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Uptake and Excretion of Dissolved Organic Phosphorus by Two Toxic Dinoflagellates, *Alexandrium tamarense* Lebour (Balech) and *Gymnodinium catenatum* Graham

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We performed experiments on the uptake and excretion of dissolved organic phosphorus (DOP) using two toxic dinoflagellates, *Alexandrium tamarense* Lebour (Balech) and *Gymnodinium catenatum* Graham, isolated from Hiroshima Bay, Japan. ATP (adenosine triphosphate), UMP (uridine-5-monophosphate), G-6-P (glucose-6-phosphate) and Glycero-P (glycerophosphate) were used as DOP sources in preliminary uptake experiments. ATP was selected as the DOP species for the short-term uptake experiment because preliminary experiments showed it to be the DOP source used by both species. Although the K_s values of *A. tamarense* and *G. catenatum* (5.63 and 7.61 μM, respectively) obtained from the short-term experiments for ATP were only slightly higher than those reported for dissolved inorganic phosphorus (DIP), the ρ_{max} values (5.04 pmol/cell/h and 13.4 pmol/cell/h, respectively) were much higher. The DOP excretion rate in batch-culture experiments was estimated at 0.084 pmol/cell/h for *A. tamarense* and 0.012 pmol/cell/h for *G. catenatum*, accounting for about 30% and 25%, respectively, of the assimilated phosphorus. Our results suggest that the DIP-depleted conditions of Hiroshima Bay favor these two species by supporting their ability to use DOP.

Key words: *Alexandrium tamarense*, Dissolved organic phosphorus, Excretion, *Gymnodinium catenatum*, Nutrient uptake

Introduction

Dissolved organic phosphorus (DOP) can be a significant fraction of the total dissolved phosphorus (TDP) pool in surface seawaters (Shan et al., 1994) and may be an important phosphorus source for phytoplankton in waters depleted of dissolved inorganic phosphorus (DIP; Oh et al., 2005). Seawater DOP is not only supplied by excretion from actively metabolizing microalgae, bacterial cells, and sloppily feeding zooplankton (Pomeroy et al., 1963; Kuenzler, 1970; Sharp, 1977), but is also delivered from land (Beusekom and Brockmann, 1998). Thus, the composition of DOP in natural seawater is complex and diverse (Shan et al., 1994; Monaghan and Ruttenberg, 1999).

The major compounds of high-molecular-weight DOP (HMW-DOP) from the Pacific Ocean, Atlantic Ocean, and North Sea waters have been identified (Clark et al., 1998; Kolowith et al., 2001). The dominant compounds are P esters (75% of DOP) and phosphonates (25%), a chemically stable group. Of the P esters identified, phosphomonoester and phosphodiester, which are potential nutrient sources for microalgae, use two different kinds of phosphorhydrolytic enzymes, alkaline phosphatase (AP) and phosphodiesterase (Cembella et al., 1984; Jackson and Williams, 1985; Suzumura and Ishikawa, 1998). Although labile low-molecular-weight DOP (LMW-DOP) in open ocean and coastal waters seems to contain a relatively high biologically reactive fraction, most parts of coastal water LMW-DOP are not biologically reactive because river water contains a significant amount of artificially synthesized LMW-DOP, which does not decompose easily (Suzumura

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and Ishikawa, 1998). Therefore, the P esters in HMW-DOP are likely to be an important fraction in the phosphorus cycle of coastal marine ecosystems.

Long-term monitoring of the abundance and composition of red tides in Hiroshima Bay has shown that dinoflagellates have become more abundant than diatoms (Oh et al., 2005). Researchers have suggested that a probable cause of this phenomenon is the depletion of seawater DIP, which is often below the detection limit (<0.03 μM). This is probably due to the effect of the directive for the reduction measure of phosphorus load by the Japanese government since 1980 (Yamamoto et al., 2002a). Because diatoms generally have a higher DIP demand than do dinoflagellates, they cannot dominate if DIP is depleted, even under high concentrations of silicate and nitrate (Egge, 1998), which indicates preferential DIP use. On the other hand, some dinoflagellates can grow using reactive DOP, such as P esters, in the environment (Cembella et al., 1984; Yamaguchi, 1999; Yamaguchi and Itakura, 1999). In addition, the characteristic feature of dinoflagellate migration enables them to increase the chance of encountering nutrients in the lower layer during the stratified season.

