisms of AP are known to differ between phytoplankton species. This enzyme generally appears to be induced via *de novo* synthesis when the orthophosphate concentration in the water column decreases below a certain threshold level (Cembella et al. 1984a). The threshold concentration of red tide phytoplanktons typically ranges from 0.2 to 0.4 μM (Dyhrman & Palenik 1999; Yamaguchi et al. 2004a). In contrast, that of *Gymnodinium catenatum* Graham is relatively higher (3.3 μM) than the above concentrations (Oh et al. 2002). *Heterosigma akashiwo* (Hada) Hada has no ability to produce AP even under various phosphate-starved conditions (Yamaguchi et al. 2004a). Therefore, AP production reflects differences in utilization efficiency of organic phosphorus by phytoplankton, and the production probably contributes to a process of the competition between phytoplankton species.

The purpose of present study was to evaluate AP production for organic phosphorus utilization of different phytoplankton species. We determined AP production and organic phosphorus utilization of three phytoplankton species, *Heterocapsa circularisquama* Horiguchi (Dinophyceae) *Fibrocapsa japonica* Toriumi et Takano (Raphidophycea) and *Chaetoceros ceratosporum* Ostenfeld (Bacillariophyceae). *H. circularisquama* and *F. japonica* are known as red tide phytoplankton (Horiguchi 1995; Kooistra et al. 2001; Fukuyo et al. 2004). Especially, *H. circularisquama* is known as one of the harmful red tide phytoplankton species which poses a serious economic impact to aquaculture in Japan (Yamaguchi et al. 2001). *C. ceratosporum* is a non-red tide species that is extensively used as a food organism in larval rearing (Tanaka 1982; Fukami et al. 1992). We also examined the characteristics of AP production in detail for the species which produce AP.

Materials and Methods

Algal culture

Clonal culture of *Heterocapsa circularisquama* HCHS-95 was obtained from Dr. Haruyoshi Takayama (Hiroshima Fisheries Experimental Station). The culture was washed repeatedly using the micropipette isolation method. No bacterial cells stained with DAPI were found in the medium and algal cytoplasm. However, *H. circularisquama* cells are generally known to contain small bacteria cells in the cytoplasm and around the algal nucleus (Horiguchi 1995; Maki & Imai 2001; Maki et al. 2004). The culture used in present study is not axenic in the strict sense. Clonal and axenic culture of *Chaetoceros ceratosporum* and *Fibrocapsa japonica* NIES-605 were obtained from Japan Marine Science and Technology Center (Japan Agency for Marine-Earth Science and Technology) and National Institute for Environmental Studies in Japan, respectively.

Stock culture of *H. circularisquama* was grown at 25°C under a 12 hr light : 12 hr dark photocycle. *C. ceratosporum* and *F. japonica* were grown at 20°C under a 14 hr light : 10 hr dark photocycle. Light was provided by cool-white fluorescent illumination (120 μmol photons m^{-2} s^{-1}). Cultures of *H. circularisquama* and *F. japonica* were maintained in modified SWM-III medium based on natural seawater (Imai et al. 1996). Background inorganic phosphate concentration in the natural seawater was consistently less than 0.02 μM. Culture of *C. ceratosporum* was maintained in ASP_2-NTA medium (Provasoli et al. 1957) which contains 10% natural seawater.

Utilization of phosphorus compounds

Stock cultures (*ca.* 1–5 mL) were inoculated into 300 mL flask containing 100 mL phosphorus-deficient medium (no addition of phosphorus sources) and were pre-incubated for 8–14 days under the same conditions for maintaining stock cultures. Utilization of phosphorus compounds by the phytoplankton was examined using 4 inorganic phosphate and 11 organic phosphate compounds shown in Table 1. These phosphorus compounds were added to the autoclaved phosphorus-deficient media as a sole phosphorus source in PP capped test tubes (13×92 mm) used for *C. ceratosporum* or 300 mL flasks used for *H. circularisquama* and *F. japonica* after filter sterilization (0.22 μm, Millipore). The concentration of a phosphorus compound was adjusted to 50 μM for SWM-III or 30 μM for ASP_2-NTA. Phosphorus-starved cells were inoculated into triplicate PP capped test tubes or

Table 1. The phosphorus compounds used in the experiment.

