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Alkaline Phosphatase Activity and Phosphatase Hydrolyzable Phosphorus for Phytoplankton in Hiroshima Bay, Japan

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Abstract – We investigated the seasonal variability of free alkaline phosphatase activity in seawater and alkaline phosphatase hydrolysable phosphorus (APHP) at 3 stations in Hiroshima Bay using alkaline phosphatase extracted from the dinoflagellates *Alexandrium tamarense* and *Gymnodinium catenatum*. The dissolved inorganic phosphorus (DIP) was lower than 1 μM in all samples; the lowest values were in May. The amount of APHP was high at the surface and bottom waters of all stations in May, showing DIP-depleted conditions. In August and November, the amount of APHP was much less than the amount of APHP in May, indicating that the availability of dissolved organic phosphorus (DOP) for these species was low and/or uptake during the dinoflagellate blooming might have occurred in the area. The results obtained from short-term variations of AP activity might suggest that the growth of dinoflagellates in this season may be partly supported by the AP produced by other diatoms.

Key words – Alkaline phosphatase, *Alexandrium tamarense*, *Gymnodinium catenatum*, DIP, DOP, APHP

1. Introduction

Hiroshima Bay is a semi-enclosed estuary located in the western part of the Seto Inland Sea, Japan, *ca.* 50 km long and 30 km wide with a surface area of *ca.* 1,000 km^2 and an average depth of *ca.* 26 m. The Ohta River (annual average volume of inflow: *ca.* 7.14×10^6 m^3 day^{-1}), which empties into the innermost northern part of the bay, may be a major source of both organic and inorganic matter for the bay (SECA 1998). Since the Law Concerning Special Measures for Conservation of the Environment of the Seto Inland Sea for phosphorus reduction was enacted in 1978, the concentration of dissolved inorganic phosphorus (DIP) in the Ohta River water has decreased markedly (Yamamoto *et al.* 2002a). Thus, the proportion of dissolved organic phosphorus (DOP) to total dissolved phosphorus (TDP) in Hiroshima Bay has been increasing and is almost at the same level as that of DIP (Yamamoto *et al.* 2002a). Actually, the DIP concentration in the surface water in spring and summer has actually fallen below the detection limit, indicating the relative seasonal importance of DOP.

In a number of phytoplankton species, alkaline phosphatase (AP) hydrolyzes external phosphomonoesters (Kuenzler 1965), and its activity is inducible by phosphorus depletion (Kuenzler and Perras 1965; Boni *et al.* 1989). This enzyme is a dimeric molecule of a high molecular weight of about 160 kDa (Chróst 1991), and is usually associated with the cell surface of microbial organisms (Cembella *et al.* 1984; González-Gil *et al.* 1998). Microbial utilization of DOP by means of enzymatic hydrolysis activity is considered one of the most important pathways in phosphorus cycling in marine ecosystems (Maeda and Taga 1973; Cembella *et al.* 1984; Suzumura *et al.* 1998). The amount of DOP hydrolyzed by phytoplankton is probably higher than that of bacteria in coastal areas

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judging from their biomasses (Valiela 1995; Nausch 1998; Tada *et al.* 1998). Pollehne *et al.* (1995) reported that the carbon of phytoplankton accounted for up to 64% of the particulate organic carbon. Free AP can be liberated into the environment from the lysis of dead phytoplankton cells and cells damaged by zooplankton grazing (Chróst 1991).

The goals of this study were to investigate concentrations of DIP and Chlorophyll *a* (Chl *a*) responsible for production of AP and to characterize the seasonal variations of DOP compounds hydrolyzed by AP (Alkaline Phosphatase Hydrolysable Phosphorus; APHP) in Hiroshima Bay. The amount of APHP was determined using AP extracted from phosphorus-deficient cells of *Alexandrium tamarense* and *Gymnodinium catenatum*. These two species were selected for the study because the toxic dinoflagellate *A. tamarense* has been reported to cause paralytic shellfish poisoning (PSP) every year in Hiroshima Bay since 1992 (Yamamoto and Tarutani 1997) and the alarming spread of *G. catenatum* is likely to harm the oyster culture in the near future (Oh *et al.* 2002). Moreover, the short-term monitoring of free AP was carried out to learn how it varied along with phytoplankton species succession during spring.