Alexandrium tamarense Lebour (Balech) and *Gymnodinium catenatum* Graham are two dinoflagellates responsible for paralytic shellfish poisoning (PSP). Since 1992, shellfish poisoning caused by *A. tamarense* has become an annual spring event in Hiroshima Bay. Although PSP caused by *G. catenatum* has not yet been reported in the bay, recent monitoring shows an increase in the species population size (Y. Matsuyama, Harmful Algal Division, National Research Institute of Fisheries and Environment of the Inland Sea, personal communication). Yamamoto et al. (2002b) have suggested that spring and autumn temperature, salinity, and light conditions in Hiroshima Bay may provide suitable conditions for *G. catenatum* outbreaks.

In this study, we conducted experiments on the DOP uptake and excretion kinetics of *A. tamarense* and *G. catenatum* isolated from Hiroshima Bay. The results showed differences and similarities in DOP use in these species. We discuss the environmental conditions, particularly the concentration of bioavailable phosphorus in the seawater, that may induce blooms of these species.

Materials and Methods

Strains and medium conditions

A. tamarense and *G. catenatum* cells were isolated from Hiroshima Bay in March 1997 and December 1998. The cells were cultured in f/2 medium (Guillard and Ryther, 1962), with no added silicate, which was sterilized through a membrane filter (Millipore, Sterivex-GS; 0.22 μm filter unit with a filling bell). The dinoflagellates were maintained at optimum growth conditions for each species (Yamamoto and Tarutani, 1997; Yamamoto et al., 2002b; Oh and Yoon, 2004). The temperature was maintained at 15°C for *A. tamarense* and 25°C for *G. catenatum*. The salinity, pH, and light intensity were 30 psu, 8.0, and 300 μmol photons/m^2/s (cool-white fluorescent lamp, 12L:12D, 6:00-18:00L), respectively, for both species.

To reduce bacterial contamination, the following procedures were used during pre-experimental culture maintenance: *A. tamarense* and *G. catenatum* cells were repeatedly rinsed with sterile seawater during the log-growth phase, and both species were cultured in AM9 (25%; Provasoli et al., 1959), an antibiotics mixture, for 48 h. We found no bacterial or other microbial contamination using either Marine Agar 2216 (DIFCO) or microscopic observation with fluorochrome 4', 6-diamidino-2-phenylindole (DAPI) staining (Porter and Feig, 1980).

Short-term DOP uptake experiments

Prior to the experiments, P-deficient cells were prepared by culturing *A. tamarense* and *G. catenatum* for 7 days in artificial seawater L1 medium (Keller et al., 1987; Guillard and Hargraves, 1993) with no added P. On day 7, we confirmed that the orthophosphate concentration was below the detection limit (0.03 μM), with no cell growth.

ATP (adenosine triphosphate), UMP (uridine-5-monophosphate), G-6-P (glucose-6-phosphate), and glycero-P (glycerophosphate) were used as DOP sources in preliminary experiments because we had determined in previous experiments that *A. tamarense* uses nucleotides (ATP, UMP), whereas *G. catenatum* is able to use various DOP compounds (Oh et al., 2002). Because phytoplankton nutrient uptake exeriments should be completed during a constant uptake rate period (Harrison et al., 1989), we added a final concentration of 3 μM DOP and P-deficient cells, as evaluated above, in L1 medium to 2-L Erlenmeyer flasks, and monitored DIP and total dissolved phosphorus (TDP) concentrations at 0, 15, 30, 45, 60, 90, 120, 150, and 240 min to determine the uptake period. DIP and TDP concentrations were determined using the methods of Strickland and Parsons (1972) and Koroleff (1983), respectively. The DOP concentration was estimated by subtracting the DIP concentration from the TDP concentration. The

preliminary experiments showed that only ATP was taken up significantly by *A. tamarense* and *G. catenatum* (Figs. 1, 2). Thus, we used ATP in the following short-term uptake experiments. The experimental periods were set at 90 min for *A. tamarense* and 60 min for *G. catenatum* because the preliminary experiments showed a constant uptake rate within these periods.