Group	Symbol	Chemical name
Inorganic phosphate		
	PO4	Orthophosphate
	PP	Pyrophosphate
	TPP	Tripolyphosphate
	MP	Metaphosphate
Phosphate monoester		
Monophosphate monoester	GMP	Guanosine-5′-monophosphate
	CMP	Cytidine-3′-monophosphate
	UMP	Uridine-5′-monophosphate
	AMP	Adenosine-5′-monophosphate
	G1P	α-D-Glucose 1-phosphate
	G6P	D-Glucose 6-phosphate
	F6P	D-Fructose 6-phosphate
	NPP	*p*-Nitrophenylphosphate
	GYP	β-Glycerophosphate
Diphosphate monoester	ADP	Adenosine-5′-diphosphate
Triphosphate monoester	ATP	Adenosine-5′-triphosphate

the flasks containing the phosphorus sources to be examined. Cultures were incubated under the same conditions as for the pre-incubation. Initial cell number of *H. circularisquama*, *C. ceratosporum* and *F. japonica* was 100 cells mL^{-1}, 5000–7500 cells mL^{-1} and 50–100 cells mL^{-1}, respectively. Maximum cell yield was determined by *in vivo* chlorophyll *a* fluorescence with Turner Designs 10-AU Fluorometer (Sunnyvale CA, USA).

Alkaline phosphatase production

Stock cultures of *H. circularisquama*, *F. japonica* and *C. ceratosporum* were inoculated into 300 mL flasks each containing 100 mL of phosphate-replete media and were pre-incubated for 10–14 days under the same conditions for maintaining cultures. The orthophosphate concentration in phosphate-replete media was adjusted to 50 μM for SWM-III or 60 μM for ASP_2-NTA. After the pre-incubation, the cell suspensions were inoculated into two 1 L flasks containing 660 mL phosphate-replete or phosphorus-deficient media individually. Initial cell number of *H. circularisquama*, *F. japonica* and *C. ceratosporum* was 10000 cells mL^{-1}, 200 cells mL^{-1} and 10000 cells mL^{-1}, respectively. The flasks were incubated under the same condition as for the pre-incubation. A portion (*ca.* 30–50 mL) of the cultures was taken at 2 day intervals for *H. circularisquama* or 3 day intervals for the others, and was filtrated through a nuclepore filter (0.8 μm, Whatman) without pressure. Cell density, AP activities in cell suspension and filtrate, and orthophosphate concentration in filtrate were determined. Orthophosphate concentration in the medium was analyzed by the molybdenum-blue method of Strickland & Parsons (1972) using auto-analyzer TRAACS-800 (BRAN+LUEBBE).

AP activities of *H. circularisquama* and *F. japonica* were not found in the above experiment. Thus, AP production of these phytoplankton species was re-analyzed in detail. Cell suspensions of stock culture were inoculated into 1 L flask containing 600 mL of phosphorus-deficient media and were pre-incubated for 15 days until a phosphorus-starved state was achieved. Initial cell number of *H. circularisquama* and *F. japonica* was 10000 cells mL^{-1} and 500 cells mL^{-1}, respectively. During the period of pre-incubation of *H. circularisquama* and *F. japonica*, the time-course of cell density, AP activity in cell suspension, and orthophosphate concentration in filtrate were determined. After the pre-incubation, 300 mL of the cell suspensions were removed and a 100 mL suspension was individually transferred into each of three 300 mL flasks. Orthophosphate, β-glycerophosphate (GYP) and milli-Q water were added to the each flask after filter-sterilization (0.22 μm Millipore). The phosphorus concentration of phosphate-replete (orthophosphate), glycerophosphate-replete (GYP), and phosphorus-deficient (milli-Q) medium was adjusted to 50 μM, 50 μM, and less than 0.02 μM, respectively. Time-course of cell density and AP activity in the each culture of *H. circularisquama* or *F. japonica* were monitored.

Assay of alkaline phosphatase activity

AP activity was measured based on an increase in fluorescence intensity of 4-methylumbelliferone (MUF). After being hydrolyzed by AP, nonfluorescent 4-methylumbelliferyl phosphate (MUF-P) produces the highly fluorescent molecule MUF (Hoppe 1983; Chróst 1991; Hoppe 1993).

MUF-P was dissolved in 2-methoxyethanol (20 mM) and was stored below −5°C. Prior to addition to the sample, the stock MUF-P solution was diluted with 2-methoxyethanol to yield a range from 10 μM to 10 mM. Working substrate-solutions were prepared adding 1.0 M Tris buffer (pH 10.7 at 25°C) at a ratio of 1 : 5. Twelve μL of the working substrate-solution and 188 μL of cell suspension or filtrate sample were added into wells of a 96-well fluor-microplate (Nunc or Greiner) to yield a final concentration of 0.1–200 μM. The plate was incubated in the dark at 25°C. Changes in fluorescence intensity were measured with a microplate reader (Spectrofluor-Plus, Tecan) at excitation 360 nm and emission 450 nm at 0 time and approximately 20–30 min intervals for at least 1 hour. Autoclaved samples and 2-methoxyethanol were used for sample and reagent controls, respectively. Assays were conducted in triplicate.