2. Materials and Methods

Field sampling and pretreatment

Seawater samples were collected with a Van Dorn water sampler at 3 stations in Hiroshima Bay in January, May, August and November 2000 (Stn 1-3; Fig. 1).

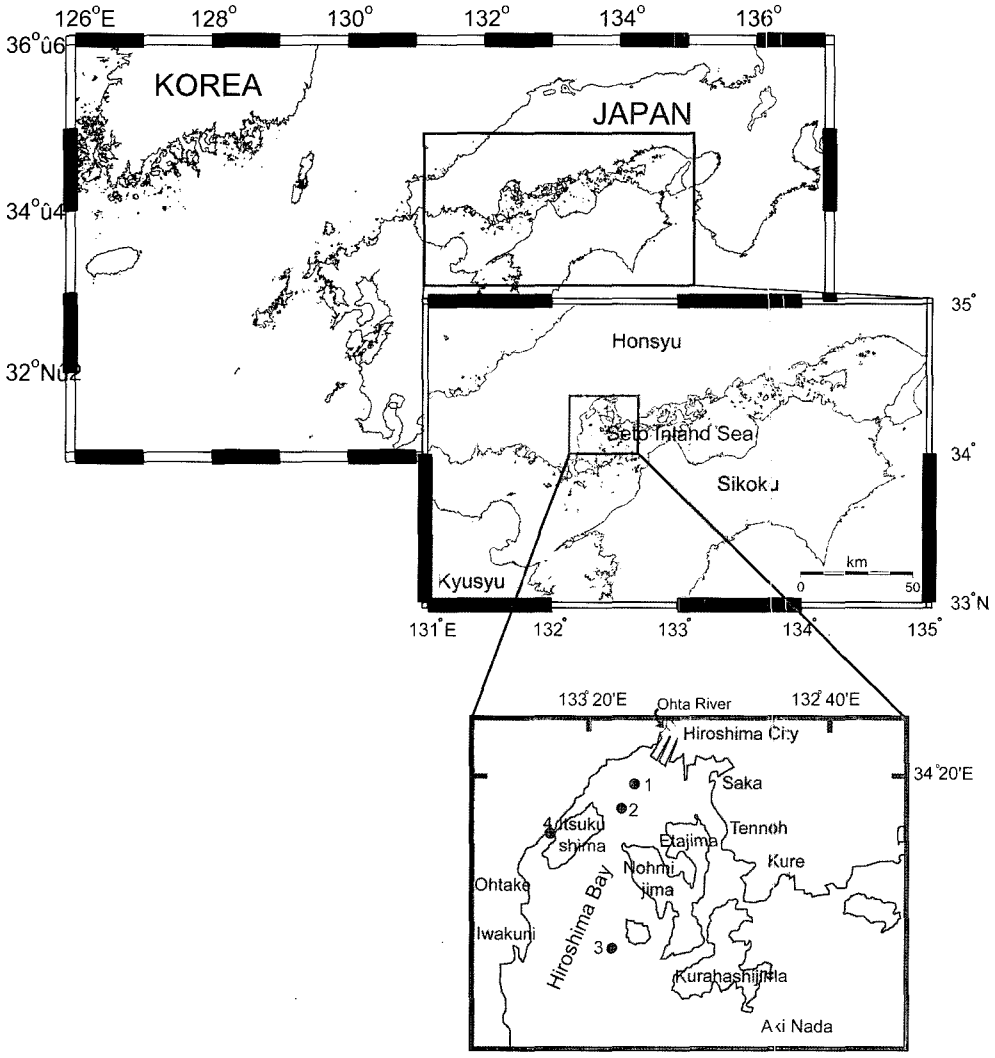

Fig. 1. Map showing the location of the sampling stations in Hiroshima Bay, Japan.

Seawater samples were also collected every week from the 1st of March to the 17th of May 1999, targeting the short-term variations in AP activity associated with phytoplankton species succession (Stn 4; Fig. 1). These samples were filtered through membrane filters (0.22 μm pore size, Millipore GSWP) under a low vacuum pressure (<15 cm Hg) to minimize physical damage to the fragile organisms. For analysis of the nutrients, 5 ml of $HgCl_2$ was added to 500 ml aliquot of the filtered sample (final conc. 100 ppm) to prevent microbial enzymatic activity after the filtration. All samples were stored at -20°C until the analysis. The DIP concentration was determined by the method described by Strickland and Parsons (1972). The TDP concentration was measured according to Koroleff's method (1983), and the DOP concentration was estimated by subtracting the amount of DIP from the amount of TDP. The Chl *a* concentration was determined with a fluorometer (Turner Designs Model 10-100R) according to the method of Holm-Hanson *et al.* (1965).

