Marine Biology 49, 223-229 (1978)

MARINE BIOLOGY

Phosphatase Activity in Eutrophic Tokyo Bay

N. Taga and H. Kobori

Ocean Research Institute, University of Tokyo; Nakano, Tokyo, Japan

Abstract

Alkaline phosphatase activity in seawater samples taken from Tokyo Bay was measured by both spectrophotometric and fluorometric methods. A stratified distribution pattern of the enzyme was observed in August, a vertically mixed pattern in December. The distribution of phosphatase activity in the eutrophic seawater was paralleled by variations in other parameters, such as viable counts of bacteria, chlorophyll *a* content, inorganic and total phosphorus concentrations, amounts of seston, particulate deoxyribonucleic acids (DNA) and protein. A significant correlation between phosphatase activity and these measurements indicated that the enzyme was a good indicator of the degree of eutrophication. The positive relationship between phosphatase and inorganic phosphorus indicates that enzyme activity was not inhibited at inorganic phosphorus levels present in the bay and that production of phosphatase by microorganisms inhabiting the bay was not repressed at the inorganic phosphorus levels in the bay. Culture experiments revealed that the formation of repressible phosphatase by bacteria isolated from the bay was not affected by the inorganic phosphorus levels in the bay.

Introduction

There has been much discussion about eutrophication of natural waters, arising from the recent growing concern for environmental problems. It is increasingly evident that not only chemical factors but also enzyme activities are instrumental in determining the degree of eutrophication of waters. In lakes, Stewart *et al.* (1971) indicated that nitrogenase activity was high in eutrophic waters, but low in oligotrophic waters. Jones (1972a, b) demonstrated a positive correlation of phosphatase activity with degree of eutrophication in 16 eutrophic lakes in England. For the marine environment, however, no data is available correlating amount of enzyme activity with degree of eutrophication. Although enzyme activity, especially phosphatase activity in seawater, has been measured by several authors (Wai *et al.*, 1960; Perry, 1972), their data was obtained only from oligotrophic regions of the ocean.

Water pollution in Tokyo Bay has resulted in drastic changes in the phytoplankton and bacterial communities. Marumo and Murano (1973) studied the succession of plankton diatoms in Tokyo Bay, and concluded that the water of the bay was slightly eutrophic in 1947 and has become extremely so in recent years. According to these investigators, frequent red tides of diatoms in Tokyo Bay have been observed since the 1950's. As a result, during the period 1947 to 1973, the diatom standing crop has abnormally increased in the inner Tokyo Bay, whereas the number of diatom species, especially oceanic forms, has decreased rapidly.

In contrast, numbers of marine bacteria have increased with the degree of eutrophication in Tokyo Bay, and the number of heterotrophic bacteria has attained the order of 10^5 ml^{-1} (Taga *et al.*, unpublished data). The generic composition of the heterotrophic bacteria has also been investigated (Simidu *et al.*, 1977): *Acinetobacter* spp. predominate in the inner regions, and *Vibrio* spp. at the mouth of Tokyo Bay and adjacent regions; the latter constitute only a small population in the inner bay.

The present paper assesses the eutrophication of Tokyo Bay in terms of alka-

0025-3162/78/0049/0223/$01.40

line phosphatase activity. In addition, the factors contributing to the positive relationship observed between phosphatase activity and phosphorus content in the bay are discussed.

Materials and Methods

Water Samples

Seawater samples were collected on cruises from July 1971 to August 1973 by the R.V. "Tansei-maru", Ocean Research Institute, University of Tokyo. The sampling stations are shown in Fig. 1. Surface-water samples were collected with sterile 500 ml glass bottles, and deeper-water samples with sterilized ORIT samplers (Taga, 1968) attached to Nansen bottles. These samples were used for microbiological analyses. Samples for phosphatase and the other chemical analyses were obtained with Nansen bottles and Van Dorn samplers, and were stored immediately at -25°C or after filtration procedures, depending on the analyses to be made.