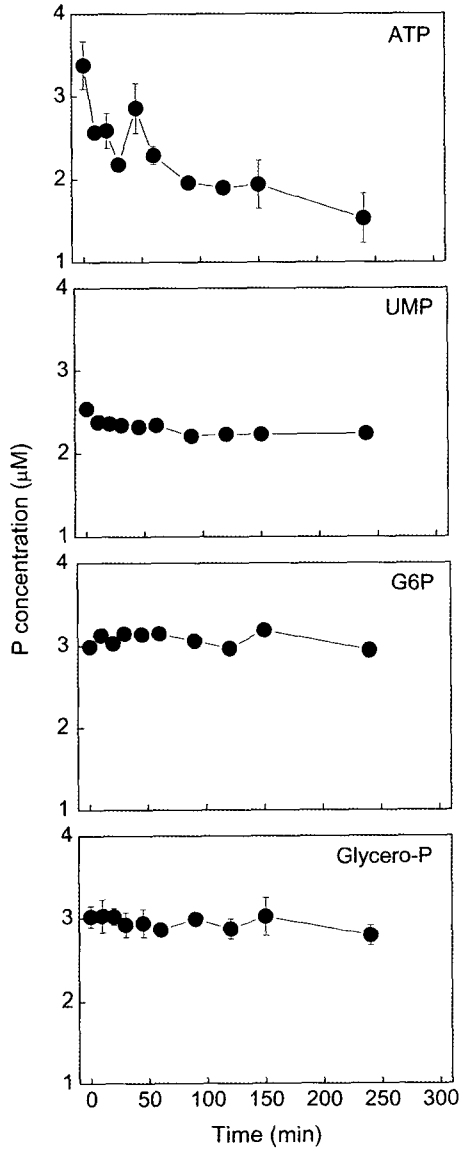

Fig. 1. Change in ATP (adenosine triphosphate), UMP (uridine-5-monophosphate), G6P (glucose-6-phosph-ate) and Glycero-P (glycerophosphate) concentration after perturbing phosphate-starved culture of *A. tama-rense*. Values are represented as means and standard deviations of duplicated measurements. Experimental conditions; Temperature 15°C, salinity 30 psu, pH 8.0, 300 μmol/m/s (cool-white fluorescent lamps, 12:12 h LD cycle).

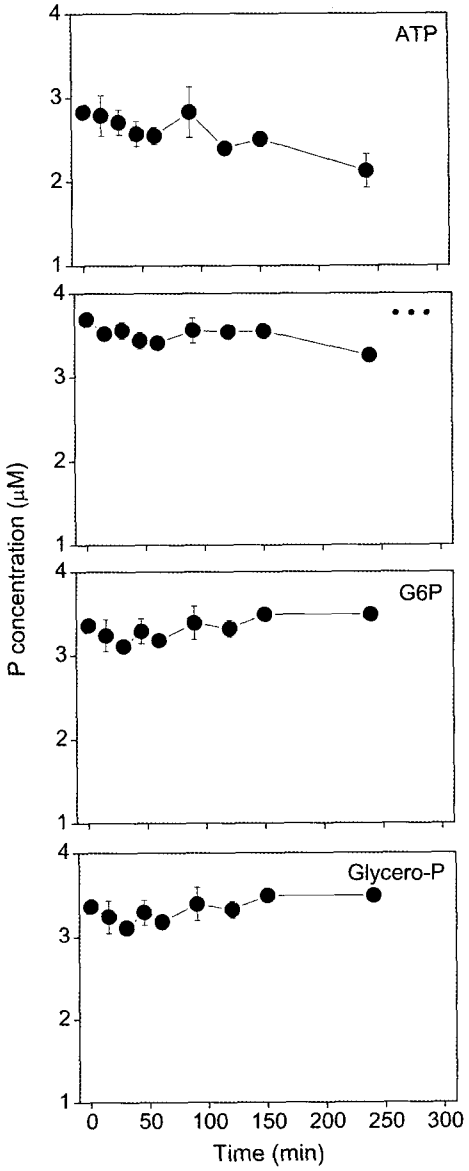

Fig. 2. Change in ATP, UMP, G6P and Glycero-P concentrations after perturbing phosphate-starved culture of *G. catenatum*. Values are represented as means and standard deviations of duplicated measurements. Experimental conditions; Temperature 25°C, salinity 30 psu, pH 8.0, 300 μmol/m/s (cool-white fluorescent lamps, 12:12 h LD cycle).