Primary standard of MUF was used to calibrate the fluorometer. MUF solution (2.0 mM) was prepared with 2-methoxyethanol and diluted with distilled water to yield final concentration 10 nM−10 μM. A significant linear relationship (r^2=0.995, n=49, p<0.001) between fluorescence intensity of MUF (F, arbitrary unit) and its concentration (C, μM) was obtained as follows; $C=35255\times10^{-8}\times F$. AP hydrolysis rates obtained at each MUF-P concentration (v) versus MUF-P concentration (s) were then used to de-

termine the substrate-saturated enzyme activity (V_{max}) for the sample employing Michaelis-Menten enzyme kinetics. V_{max} values were determined using calculations of the Lineweaver-Burke plot ('*s/v*' versus '*s*' plot) (Armstrong 1983) and were expressed as AP activity (mol L^{-1} min^{-1}).

Algal AP activity (AP bond cell membrane) was evaluated as the difference between AP activity in whole media (wAP) and that in filtrate (fAP). AP activity in filtrate was expressed as algal extracellular (free) AP activity. Cellular AP activity (cAP) was calculated using the following equation.

$$cAP=[(wAP)-(fAP)]/\text{Algal cell number}$$

Results

Utilization of phosphorus compounds

Utilization of inorganic phosphate and organic phosphorus compounds by the three phytoplankton species is shown in Fig. 1. The utilization of various phosphorus sources is expressed as the relative growth (%) in comparison to the growth obtained with orthophosphate.

Heterocapsa circularisquama, *Chaetoceros ceratosporum*, and *Fibrocapsa japonica* could utilize the four inorganic phosphate compounds, such as orthophosphate, pyrophosphate (PP), tripolyphosphate (TPP) and metaphosphate (MP) as the phosphorus source. The relative growth yields of *H. circularisquama*, *C. ceratosporum*, and *F. japonica* obtained with inorganic phosphate were 83.7–93.0%, 73.4–95.2%, and 109–134%, respectively. *Heterocapsa circularisquama* and *C. ceratosporum* could utilize phosphate monoesters as a phosphorus source. The relative growth of *H. circularisquama* and *C. ceratosporum* obtained with phosphate monoester compounds ranged from 64.1 to 100% and 60.4 to 107%, respectively. On the other hand, *F. japonica* could utilize only adenosine di- (ADP: 121%) and tri-phosphate (ATP: 87.9%) but not the monophosphate monoester compounds (less than 5%).

Alkaline phosphatase production of *Heterocapsa circularisquama*

In the culture of phosphate-replete medium after the pre-incubation in phosphate-replete medium, cell density of *H. circularisquama* increased from 1.00×10^4 cells mL^{-1} on day 0 to 4.42×10^5 cells mL^{-1} on day 12, and then the culture entered the stationary phase (Fig. 2A). Orthophosphate concentration decreased to less than 0.02 μM on day 10 with increasing cell density (Fig. 2A). No AP activities were found during this incubation period (Fig. 2A). In the phosphorus-deficient medium, there was little change of cell density ($6.97-8.83\times10^3$ cells mL^{-1}), and orthophosphate was less than 0.02 μM (Fig. 2B). AP activities of *H. circularisquama* were not found even under the orthophosphate-starved condition (Fig. 2B).

After the pre-incubation in phosphorus-deficient medium, *H. circularisquama* did not grow in the phosphorus-deficient medium, but the cell number increased rapidly