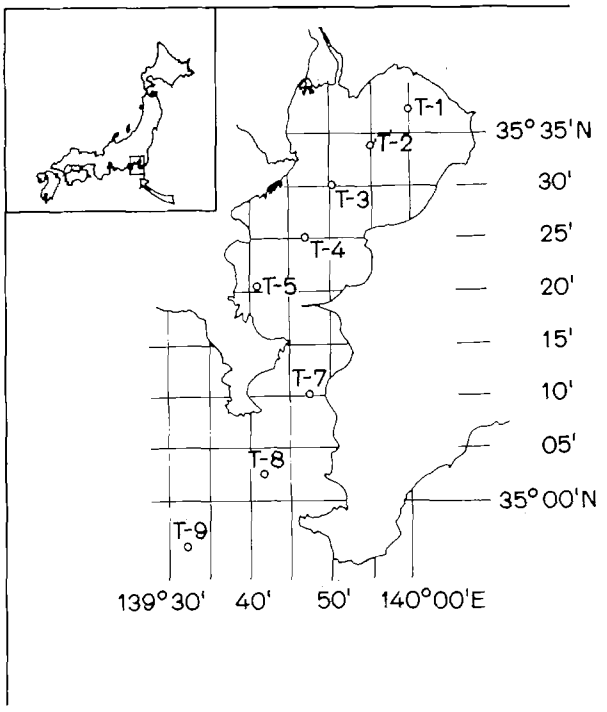

Fig. 1. Location of sampling stations in Tokyo Bay

Enumeration of Bacteria

Each 0.1 ml portion of serially diluted water samples was spread with a glass-rod spreader onto an agar-plate of PPES-II medium (Taga, 1968). The bacterial colonies that appeared on the plates were counted after incubation for 2 weeks, and the numbers were expressed as colony-forming units (cfu). For the enumeration of phosphatase-producing bacteria, the procedure outlined by Parker (1963) was employed using agar-plates of PPES-II as medium.

Measurement of Phosphatase Activity

Two assay procedures were used to determine alkaline phosphatase activity:

Method 1. The spectrophotometric method was employed for the seawater samples collected in 1971, and the assay was based on the release of phosphorus from sodium β-glycerophosphate. A 100 ml portion of unfiltered seawater was incubated with 5 x 10^5 M sodium β-glycerophosphate, 0.02 M Tris HCl buffer, pH 8.2, and 5 ml chloroform was used as bacteriostatic agent. The enzyme reaction was stopped with N NaOH after incubation for 24 or 48 h at 28°C, and the inorganic phosphorus released was estimated by the method described below.

Method 2. The fluorometric method was used for the unfiltered samples collected in 1972 and in 1973. The assay was based on the hydrolysis of the monophosphate-ester of 3-O-methylfluorescein phosphate, and the procedure was essentially that described by Perry (1972)

The rate of reaction was linear both with respect to time and substrate concentration under these assay conditions. Dissolved phosphatase activity was determined by the fluorometric method described above, after seawater samples had been filtrated through a Millipore filter (HA, 0.45 µm pore size).

Measurements of Other Factors

Inorganic phosphorus (Pi) and total phosphorus (total P) determinations were carried out using the procedure outlined by Murphy and Riley (1962). Chlorophyll *a* concentrations were measured by fluorometry (Yentsch and Menzel, 1963). Amounts of seston collected on Millipore filters (HA-type) after filtering seawater samples were measured by the method of Strickland and Parsons (1972). Amounts of particulate DNA and protein collected on the Millipore filters were estimated by the methods of Holm-Hansen (1969) and Lowry *et al.* (1951), respectively.

Culture Experiments on Formation of Repressible Phosphatase

Experiments were carried out using *Vibrio* spp. isolated from Tokyo Bay. Organisms were cultivated on media of the following composition per liter. Medium A was composed of casamino acid, 0.5 g; yeast extract, 0.2 g; sodium succinate, 0.5 g; $(NH_4)_2SO_4$, 1 g; NaCl, 12.9 g; KCl, 0.75 g; $MgCl_2 \cdot 6H_2O$, 2.6 g; ferric citrate, 5 x 10^{-5} g; $ZnCl_2$, 5 x 10^{-5} g; $NaHPO_4$, 0.3 g. Medium B was identical to Medium A except that 5 g of sodium succinate was added, and 0.1 M Tris was substituted for phosphate.