After subdividing the P-depleted culture into five 100-mL flasks, ATP was added to give final concentrations of 1, 3, 5, 30, and 50 μM. The cell densities of *A. tamarense* and *G. catenatum* were about 532 cells/mL and 330 cells/mL, respectively. After incubating the cultures (90 min for *A. tamarense*; 60 min for *G. catenatum*), the ATP uptake rate was estimated from the decrease in ATP concentration.

which was calculated by subtracting the DIP concentration from the TDP concentration as described above. The results were substituted into the Michaelis-Menten equation,

$$\rho=\rho_{max}\frac{S}{(K_S+S)} \quad (1)$$

Where, K_s is the half-saturation constant (μM), S is the ambient concentration (μM), and ρ_{max} is the maximum uptake rate (pmol/cell/h). Ks and ρ_{max} were estimated using the non-linear least squares method (Abe, 1985). The experiments were conducted in duplicate.

DOP excretion experiments

A batch-culture method was used to estimate the DOP excretion rate by *A. tamarense* and *G. catenatum*. Prior to the experiments, cells in log-growth phase were harvested from the stock culture and inoculated into a 2-L Erlenmeyer flask that contained 1 L of artificial seawater and L1 of medium with a 10-μM orthophosphate concentration. The size of the inoculum was adjusted to 150 cells/mL for *A. tamarense* and 40 cells/mL for *G. catenatum*, based on their cell volume (2.2×10^{-5} mm^3, *A. tamarense*; 7.4×10^{-5} mm^3, *G. catenatum*). The mixtures were maintained at optimal growth conditions, as described above. During the experiments, a 100 mL sample was drawn from the vessel every 2 days, and the number of cells was counted under a light microscope (Nikon; TS 100). DIP and TDP concentrations were determined as described above for membrane-filtered samples (0.45 μm pore size; Millipore HA).

Beginning at late-phase exponential growth, cells were dyed with 0.4% Trypan Blue to distinguish dead from living cells. Although DOP may be released by the autolysis of dead cells, we only considered active DOP release from living cells active "excretion". Therefore, the excretion rate was estimated from the increase in DOP concentration in the medium during the pre-autolysis phase.

Measurement of DOP and DIP concentrations in Hiroshima Bay water

To investigate the availability of phosphorus compounds among the environmental conditions that induce *A. tamarense* and *G. catenatum* blooms, we investigated the seasonal and spatial variation of DIP and DOP in seawater collected at 11 Hiroshima Bay stations during 1996-2000. The DIP and DOP concentrations were determined using the method described above for filtered seawater samples (0.45 μm pore size, vacuum pressure <15 cm Hg).

Results and Discussion

Of the DOP compounds used in the preliminary experiment, ATP proved to be the most efficient phosphorus source for both *A. tamarense* and *G. catenatum* (Figs. 1, 2). The concentration of ATP in natural seawater is low, but ATP is a ubiquitous component of DOP (Hodson et al., 1976). ATP is thought to cycle rapidly via phytoplankton and bacteria (Hodson et al., 1981) because triphosphates have two phosphoanhydride bonds, which are prone to hydrolysis (Monaghan and Ruttenberg, 1999). In fact, Björkman and Karl (1994) showed that the bioavailability of ATP in microbial assemblages is higher than that of phosphomonoesters, such as glucose-1-phosphate (G-1-P), fructose-1, 6-diphosphate (F-1,6-P) and glycero-P.

Oh et al. (2002) observed the growth of *A. tamarense* and *G. catenatum* with phosphomonoester in long-term growth experiments, but we did not detect the uptake of phosphomonoesters by these two species in our short-term experiments. This discrepancy may reflect the low hydrolysis rate of phosphormonoesters by alkaline phosphatases (AP; Björkman and Karl, 1994). Our constraint was the necessity to complete the experiments quickly during active uptake to avoid any change in cellular physiological conditions. Other methods, such as the use of isotopes, are required to obtain an accurate phosphomonoester uptake rate (Harrison et al., 1989).