Measurement of free alkaline phosphatase activity in seawater

Free AP activity was measured by the standard colorimetric method recommended by Hernández and Whitton (1996). Ten milliliters of 0.22 μm-filtered seawater was incubated at 35°C for 1 hr after adding 1 ml mixed reagent (2500 μM *p*-nitrophenyl phosphate in 100 mM Tris-buffer of pH 8.0 and 0.2 M in $MgSO_4$). Measurement of the AP activity for the August samples failed due to a malfunction of the incubator during incubation. The reaction proceeding from the sample was terminated by cooling in an ice bath. The *p*-nitrophenol concentration accumulation after hydrolysis of *p*-nitrophenyl phosphate was determined as absorbance at 410 nm using a spectrophotometer (Hitachi, U-2001).

Phosphorus hydrolyzed by AP extracted from *A. tamarense* and *G. catenatum*

The P-deficient cells of the two toxic dinoflagellates isolated from Hiroshima Bay, *A. tamarense* and *G. catenatum*, were prepared for AP extraction by culturing the cells in an artificial seawater L1 medium (Keller *et al.* 1987; Guillard and Hargraves 1993) without phosphorus for 7 days. In the culture media, the orthophosphate concentration was less than the detection limit of 0.02 μM on the fourth day, and no cell growth was observed after the fifth day of incubation. Optimal growth culture conditions were set (Yamamoto and Tarutani 1997; Yamamoto *et al.* 2002b; Oh and Yoon 2004): temperature (15°C for *A. tamarense* and 25°C for *G. catenatum*), salinity (30 psu for both species) and light intensity (300 μmol m^{-2} s^{-1}; 12:12 hr light-dark cycle for both species). The P-deficient algal cultures (*ca.* 5×10^3 and 2×10^3 cells ml^{-1} for *A. tamarense* and *G. catenatum*, respectively) were centrifuged at 3000 rpm for 10 min, and then the supernatant was ultrafiltered with an Amicon Centricon Plus-80 (100 kDa) at 2500 rpm for 20 min. To remove the remaining low molecular weight compounds, especially DIP, the ultrafiltered solution was again dialyzed with a cellulose ester membrane (Spectrum Lab. Inc., 100 kDa), using 0.1 M ammonium sulfate and 1 mM magnesium chloride as the exterior solute. The dialysis period was 24 hrs at room temperature (*ca.* 20°C). The extracted alkaline phosphatase from *A. tamarense* and *G. catenatum* are called At-AP and Gc-AP, respectively. To determine the incubation time for degradation of DOP by the extracted AP, the following pre-examination was carried out (Fig. 2). After the addition of 1 ml of Gc-AP to 10 ml glycerophosphate-enriched (3 μM) Kuroshio seawater (DIP concentration of *ca.* 0.2 μM), the time change in the DIP concentration was monitored for 120 min. The increase in DIP after an incubation time of 60 min was used as a marker of hydrolyzed DOP. The liberated DIP concentration did not significantly increase at 90 min and 120 min. Thus, the incubation time of the APHP experiment was set at 60 min in the present study.

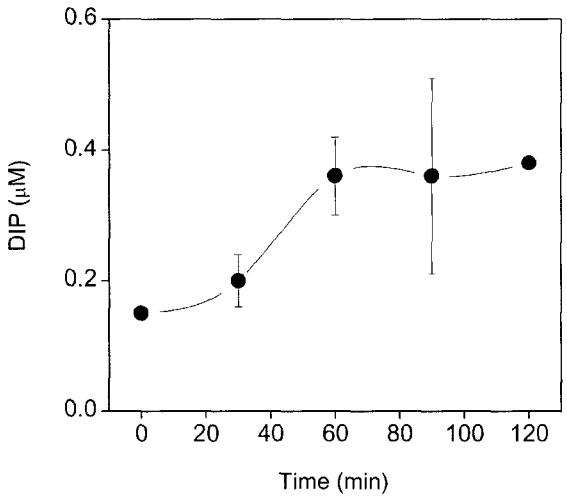

Fig. 2. Increase in the DIP concentrations following the addition of alkaline phosphatase, which was extracted from *Gymnodinium catenatum*, into Kuroshio water containing 3 μM glycerophosphate.