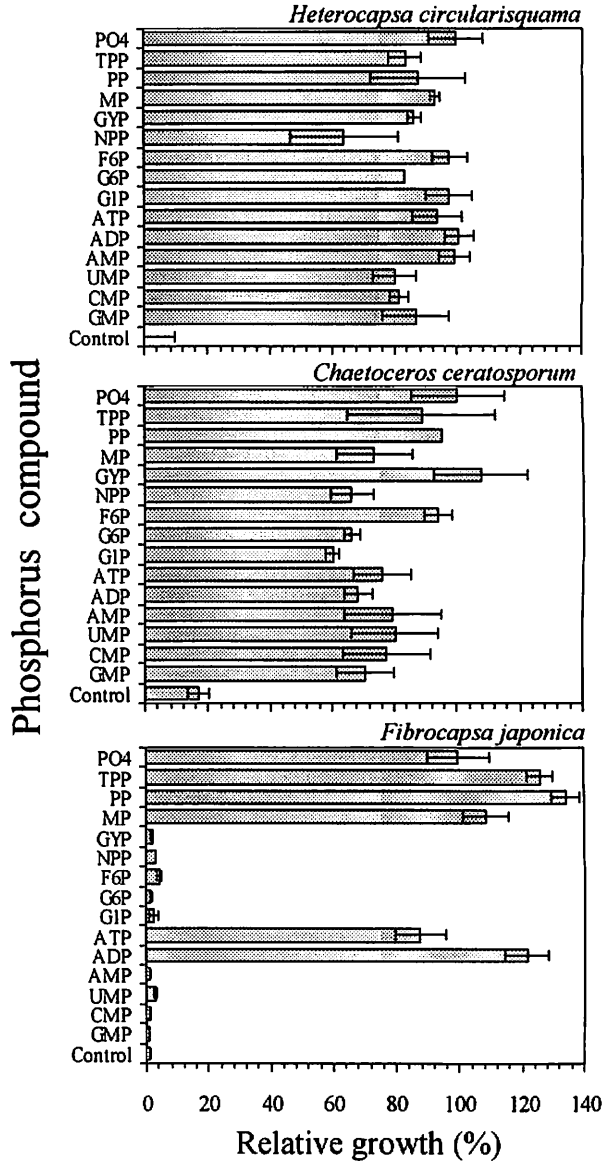

Fig. 1. Relative growth (%) of marine phytoplankton with various phosphorus compounds. Growth is expressed as a relative percentage to the maximum yield obtained with orthophosphate (PO4). Control represents no addition of phosphorus compounds in the medium. Bars show the standard deviation.

from 3.07×10^3 to 1.46×10^5 and 1.21×10^5 cells mL^{-1} on day 15 in orthophosphate-replete and glycerophosphate-replete media, respectively (Fig. 3). Cellular AP activities were found in only glycerophosphate-replete medium, at exponential phase on day 3 (3.44 fmol $cell^{-1}$ min^{-1}) (Fig. 3). The activities declined to 0.139 fmol $cell^{-1}$ min^{-1} on day 6. After that, the activities changed in the range from 0.007 fmol $cell^{-1}$ min^{-1} to 0.150 fmol $cell^{-1}$ min^{-1}. All AP activities appeared to be associated with the algal cell-membrane during the incubation periods (data not shown). This result indicated that *H. circularisquama* produces AP under the phosphate-starved condition but it required the presence of organic phosphorus.

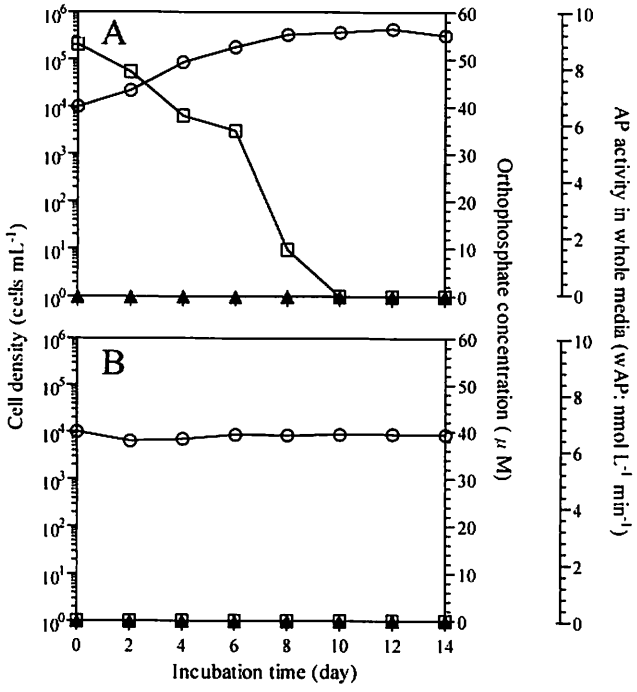

Fig. 2. Changes of cell density, orthophosphate concentration, and AP activity in the culture of *Heterocapsa circularisquama* growing in orthophosphate (A) and phosphorus-deficient (B) media. ○, Cell density; □, Orthophosphate concentration; ▲, AP activity in whole media (wAP: nmol L^{-1} min^{-1}).

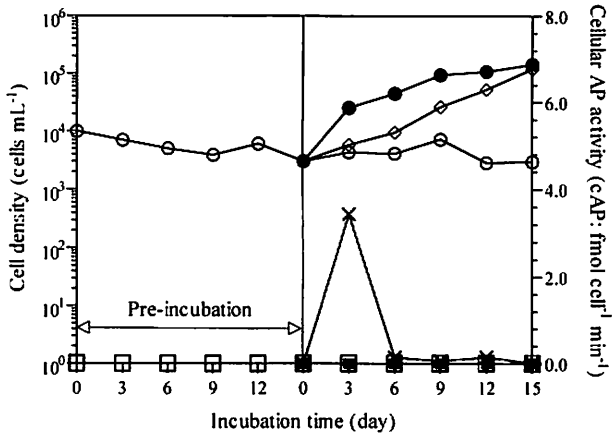

Fig. 3. Changes of cell density (cells mL^{-1}) (○, ●, and ◇) and cellular AP activity (cAP: fmol cell^{-1} min^{-1}) (□, ■, and ×) in the culture of *Heterocapsa circularisquama* growing in each medium. The center line shows the end time of pre-incubation in phosphorus-deficient media. Symbol ○ and □ in phosphorus-deficient, ● and ■ in orthophosphate, and ◇ and × in glycerophosphate media, respectively.