After overnight incubation on a reciprocal shaker in Medium A, the cells were centrifuged at 15,000 x *g* at 0°C and then the cells were resuspended in a new Medium A for 18 h. After centrifugation, they were inoculated into a 500 ml Sakaguchi flask containing Medium B. The cells were then incubated at room temperature (ca. 20°C), with reciprocal shaking to ensure adequate aeration. At intervals, a 20 ml portion was extracted for measurement of bacterial growth, inorganic phosphorus concentration, and phosphatase activity. Growth was followed at 660 nm in the spectrophotometer. Inorganic phosphorus was measured by the method of Murphy and Riley (1962). The enzyme activity was measured as the release of p-nitrophenol from P-nitrophenylphosphate. One milliliter of culture medium was incubated with 1 ml of 5 mM substrate solution and 2 ml of 0.5 M Tris HCl buffer, pH 8.2. After incubation for 30 or 60 min at 28°C, p-nitrophenol content was measured at 400 nm in the spectrophotometer.

Results

Distribution of Phosphatase Activity

Alkaline phosphatase activity of unfiltered seawater samples taken from Tokyo Bay was determined, using sodium β-glycerophosphate as a substrate (Method 1), in August and December 1971. Fig. 2 shows the profile of phosphatase activity in the bay. Different distributional patterns were observed for these two months: a stratified pattern was observed in August, a vertically mixed pattern in December. This may reflect changes in the physical condition of the seawater. In fact, water temperature and salinity profiles displayed the same trend as that of phosphatase in both August and December (Fig. 3). Phosphatase activity in August was approximately five times higher than that in December

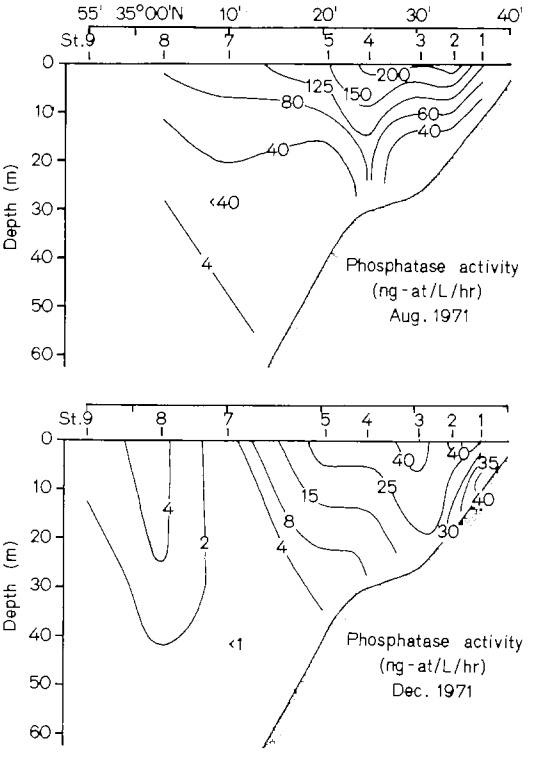

Fig. 2. Vertical distribution of phosphatase activity in seawater of Tokyo Bay during summer and winter, 1971. Phosphatase activity was determined using sodium β-glycerophosphate (Method 1)

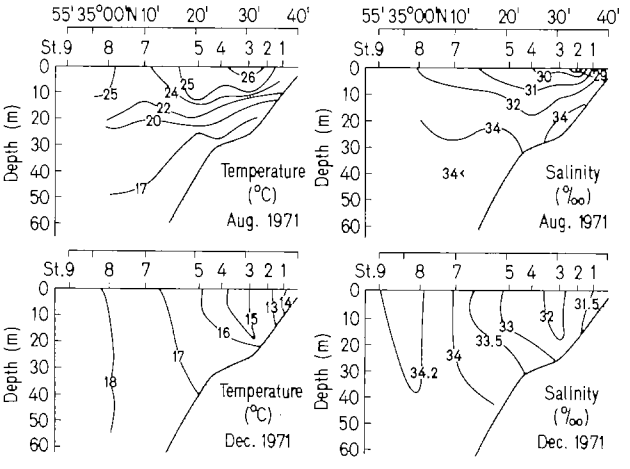

Fig. 3. Profiles of water temperature and salinity in Tokyo Bay during summer and winter, 1971