Although the K_s values of *A. tamarense* and *G. catenatum* obtained from the short-term experiments for ATP (5.63 and 7.61 μM, respectively) were higher than the DIP values, the ρ_{max} values (5.04 pmol/cell/h for *A. tamarense*; 13.4 pmol/cell/h for *G. catenatum*) were much higher than those for DIP (Fig. 3, Table 1). Using these ρ_{max} values and the minimum phosphorus cell quota (1.83 pmol/cell) reported by Yamamoto et al. (2004), V_{max}, the maximum specific uptake rate, was estimated at 216/day for *A. tamarense* and 175/day for *G. catenatum* (Fig. 4). The α value (V_{max}/K_s) represents the initial slope of uptake as a function of the external ATP concentration, and thus, is an indicator of the competitive uptake status (Healey, 1980). The α was calculated at 38.4 in *A. tamarense* and 23.0 in *G. catenatum*. The V_{max} and α were higher than those for DIP (Fig. 5, Table 1), indicating that both *A. tamarense* and *G. catenatum* have a higher affinity for DOP than DIP. In particular, the α of *G. catenatum* was about four times higher than that of DIP. Doblin et al. (1999)

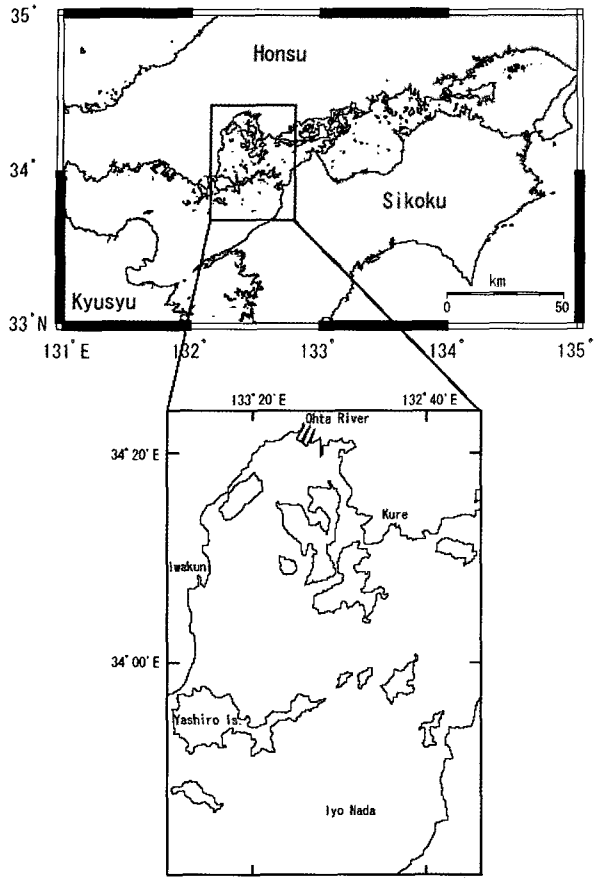

Fig. 3. Map showing sampling stations in Hiroshima Bay.

Table 1. Summary of reported half-saturation (K_s), maximum uptake rate (ρ_{max}), specific maximum uptake rate (V_{max}), and affinity index (α) for dissolved inorganic phosphorus (DIP) uptake by *Alexandrium tamarense* and *Gymnodinium catenatum*. The specific DIP uptake rate of *A. tamarense* is from Yamamoto and Tarutani (1999), and that of *G. catenatum* is from Yamamoto et al. (2004)

Species	Constants			
	K_s (μM)	ρ_{max} (pmol/cell/hr)	V_{max} (/day)	α
A. tamarense	2.60	1.40	60.0	23.1
G. catenatum	3.40	1.42	18.6	5.47

observed that DOM extracted from natural seawater stimulated the growth of an Australian strain of *G. catenatum*. Oh et al. (2002) showed that the maximum AP activity of *G. catenatum* was about two-fold higher than that of *A. tamarense*. They also suggested that *G. catenatum* presumably possesses as much affinity to mono-, di- and triphosphorus as to DIP, judging from the absence of a significant difference in the growth rate (range: 0.24-0.37/day) in the long-

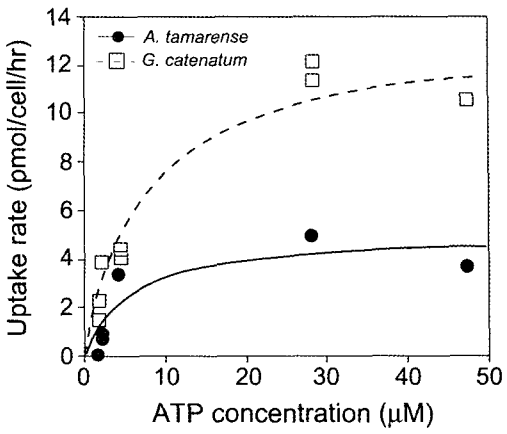

Fig. 4. ATP uptake rates of *A. tamarense* and *G. catenatum*, the Hiroshima Bay strains, as a function of ambient ATP (see Figs. 1 and 2 for incubation conditions).