Alkaline phosphatase production of *Chaetoceros ceratosporum*

Cell density of *C. ceratosporum* growing in orthophosphate-replete medium increased from 1.00×10^4 cells mL^{-1} to 1.17×10^7 cells mL^{-1} on day 21 (Fig. 4A). Orthophosphate concentration decreased with increasing cell density, and reached 5.97 μM on day 21 (Fig. 4A). No AP activity was found during this incubation period.

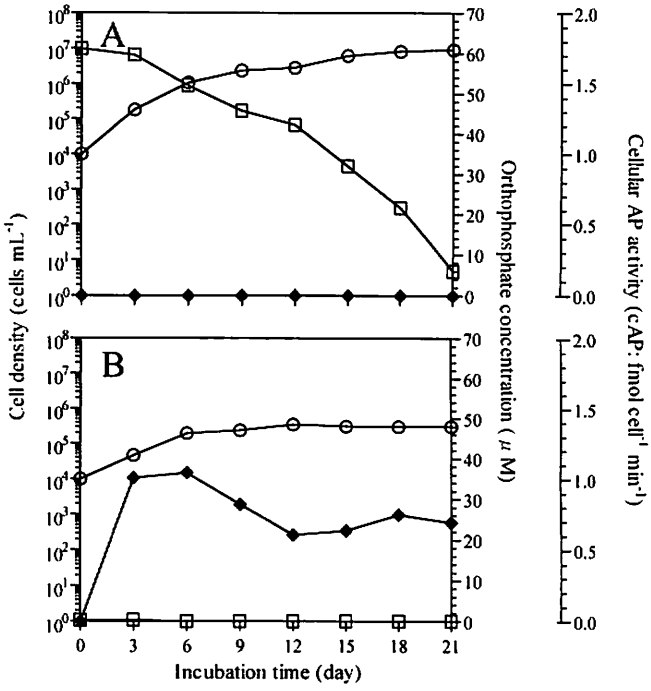

Fig. 4. Changes of cell density, orthophosphate concentration, and AP activity in the culture of *Chaetoceros ceratosporum* growing in orthophosphate (A) and phosphorus-deficient (B) media. ○, Cell density; □, Orthophosphate concentration; ◆, Cellular AP activity (cAP: fmol cell^{-1} min^{-1}).

In phosphorus-deficient medium, the cell density increased after inoculation, and the culture entered the stationary phase on day 12 (3.55×10^5 cells mL^{-1}) (Fig. 4B). The orthophosphate concentration was 0.41 μM on day 3 and below 0.05 μM after the days (Fig. 4B). AP activity appeared at log phase on day 3 and its cellular activity was 1.01 fmol cell^{-1} min^{-1}. The activity was maximal on day 6 (1.04 fmol cell^{-1} min^{-1}) (Fig. 4B). Cellular AP activity showed a tendency to decrease after that. Most of the AP activity (over 92%) appeared to be associated with the algal cell-membrane (Fig. 5).

Alkaline phosphatase production of *Fibrocapsa japonica*

Cell density of *F. japonica* in orthophosphate-replete medium increased to 5.10×10^4 cells mL^{-1} on day 15 with decreasing orthophosphate concentration (Fig. 6A). Also, in orthophosphate-deficient medium, cell density increased to 9.05×10^3 cells mL^{-1} on day 15 (Fig. 6B), and orthophosphate concentrations were below 0.02 μM during this incubation period. AP activity of *F. japonica* was not found in both orthophosphate-replete and phosphorus-deficient media.

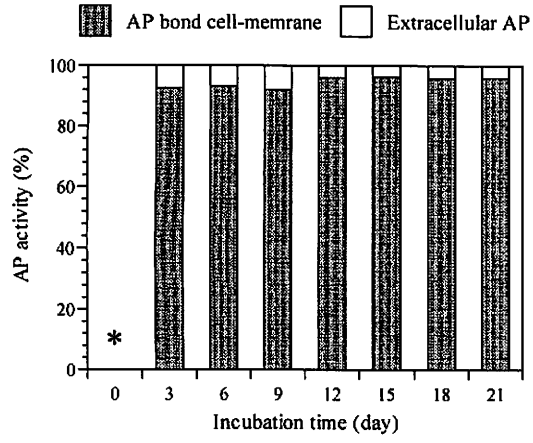

Fig. 5. Distribution of AP activity of *Chaetoceros ceratosporum* growing in phosphorus- deficient media. An asterisk (*) represents no AP activity detected.