(Fig. 2). Fig. 2 also indicates that phosphatase activity at the head of the bay differed from that of the mouth of the bay (i.e., activity in the inner region at Stations T-1, T-2, T-3, T-4 and T-5 was approximately four times higher in August and ten times higher in December, compared with the mouth-of-the-bay Stations T-7, T-8 and T-9). Phosphatase

activity decreased as the waters became deeper, and also closer to the mouth of the bay.

The distribution of phosphatase activity was also determined by the fluorometric method, using stagnant seawater, in July 1973 (Fig. 4A). Similar distributional patterns were obtained using the two methods, although measured phosphatase activity was different between the two methods.

Distributions of the other parameters, such as phosphatase-producing bacteria, heterotrophic bacteria, chlorophyll *a*, and Pi and total P concentrations were also examined during the period of stagnation in July 1973 (Fig. 4). The viable counts of phosphatase-producing bacteria was as high as 10^5 ml^{-1} in surface waters of the inner regions of the bay, which is 1000-fold higher than in waters at the mouth of the bay. On the average, 40% of the heterotroph population were found to be phosphatase producers. The distributions of both heterotrophs and phosphatase producers were quite similar to that of phosphatase activity shown in Fig. 4A.

An extremely high value of chlorophyll *a* was observed in the inner region of the bay (Fig. 4D). The mean chlorophyll *a* content in the inner region of the bay (Stations 1-5) was 498 µg l^{-1}, 300-fold higher than that at the mouth of the bay. The distributional pattern of chlorophyll *a* parallels the pattern of phosphatase activity. The amounts of chlorophyll *a* and the numbers of heterotrophic bacteria in the inner bay suggest that excessive biological production occurs in the inner Tokyo Bay. Pi and total P concentrations in the bay were also higher in the inner regions, and their distributional patterns were also fairly similar to that of phosphatase (Fig. 4E, F).

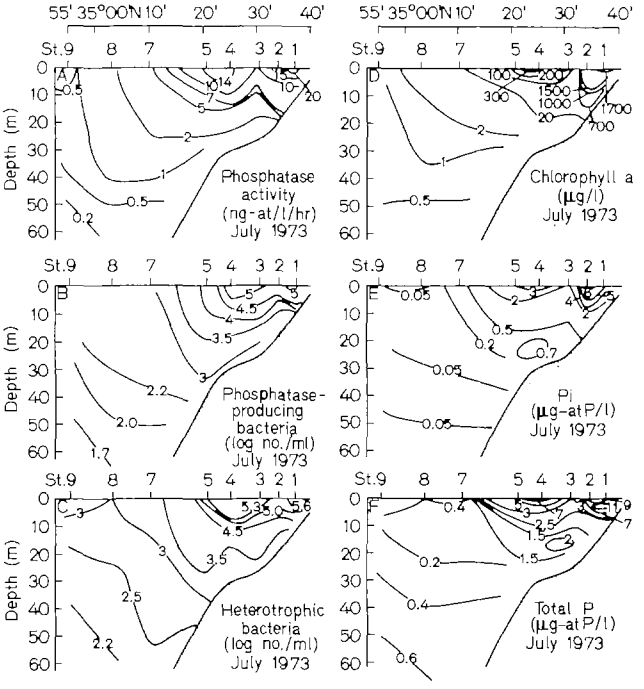

Fig. 4. Vertical distribution of phosphatase activity and other parameters in stagnant seawater from Tokyo Bay in July, 1973. Phosphatase activity was estimated using 3-O-methylfluorescein phosphate (Method 2)