Thus, for the degradation of DOP, 0.1 ml of extracted AP solution was added to 10 ml of the filtered samples, and then was incubated at 35°C for 60 min as determined above. The APHP concentration was estimated from the difference in the DIP concentrations of the sample before and after the addition of AP. Measurements were carried out in duplicate. Hereafter, we will call the phosphorus fraction hydrolyzed by the At-AP and Gc-AP the At-APHP and Gc-APHP, respectively.

3. Results and Discussion

Seasonal variation of DIP, DOP, chlorophyll a and free AP activity

Both DIP and DOP showed different patterns in seasonal variation (Fig. 3). The concentration of DIP was low in May (0.00–0.13 μM) and relatively high in January, August and November (0.36±0.26 μM). The concentration of DOP was relatively high in August (0.75–2.12 μM) and low in January (ND–0.32 μM). The DOP in May and November ranged from 0.28 to 0.49 μM and from 0.15 to 1.22 μM, respectively. Thus, the amount of DOP as a part of TDP accounted for 32% in January; however, DOP was a significant part in May (88%), August (78%) and November (70%). The Chl *a* concentration showed a similar distribution pattern with AP activity (Fig. 4). It was quite low and homogeneous in January (1.03–2.02 μg l^{-1}) and May except at Stn. 1 (4.51 μg l^{-1}). In November, the Chl *a* concentration was high at the surface of Stns 1 and 2 (8.99 and 7.41 μg l^{-1}, respectively).