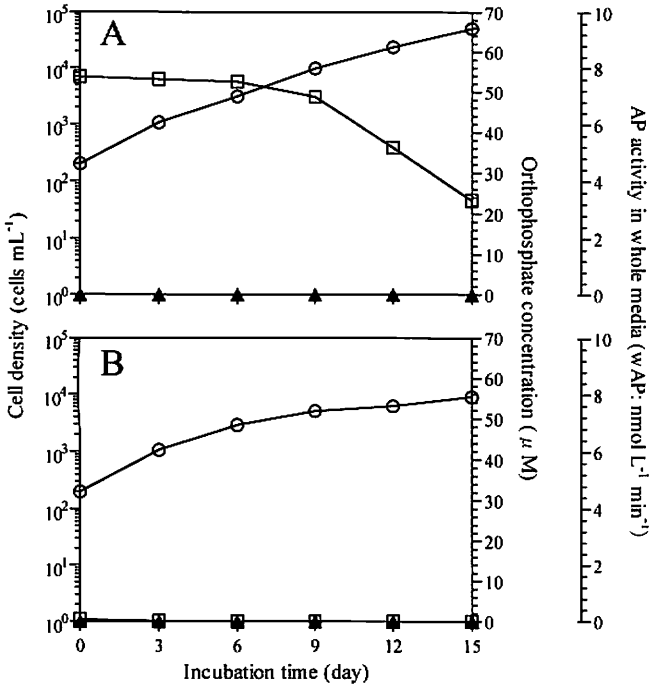

Fig. 6. Changes of cell density, orthophosphate concentration, and AP activity in the culture of *Fibrocapsa japonica* growing in orthophosphate (A) and phosphorus-deficient (B) media. ○, Cell density; □, Orthophosphate concentration; ▲, AP activity in whole media (wAP: nmol L^{-1} min^{-1}).

Discussion

The present results showed that inorganic phosphate compounds used in this experiment, such as orthophosphate (PO4), pyrophosphate (PP), tripolyphosphate (TPP), and metaphosphate (MP), were used by *Heterocapsa circularisquama*, *Fibrocapsa japonica* and *Chaetoceros ceratosporum* (Fig. 1). Also diphosphate monoester (ADP) and triphosphate monoester (ATP) were used by these phytoplanktons (Fig. 1). Yamaguchi et al. (2004a) reported the utilization of PO4, PP, TPP, MP, ADP and ATP by 3 species of red tide phytoplankton, *Karenia mikimotoi* (Miyake et Kominami ex Oda) G. Hansen et Moestrup, *Skeletonema costatum* (Greville) Cleve and *Heterosigma akashiwo*. Oh et al. (2002) also reported the same utilization by *Alexandrium tamarense* (Lebour) Balech and *Gymnodinium catenatum*. These results support the idea that utilization of inorganic phosphate compounds, ADP and ATP as a sole phosphorus source for the algal growth is general characteristic among marine phytoplankton species.

Algal AP production reflected the difference of utilization of monophosphate monoester compounds. Among the 3 phytoplankton species tested in this experiment, *H. circularisquama* and *C. ceratosporum* were able to use phosphate monoester compounds, but *F. japonica* was able to use only ADP and ATP. Furthermore, *H. circularisquama* and *C. ceratosporum* have the ability to produce AP (Figs. 3, 4), but *F. japonica* has no ability of the production (Fig. 6). Non-AP-producing phytoplankton such as *F. japonica* (present study) and *H. akashiwo* (Yamaguchi et al. 2004a) are not able to use monophosphate monoester compounds as a phosphorus source. On the other hand, AP-producing phytoplankton such as *H. circularisquama*, *C. ceratosporum* (present study), *Pyrocystis noctiluca* Murray (Rivkin & Swift 1980), *A. tamarense*, *G. catenatum* (Oh et al. 2002), *K. mikimotoi* and *S. costatum* (Yamaguchi et al. 2004a) are able to use monophosphate monoester. Actually, AP activities were found in the culture *H. circularisquama* (Fig. 3) and *C. ceratosporum* (data not shown) cultures growing in glycerophosphate-replete medium. These results confirm that utilization of monophosphate monoester by marine phytoplankton is determined by AP production, and suggest that marine phytoplankton use ADP and ATP using other phosphatases such as ADPase and ATPase.