Correlation between Phosphatase Activity and Other Parameters

In order to clarify the relationship between phosphatase and other parameters, correlation coefficients of the data collected from the bay for all depths and locations during the periods of stratification in 1972 and 1973 were calculated. The logarithm of phosphatase activity was found to correlate at the 0.1% significant level with the logarithm of numbers of phosphatase-producing bacteria and that of chlorophyll *a* content in the bay, as shown in the scatter diagrams of Fig. 5A and B, respectively. This indicates that a considerable portion of the phosphatase present

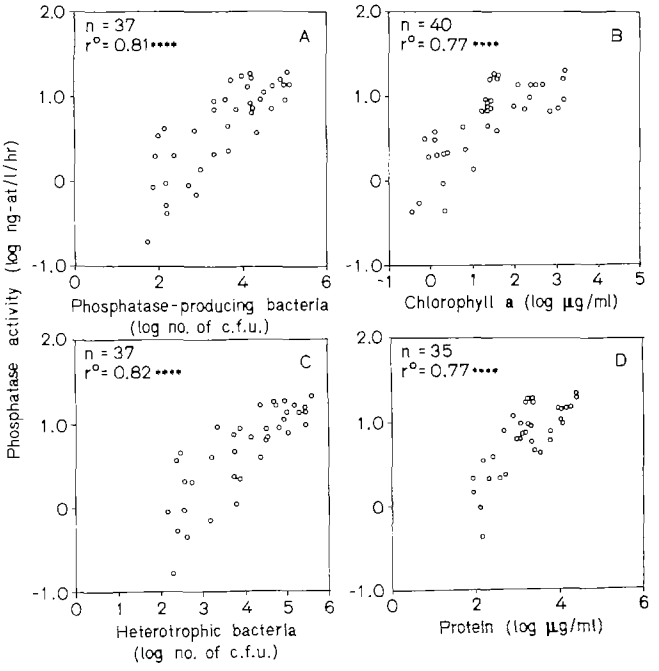

Fig. 5. Relationship between phosphatase and (A) viable counts (c.f.u.) of phosphatase-producing bacteria, (B) chlorophyll *a* content, (C) viable counts of heterotrophic bacteria, (D) particulate protein. Asterisks indicate significance at 0.1% level and *n* indicates sample numbers

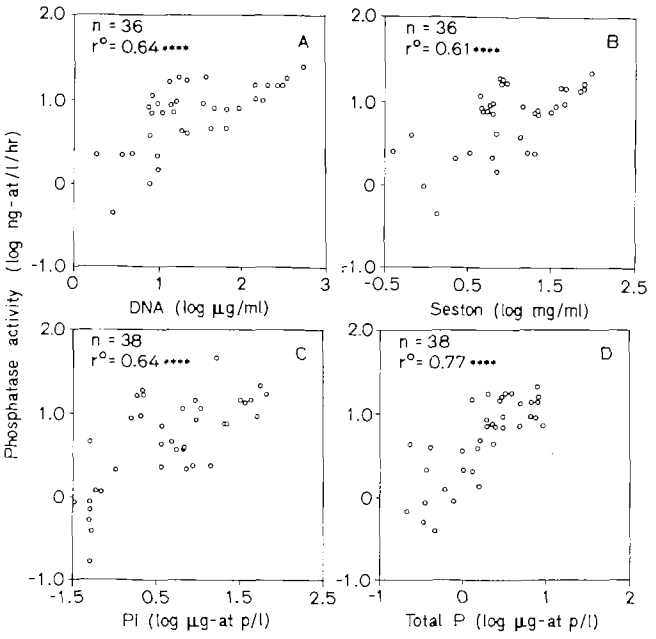

Fig. 6. Relationship between phosphatase and (A) particulate DNA, (B) seston, (C) Pi, (D) total P