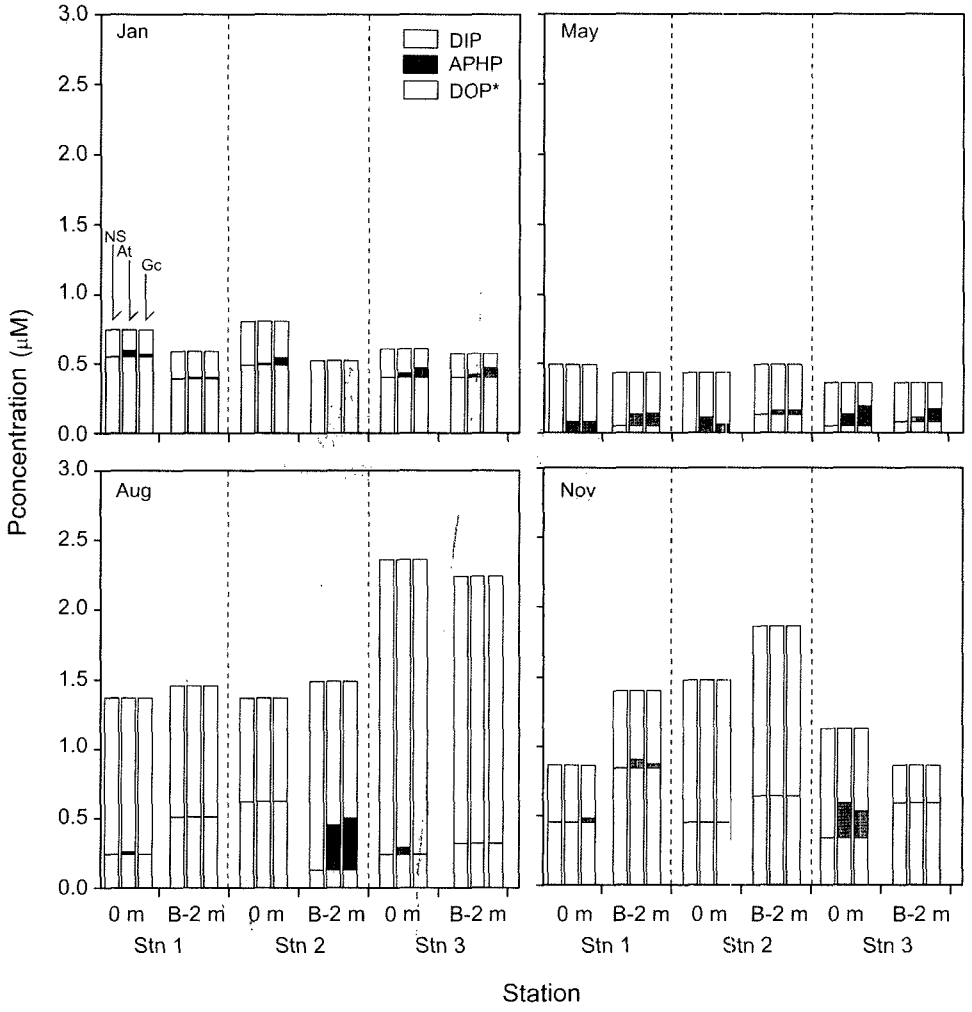

Fig. 3. The spatio-temporal variability in phosphorus hydrolyzed by alkaline phosphatase, which was extracted from *Alexandrium tamarense* and *Gymnodinium catenatum*. Values are represented as the average of two replicates. DOP* indicates the refractory fraction in DOP (total DOP minus hydrolyzed phosphorus). NS: natural seawater, At: *A. tamarense*, Gc: *G. catenatum*.

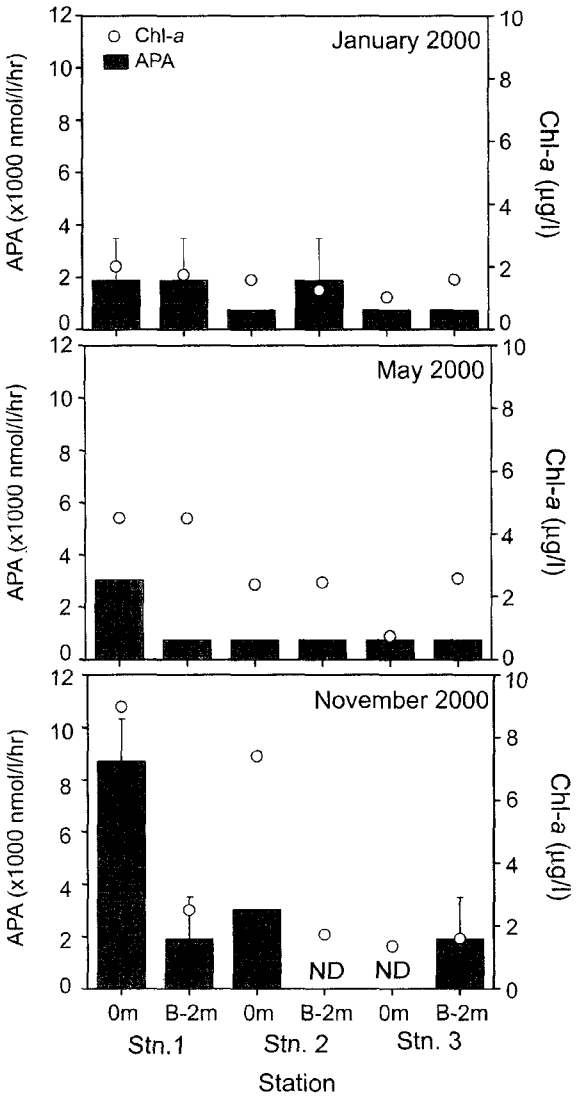

Fig. 4. Free alkaline phosphatase activity and concentration of chlorophyll *a* at each station. Values are represented as the mean and standard deviations of two replicates (ND: not detected).

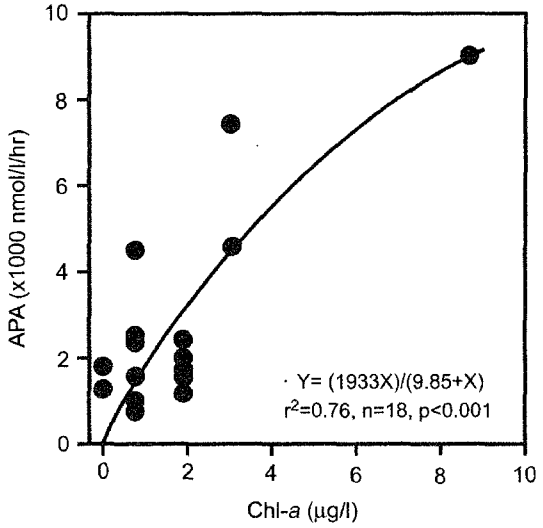

Fig. 5. Relationship between the free alkaline phosphatase activity and the concentration of chlorophyll *a*.