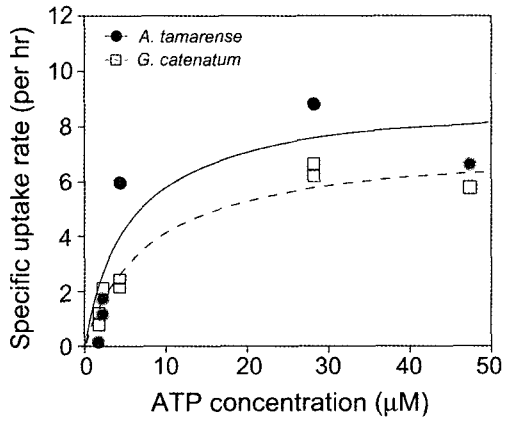

Fig. 5. Specific ATP uptake rates of *A. tamarense* and *G. catenatum* Hiroshima Bay strains.

term experiments (Oh et al., 2002). These results from a series of culture studies suggest that *G. catenatum* would be more likely than *A. tamarense* to survive in DIP-depleted conditions.

In the excretion experiments, a large amount of DOP was detected at the late log-growth phase (day 9) in *A. tamarense*, at which time the DIP concentration was 0.56 μM (Fig. 6). Significant DOP excretion was also detected in *G. catenatum*, but the DIP concentration in the medium (2.56 μM) was much higher. These results agree with the findings of Kuenzler (1970), in which vigorous DOP excretion occurred in the period from the late log-growth to the early stationary-growth phase. The DOP excretion rate was estimated at 0.084 pmol/cell/h for *A. tamarense* from day 7 to 9, and at 0.012 pmol/cell/h for *G. catenatum* from day 9 to 11. These rates

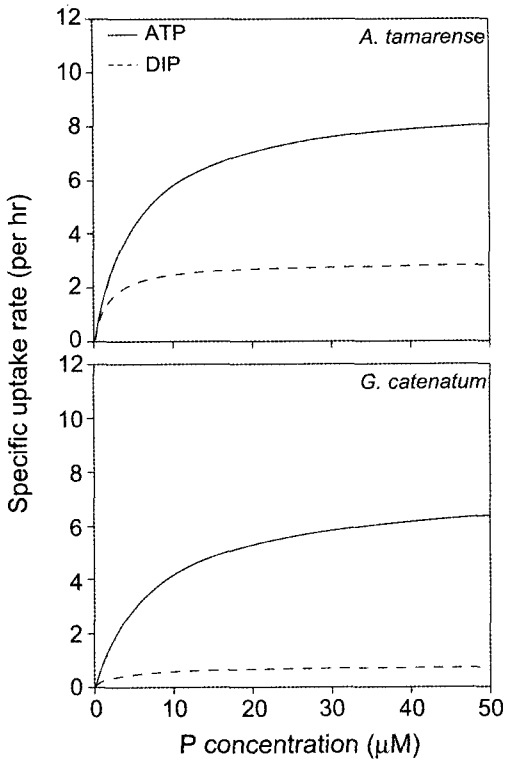

Fig. 6. Comparison of specific uptake rates of DIP and ATP in *A. tamarense* and *G. catenatum*. The specific DIP uptake rate of *A. tamarense* was cited from Yamamoto and Tarutani (1999) and that of *G. catenatum* was cited from Yamamoto *et al* (2004).

account for approximately 30% and 25% of the cumulative amount of DIP taken up from the beginning of the experiments for *A. tamarense* and *G. catenatum*, respectively. Because Trypan Blue dye revealed dead cells in the cultures from day 10 onward for *A. tamarense* and day 12 onward for *G. catenatum*, data from the late culture periods were omitted from the calculation. However, the dead cells did not release any detectable DOP because the DOP concentration did not increase significantly in the late-growth phase (Fig. 6). These findings also indicate that the cultures were not contaminated, although we did not determine whether they were axenic in the latter phase of the experiments.