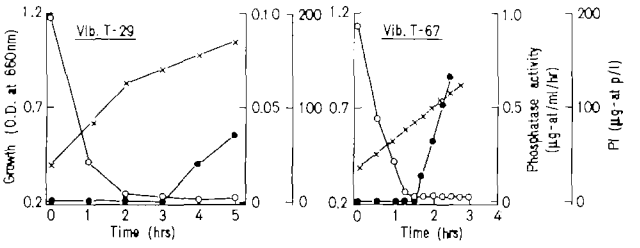

Fig. 7. Influence of inorganic phosphorus concentration in the medium on phosphatase formation of two marine *Vibrio* isolated from Tokyo Bay. Crosses: optical density (O.D.) of growth at 660 nm; filled circles: phosphatase activity in culture medium; open circles: Pi concentration in supernatant of culture medium. Phosphatase activity was measured by using p-nitrophenylphosphate as a substrate. Enzyme activity is expressed as µg-at P l^{-1} h^{-1}

in the seawater is produced by bacteria and phytoplankton inhabiting the bay. Phosphatase activity was also highly correlated (0.1% significance level) with both phytoplankton biomass and bacterial biomass estimated from chlorophyll *a* content and heterotrophic bacteria (Fig.5B,C).

Phosphatase activity was also correlated with chemical parameters such as particulate protein (Fig. 5D), DNA (Fig. 6A), and seston (Fig. 6B), Pi (Fig. 6C) and total P (Fig. 6D).

Influence of Pi on Formation of Repressible Phosphatase by Bacteria Isolated from Tokyo Bay

The positive relationship between phosphatase and Pi suggests that phosphatase did not exhibit end-product repression at Pi levels in Tokyo Bay. To confirm that repression of phosphatase by microorganisms does not occur at the Pi levels present in eutrophic seawater, the formation of repressible phosphatase by bacteria isolated from Tokyo Bay was examined under culture conditions. Fig. 7 shows that phosphatase formation by two marine *Vibrio* spp. was repressed at the beginning of incubation when excess phosphorus was present in the medium. Phosphorus concentration was reduced along with the bacterial growth, and formation of the enzyme began when the phosphorus concentration in the medium reached 5 to 6 µg-at P l^{-1}. This experiment indicates that phosphatase of bacteria in the bay was repressed at phosphorus levels above 7 µg-at P l^{-1}. As the phosphorus concentrations in Tokyo Bay do not exceed 6 µg-at P l^{-1}, repression of phosphatase by bacteria might not occur in the bay. However, there is some possibility that repression partially occurs in natural seawaters where Pi concentrations exceed 7 µg-at P l^{-1}.

Dissolved and Particulate Phosphatase in Seawater

The activity of phosphatase in both a dissolved and particulate state was measured separately in the inner region of Tokyo Bay (Table 1). The average activity of the dissolved enzyme comprised only 15% of the total activity. The proportion of dissolved enzyme to total phosphatase increased with depth at all stations, except at Station 2. At all stations, however, particulate phosphatase activity closely paralleled total phosphatase activity.

Discussion

Phosphatase activity was detected in all seawater samples examined. The results indicate that seawater has a potential ability to mineralize monophosphate esters. Since concentrations of monophosphate esters ranged from 0.08 to 0.35 µg-at P l^{-1} at Station 3 in Tokyo Bay, which accounted for 13 to 32% of the total organic phosphorus present in seawater (Kobori and Taga, unpublished data),

Table 1. Dissolved and particulate phosphatase activity in inner regions of Tokyo Bay, July 1972. Phosphatase activity was measured using 3-O-methylfluorescein phosphate as a substrate

Station no.	Depth (m)	Phosphatase activity (ng-at P l^{-1} h^{-1}) Total	Particulate	Dissolved	Dissolved activity Total activity (%)
1	0	19.03	17.43	1.60	8.4
	8	16.49	14.30	2.19	13.3
2	0	18.18	16.90	1.28	7.0
	10	16.87	16.11	0.76	4.5
3	0	9.28	8.12	1.16	12.5
	5	9.63	7.84	1.79	18.6
	20	11.62	7.67	3.95	34.0
4	0	7.01	6.58	0.43	6.1
	5	7.88	7.05	0.84	10.5
	25	2.31	1.51	0.80	34.6
				Average:	15.0

phosphatase in Tokyo Bay would play a role in mineralization of organic phosphorus present in seawater.