The free AP activity was low, ranging from 800 to 2500 nmol l^{-1} hr^{-1} at all the stations and at all the depths in January and May (Fig. 4). No free AP activity was detected at the bottom of Stn 2 and surface of Stn 3 in November, although very high free AP activity was observed at the surface of Stn 1 (8700 nmol l^{-1} hr^{-1}). These spatial and temporal patterns in the free AP activity and the Chl *a* concentration were validated statistically as shown in Fig. 5. The free AP activity was positively correlated with the Chl *a* concentration (r^2=0.76, n=18, P<0.001). In general, phytoplankton and bacteria are considered to be the major organisms that produce free AP in natural seawater (Nausch 1998). The AP of phytoplankton and bacteria is loosely bound to the cell surface and easily shed by simple filtration (Uchida 1992). In the present study, we filtered the seawater on-board; therefore, the bound AP of phytoplankton and bacteria would pass through the 0.22 μm filters. However, the biomass of phytoplankton was significantly higher than that of bacteria in Hiroshima Bay, where the nutrient condition is eutrophication (Tada *et al.* 1998), as found in other coastal areas (Nausch 1998). Our results showed that *ca.* 76% of the variation in free AP activity could be explained by the variation in Chl *a* concentration (Fig. 5), although a difference in reactions between bacteria and phytoplankton was not exactly distinguished because bacteria as well as phytoplankton can shed AP as a result of filtration. These findings suggest that a major part of the AP activity in the Hiroshima Bay seawater is coming from phytoplankton.

Amount of phosphorus hydrolyzed by At-AP and Gc-AP

At-APHP and Gc-APHP exhibited similar spatial and temporal variations (Fig. 3). In January and May, At-APHP and Gc-APHP were detected in both the surface and bottom waters at almost all stations and were substantially higher in May when DIP was depleted. On the other hand, in August and November, APHP was sporadically detected in samples. However, the APHP fractions were

much smaller compared to those in May, indicting that the availability of DOP for these species was low during these months. Even though the preference of DOP utilization might vary depending on the species composition in the phytoplankton assemblage, it could be supposed that some portion of DOP was not bioavailable in August and November. The freshwater discharge in the rainy season from June to July could be one of the causes increasing the concentration of non-bioavailable DOP.

However, dinoflagellate often blooms in the surface water during these periods because the stratification caused by freshwater discharge along with the increase in solar radiation would provide suitable conditions for dinoflagellates blooms. Moreover, bioavailable DOP in the bottom layer would support dinoflagellate blooms in these periods due to their swimming ability. In fact, *Karenia mikimotoi* formed a dense red tide in July 2000 just before sampling. Since *K. mikimotoi* is capable of utilizing phosphomonoesters as well as DIP (Yamaguchi and Itakura 1999), the reactive part of DOP in August 2000 might have been consumed by this species.

In the batch culture, both *A. tamarense* and *G. catenatum* showed maximum AP activity in the late log growth phase: 0.054 nmol cell^{-1} hr^{-1} and 0.11 nmol cell^{-1} hr^{-1}, respectively (Oh *et al.* 2002). In the present study, the cell density when AP was extracted was 5x10^3 cells ml^{-1} for *A. tamarense* and 2×10^3 cells ml^{-1} for *G. catenatum*. (See the Materials and Methods section.) Assuming that the cellular AP activity was at its maximum since we prepared the culture of *A. tamarense* and *G. catenatum* from the late log phase, the total AP activity is estimated to be *ca.* 270 μmol l^{-1} hr^{-1} and 220 μmol l^{-1} hr^{-1}, respectively. Since 0.1 ml portion of these AP solutions was added to a 10 ml seawater sample, the final AP activity of *A. tamarense* and *G. catenatum* is estimated to be *ca.* 2.7 μmol l^{-1} hr^{-1} and 2.2 μmol l^{-1} hr^{-1}, respectively. The AP activity of *A. tamarense* and *G. catenatum* at the peak of their blooms is estimated to be *ca.* 10^2 μmol l^{-1} hr^{-1}, if we assume the cell densities at the peak of the bloom to be 10^3 cells ml^{-1} (Hiroshima Prefecture 1995; Hallegraeff *et al.* 1989). Hence, much higher amounts of APHP would be expected to be available at their bloom peak in the field compared to the AP activity found in our experiment. Thus, in DIP-depleted conditions such as those of as Hiroshima Bay, the APHP fraction may play an important role for the survival and bloom formation in the noxious dinoflagellates.