Condition of algal AP-production was obviously different between phytoplankton species. AP activity of *C. ceratosporum* appeared in the early log phase and the activity was induced by a deficiency of orthophosphate (Fig. 4). Yamaguchi et al. (2004a) previously reported the threshold concentration of orthophosphate of some red tide phytoplanktons which have inducible-AP typically ranges from 0.2 to 0.4 μM (average 0.29 μM). The threshold concentration of *C. ceratosporum* was close to this range. In contrast, AP activities of *H. circularisquama* and *F. japonica* were not found even under orthophosphate-starved conditions (Figs. 2, 6). In the case of *H. circularisquama*, production of AP required the conditions of a deficiency of orthophosphate and the presence of APHP (Fig. 3). Therefore, AP-production of marine phytoplankton can be classified into at least 3 groups. The first group including *F. japonica* (present study) and *H. akashiwo* (Yamaguchi et al. 2004a) has no the ability to produce AP. The second group including *C. ceratosporum* (present study), *Prorocentrum minimum* (Pavillard) Schiller (Dyhrman & Palenik 1999), *G. catenatum* (Oh et al. 2002), *K. mikimotoi* and *S. costatum* (Yamaguchi et al. 2004a) produces AP which is induced in or-

Table 2. The potential alkaline phosphatase (AP) activity of 3 red tide phytoplankton.

Phytoplankton species	Maximum growth rate (day^{-1})	Minimum cell quota for phosphorus (fmol $cell^{-1}$)	Phosphorus uptake rate for saturated growth rate (A) (fmol $cell^{-1}$ min^{-1})	Potential cellular AP activity (B) (fmol $cell^{-1}$ min^{-1})	B/A	Reference
Karenia mikimotoi	0.67	250	2.21	115	52.0	Yamaguchi *et al.* (2004a)
Skeletonema costatum	0.71	2.5	0.023	0.273	11.7	Yamaguchi *et al.* (2004a)
Heterocapsa circularisquama	1.10[a]	89.4[a]	1.30	3.44	2.65	Present study

a: By Yamaguchi *et al.* (2001)

thophosphate-deficient or -starved conditions. But the threshold concentration for inducing AP is different between phytoplankton species. The third group including *H. circularisquama* produces AP under orthophosphate-starved and APHP-present conditions.

In case of *H. circularisquama* growing under glycerophosphate-replete medium, AP activity appeared on day 3 and rapidly decreased on day 6. After that, the activities were maintained at low level (Fig. 3). Alkaline phosphatase of phytoplankton has been known to be produced under phosphate-deficient conditions and its production is repressed in the presence of orthophosphate (Kuenzler 1965; Cembella et al. 1984a, 1984b; González-Gil et al. 1998; Yamaguchi et al. 2004a). Therefore, the decreases in AP activity after day 3 may be attributed to the rapid release of orthophosphate by AP activity.

Orthophosphate concentration in natural coastal waters, such as Uranouchi and Nomi Inlet (Kochi), and Hiroshima Bay, Japan, is usually the range from 0.2 to 0.4 μM or below 0.2 μM in especially summer (Itakura et al. 2002; Yamamoto et al. 2002; Yamaguchi et al. 2004b). Furthermore, previous reports have indicated that APHP represents a significant proportion of the algal-available phosphorus (Taga & Kobori 1978; Kobori & Taga 1979; Hernández et al. 2000; Yamaguchi et al. 2004b). Therefore, *H. circularisquama*, *C. ceratosporum* and other AP-producing phytoplankton could grow using APHP as a phosphorus source in natural coastal waters.

The maximum AP activities of *H. circularisquama* and *C. ceratosporum* were 3.44 and 1.04 fmol $cell^{-1}$ min^{-1}, respectively (Fig. 3,4). The AP activities of both species were higher than that of *S. costatum* (0.273 fmol $cell^{-1}$ min^{-1}) but lower than that of *K. mikimotoi* (115 fmol $cell^{-1}$ min^{-1}) (Yamaguchi et al. 2004a). However, there are considerable differences in cell volume and phosphorus demand among these phytoplankton species. AP activity of phytoplankton must be evaluated and compared on the basis of algal cell volume and phosphorus demand. It is difficult to discuss the contribution of AP activity for algal growth using the activity basis of algal cell or cell volume, because phosphorus demand of phytoplankton is different between phytoplankton species. Therefore, AP activity of phytoplankton should be properly evaluated on the basis of phosphorus demand. We evaluated the potential AP activity of *H. circularisquama* on the basis of phosphorus demand (Yamaguchi et al. 2004a). Under a steady-state in the phosphorus-limited condition, phosphorus uptake rate of *H. circularisquama* to maintain the saturated growth rate (95% μ_{max}) is calculated to be 1.30 fmol $cell^{-1}$ min^{-1} (u_{max}) using the Droop equation (Yamaguchi et al. 2001) (Table 2). The μ_{max} is calculated by the product of saturated growth rate (95% μ_{max}) and cell quota for phosphorus to maintain 95% μ_{max}. Maximum AP activity (3.44 fmol $cell^{-1}$ min^{-1}) of *H. circularisquama* obtained from the present study was 2.65 times higher than the u_{max} (Table 2). Although the value is lower than those of *K. mikimotoi* (52.0) and *S. costatum* (11.7) (Yamaguchi et al. 2004a), *H. circularisquama* has potentially sufficient AP activity to degrade APHP and uptake the released orthophosphate for growth. Present study indicates that AP-producing phytoplanktons have an ecological advantage in the process of algal succession under phosphate-starved conditions against non-AP-producing species.