Phosphatase activity in unfiltered seawater in Tokyo Bay would encompass both free and organism-associated enzymes of mixed populations. Some free enzymes might be secreted by extracellular phosphatase-producing microorganisms and others might be liberated from non-viable and lysed cells. Organism-associated phosphatases of bacteria and algae are localized at their surfaces and these surface phosphatases can hydrolyze organic phosphorus extracellularly (Aaronson and Patni, 1976). Therefore, phosphatase activity in unfiltered seawater may be used as an indicator of the enzymatic potential in water samples and of the eutrophic state of the water mass.

Phosphatase activity was significantly correlated with the number of heterotrophic bacteria and total P levels in the bay (Figs. 5C and 6D). The number of heterotrophic bacteria is related to the eutrophic state of Tokyo Bay (Taga *et al.*, unpublished data). The total P concentrations in seawater have been used as an index of the potential fertility of water (e.g. Armstrong and Harvey, 1950). Therefore, the phosphatase activity in Tokyo Bay also reflects the size of the bacterial population present in the bay, which in turn is closely related to the total P levels and the degree of eutrophication. Jones (1972a) reported similar results for 16 lakes of the English Lake District. He concluded that alkaline phosphatase is a good indicator of biomass and of degree of eutrophication in lake water.

Phosphatases in some bacteria (Torriani, 1959; Shah and Blobel, 1967) fungi (Kadner *et al.*, 1968) and higher plants (Hewitt and Tatham, 1960) are produced when Pi concentrations become limiting and are repressed under conditions of excess Pi. Kuenzler and Perras (1965) also showed that Pi-starved marine algae displayed greatly increased amounts or levels of alkaline phosphatases. These phosphatases exhibited end-product repression. Moreover, the activities of phosphatases are known to be inhibited by Pi (Reichardt *et al.*, 1967; Jones, 1972a). The evolutionary rationale for the development of these mechanisms is obvious, and it would therefore be of some interest to demonstrate whether the phenomena of production, repression and inhibition operate on an ecological scale or not.

Berman (1970) implied the existence of a repression-inhibition mechanism with respect to phosphorus in the natural environment, by showing that a reciprocal relationship existed between the level of phosphatase activity per unit biomass and the concentration of total phosphorus in Lake Kinneret of Israel. In the present survey, however, no such reciprocal relationship was found. On the contrary, a positive relationship was noticed between phosphatase activity and total P concentrations.

Perry (1972) measured the alkaline phosphatase activity in oligotrophic waters of the subtropical Central North Pacific Ocean and interpreted the presence of naturally produced phosphatase by microorganisms as an indication of phosphate deficiency. In this study, however, the presence of phosphatase did not reflect the absence of Pi in Tokyo Bay. On the contrary, a positive relationship was found between phosphatase levels and Pi levels in eutrophic Tokyo Bay (Fig. 6C). Therefore, alkaline phosphatase can not be used as an indicator of phosphorus deficiency of microorganisms in eutrophic seawaters.

At least three factors might be considered to explain the fact why no reciprocal relationship between phosphatase and Pi concentrations was observed in Tokyo Bay: (1) Although Pi in Tokyo Bay is present in concentrations which represent threshold levels causing repression

of phosphatase, the turnover rate of phosphorus in eutrophic waters is faster than that in oligotrophic waters, and competition for available Pi among microorganisms is also keen in eutrophic waters; therefore, repression of phosphatase might not occur in Tokyo Bay. (2) A quarter of the phosphatase-producing bacteria in Tokyo Bay was comprised of constitutive phosphatase-producers, and these bacteria were capable of producing phosphatase even when the external Pi concentration exceeded 50 mg-at P l^{-1} under culture conditions (Kobori, unpublished data); these constitutive phosphatases would probably not be repressed at the Pi levels in Tokyo Bay. (3) Phosphatase activity in Tokyo Bay would not be inhibited at the Pi levels present in the bay. Reichardt *et al.* (1967) found that phosphatase in lakes was not inhibited by Pi at a concentration up to 10 µg-at P l^{-1}; in Tokyo Bay, where the maximum Pi concentration is not likely to exceed 6 µg-at P l^{-1}, the phosphatase is conceivably not subject to phosphorus inhibition.