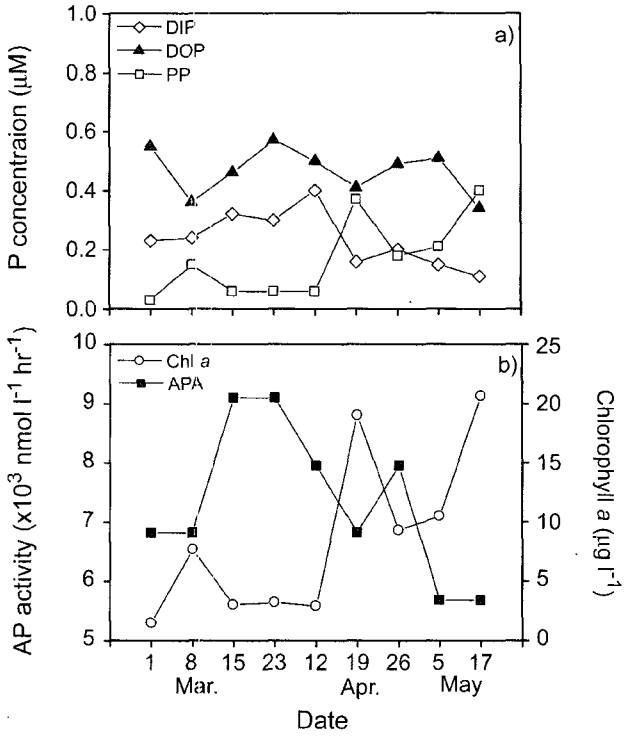

Fig. 6. (a) Short-term variation of dissolved inorganic phosphorus (DIP), dissolved organic phosphorus (DOP) and particulate phosphorus (PP) of the northern Hiroshima Bay. (b) Short-term variation of free alkaline phosphatase activity and chlorophyll *a* of northern Hiroshima Bay.

Short-term variations of free AP activity

Short-term monitoring of free AP activity along with species succession is shown in Fig. 6. The DIP and DOP concentrations ranged from 0.11 μM to 0.40 μM and from 0.34 μM to 0.57 μM, respectively. Particulate phosphate (PP), which is calculated assuming phytoplanktonic C: P = 106:1 and C: Chl *a*=25 (Parsons *et al.* 1984), showed less variation in March (*ca.* 0.08 μM), and increased with the decrease of DIP and DOP in April and May. The AP activity peak was found to have doubled from the 15th to the 23rd of March and the 26th of April (Fig. 6). At the two AP peak, the dominant species were *Eucampia zodiacus, Stephanopyxis* sp. and *Chaetoceros* spp. Although there are no reports that these species produce AP, *E. zodiacus* and *Stephanopyxis* sp. are supposed to have shed AP into the environment. The decrease of AP activity from the 12th to the 19th of April corresponded to the appearance of the dinoflagellates *A. tamarense* and *Protoperidinium* spp., and, from the 5th to the 17th of May, corresponded to the appearance of *Prorocentrum micans* and *Protoperidinium* sp.

During the periods of decreased AP activity, a simultaneous decrease in both DIP and DOP was observed. The decreases in DIP and DOP from the 12th to the 19th of April were 0.24 μM and 0.09 μM, respectively (Fig. 6), amounting to 0.33 μM in total. During the period from the 5th to 17th of May, the decreases in DIP and DOP were 0.05 μM and 0.17 μM, respectively, amounting to 0.22 μM in total. On the other hand, an increase in phytoplankton biomass in terms of Chl *a* concentration was apparent during these periods. The increases in PP during these periods are estimated to be 0.32 μM and 0.2 μM, respectively. These estimated values for the formation of PP coincide closely with the decrease in the total dissolved phosphorus fraction, indicating that the amount of decreased dissolved phosphorus could have been taken up by phytoplankton assemblages, although transportation processes such as the movement of water mass and the DIP release from organic matter by bacterial decomposition should be taken into consideration. The results of our observations might also suggest that the growth of dinoflagellates in this season may be partly supported by the AP produced by the other diatoms.

The DOP utilized by dinoflagellates in natural seawater are not well characterized and the extent to which they are utilized is poorly understood. Quantitative estimation of available P compounds in natural seawater and the DOP uptake kinetics of these phytoplankton remains to be investigated.

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