Although little is known about the chemical composition of the DOP excreted by phytoplankton, cyclic adenosine 3':5' monophosphate (cyclic-AMP) and other phosphomonoesters have been reported to be excreted (Kuenzler, 1970). Cyclic-AMP constitutes a minor fraction of the total excreted DOP (Francko and Wetzel, 1982), but seems to play an important role in the regulation of carbon, nitrogen, and phosphorus metabolism, as well as changes in the permeability of the cell to various metabolites (Lepo and Wyss, 1974). However, phosphorus excretion under the P-depleted condition observed in our experiments corresponds to a net loss in terms of the cellular phosphorus budget, rendering population maintenance difficult. The estimated maximum phosphorus cell quota (Q_{max}) using the Droop equation, $\mu=\mu_{max}$ (François and Morel, 1987), revealed that the Q_{max} of *A. tamarense* (0.96 pmol/cell) is only 1/12 that of *G. catenatum* (11.3 pmol/cell). From these values and the obtained excretion rates, we suggest that *A. tamarense* may form cysts in the late-bloom period, whereas *G. catenatum* is able to maintain its vegetative cell population under P-depleted conditions. Yamamoto et al. (2002c) simulated the bloom dynamics of *A. tamarense* in Hiroshima Bay using a model that considered cyst formation to be related to the ambient phosphate concentration.

The 1996-2000 monitoring data showed an apparent seasonal variation in the DIP concentration in Hiroshima Bay (Fig. 7). DIP was <0.6 μM throughout the year, and was almost depleted (<0.2 μM) in spring and summer. This tendency most likely resulted from the phosphorus reduction measures implemented in 1980 (Yamamoto et al., 2002a). On the other hand, DOP comprises about one-third (0.37 μM on average) of the average TDP (Yamamoto et al., 2002a; Oh et al., 2005), suggesting that DOP may be

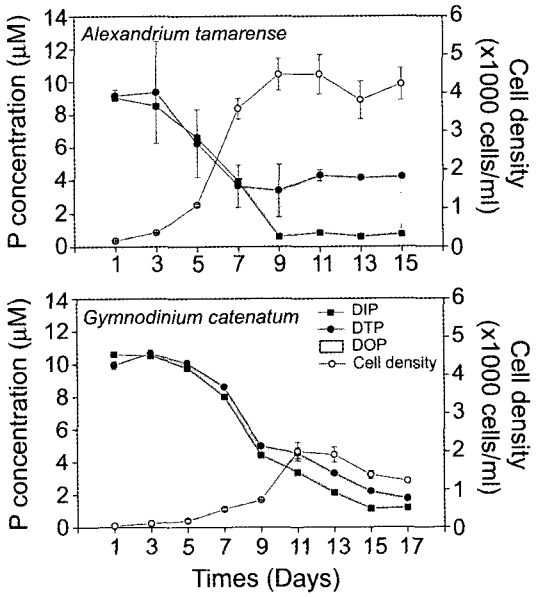

Fig. 7. Change in DOP concentration during the time-course experiments of *A. tamarense* and *G. catenatum*. Values are represented as means and standard de-viations of duplicated measurements (see Figs. 1 and 2 for incubation conditions).

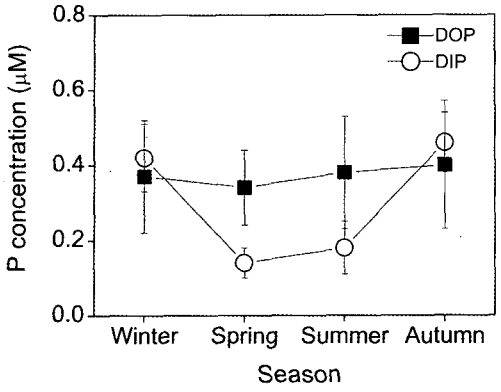

Fig. 8. Seasonal variabilities of DOP and DIP concentrations in the surface seawater of Hiroshima Bay from 1996-2000.

an alternative phosphorus source for phytoplankton in spring and summer (Fig. 7). In the uptake kinetics of this study, both *A. tamarense* and *G. catenatum* showed a higher affinity for ATP than DIP. Moreover, the phosphatase produced by *A. tamarense* and *G. catenatum* has been shown to hydrolyze labile DOPs, such as mono-, di-, and triphosphorus (Oh et al., 2002). Therefore, the spring *A. tamarense* blooms in Hiroshima Bay may depend on the availability of labile DOP. Although *G. catenatum* was first recorded in Japan in the bay (Hada, 1967), it has not yet formed blooms there. If DIP-depleted condition per-sists in the bay, *G. catenatum* will most likely form blooms because of its higher affinity for DOP than *A. tamarense*, unless other physical and biological fac-tors prevent their occurrence.

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