Heterocapsa circularisquama cells are known to contain endosymbiotic bacteria in the cytoplasm and around the algal nucleus (Horiguchi 1995; Maki & Imai 2001; Maki et al. 2004). Therefore, bacterial effects on the utilization of organic phosphorus by phytoplankton can not be ignored. In the present study, however, the above effects were probably small because we could not observe bacterial cells in the medium and not find AP activities in the filtrates (0.8 μm). Furthermore, the roles of intracellular bacteria have not been clarified yet. Therefore, we presume at present that *H. circularisquama* produce AP and can directly use APHP compounds as a phosphorus source. Further study would be necessary to examine the roles of intracellular bacteria in the utilization of phosphorus by *H. circularisquama*.

The present study found that AP production reflects the difference of phosphate monoester utilization of marine phytoplankton. Therefore, AP production of marine phytoplankton would contribute not only to the outbreak of red tides but also to the process in which phytoplankton compete to acquire a phosphorus source from APHP among phytoplankton species in coastal environments.

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Horizontal distribution of toxic *Alexandrium* spp. (Dinophyceae) resting cysts around Hokkaido, Japan

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Abstract: To clarify the distribution of toxic *Alexandrium* spp. resting cysts throughout the coastal waters around Hokkaido, sediment samples from 152 stations were examined using the primuline-staining direct count method. Cysts were found to be distributed especially in the cold current areas, from the coast of the Pacific Ocean to the Sea of Okhotsk. Large concentrations of cysts were found in Funka Bay (max. 2,568 cysts g^{-1} sediment) and the Sea of Okhotsk (max. 1,022 cysts g^{-1} sediment). On the other hand, there were no cysts in the warm current areas, from the coast of the Sea of Japan to the Tsugaru Strait. There was a significant correlation between the cyst abundance and the frequency of past PSP occurrences in each area around Hokkaido. Therefore, the cyst abundance was concluded to be a useful parameter for predictions of the frequency of PSP occurrence.

Key words: *Alexandrium tamarense*, cyst, paralytic shellfish poisoning, prediction, Hokkaido

Introduction

The toxic dinoflagellate *Alexandrium tamarense* is known as one of the typical species that cause paralytic shellfish poisoning (PSP) in Japan (Fukuyo 1985). Since 1978, PSP has often occurred because of *A. tamarense*, and has had a harmful effect on the shellfish fishery, especially for the scallop (*Mizuhopecten yessoensis*) fishery from the coast of the Pacific Ocean to the Sea of Okhotsk around Hokkaido (Nishihama 1985). Since *A. tamarense* has a resting cyst (hypnozygote) stage as well as the planktonic vegetative cell stage in the life cycle (Anderson & Wall 1978; Turpin et al. 1978; Yoshimatsu 1992), the cyst distribution is important information when considering the dynamics of blooms and the potential to predict *A. tamarense* appearances. It has been observed that the vegetative cells of *A. tamarense* show an annual regular pattern of increase from spring to summer (Shimada et al. 1996; Shimada 2000) where high concentrations of cysts (approx. 200-1,100 cysts g^{-1}) occur in Funka Bay, south western Hokkaido (Miyazono & Shimada 2000). However, surveys on the cyst distribution have only been carried out in limited areas such as Funka Bay (Fukuyo 1982; Miyazono 2000; Miyazono 2002), Lake Saroma (Fukuyo 1982), Akkeshi Bay (Fukuyo 1982) and the coast of Tokachi subprefecture (Hokkaido 1987), because cyst counting using a normal light microscope is highly labor intensive work. However, it has been possible to survey cyst distribution rapidly and extensively, after Yamaguchi et al. (1995) reported the "primuline–staining direct count method" which uses an epi-fluorescence microscope. Thus we attempted to elucidate the horizontal distribution of toxic *Alexandrium* spp. cysts all around Hokkaido as a fundamental database for the prediction of PSP occurrence, to determine if there is a relationship between the cyst abundance and the frequency of past PSP occurrences in each area.

Abbreviations: PSP, paralytic shellfish poison; FB, Funka Bay; SP, Pacific coast of southern Hokkaido; EP, Pacific coast of eastern Hokkaido; NS, Nemuro Strait; OS, Sea of Okhotsk & Soya Strait; JP, Sea of Japan; TS, Tsugaru Strait.

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Materials and Methods

Sampling *in situ*

Surveys were carried out from 1999 to 2000 at 152 sta-