Acknowledgements. The authors wish to express their sincere gratitude to Professor R.Y. Morita, Oregon State University, for reviewing the manuscript. Appreciation is also extended to Dr. M. Maeda of our laboratory for estimation of DNA, and also to Mr. M. Funakoshi, Tokyo Metropolitan University, for measuring chlorophyll *a* content. We also wish to thank the staff of the R.V. "Tansei-maru", for assistance in collecting samples. This work was supported in part by a grant from the Ministry of Education of Japan.

Literature Cited

Aaronson, S. and N.J. Patni: The role of surface and extracellular phosphatases in the phosphorus requirement of *Ochromonas*. Limnol. Oceanogr. *21*, 838-845 (1976)

Armstrong, F.A.J. and H.W. Harvey: The cycle of phosphorus in the waters of the English Channel. J. mar. biol. Ass. U.K. *29*, 145-162 (1950)

Berman, T.: Alkaline phosphatase and phosphorus availability in Lake Kinneret. Limnol. Oceanogr. *15*, 663-674 (1970)

Hewitt, E.G. and P. Tatham: Interaction of mineral deficiency and nitrogen source on acid phosphatase activity in leaf extracts. J. exp. Bot. *11*, 367-376 (1960)

Holm-Hansen, O.: Determination of microbial biomass in ocean profiles. Limnol. Oceanogr. *14*, 740-747 (1969)

Jones, J.G.: Studies on fresh water bacteria — association with algae and alkaline phosphatase activity. J. Ecol. *60*, 59-75 (1972a)

– Studies on freshwater microorganisms — phosphatase activity in lakes of differing degrees of eutrophication. J. Ecol. *60*, 777-791 (1972b)

Kadner, R.J., J.F. Nyc and D.M. Brown: A repressible alkaline phosphatase in *Neurospora crassa*. J. biol. Chem. *243*, 3076-3082 (1968)

Kuenzler, E.J. and J.P. Perras: Phosphatases of marine algae. Biol. Bull. mar. biol. Lab., Woods Hole *128*, 271-284 (1965)

Lowry, O.H., N.J. Rosebrough, A.F. Farr and R.J. Randall: Protein measurement with the Folin phenol reagent. J. biol. Chem. *193*, 265-375 (1951)

Marumo, R. and M. Murano: Succession of plankton diatoms in Tokyo Bay. La Mer (Bull. Soc. franco-jap. Océanogr.) *11*, 70-82 (1973)

Murphy, J. and J.P. Riley: A modified single solution method for the determination of phosphate in natural waters. Analytica chim. Acta *27*, 31-36 (1962)

Parker, A.B.C.: Classification of Micrococci and Staphylocci based on physiological and biochemical tests. J. gen. Microbiol. *30*, 409-427 (1963)

Perry, M.J.: Alkaline phosphatase activity in subtropical Central North Pacific waters using a sensitive fluorometric method. Mar. Biol. *15*, 113-119 (1972)

Reichardt, W., J. Overbeck and L. Steubing: Free dissolved enzymes in lake waters. Nature, Lond. *216*, 1345-1347 (1967)

Shah, D.B. and H. Blobel: Repressible alkaline phosphatase of *Stapylococcus aureus*. J. Bact. *94*, 780-781 (1967)

Simidu, U., E. Kaneko and N. Taga: Microbiological studies of Tokyo Bay. Microb. Ecol. *3*, 173-191 (1977)

Stewart, W.D.P., T. Mague, G.P. Fitzgerald and R.H. Burris: Nitrogenase activity in Wisconsin lakes in differing degrees of eutrophication. New Phytol. *70*, 497-509 (1971)

Strickland, J.D.H. and T.R. Parsons: Determination of the weight of microscopic materials in seawater. *In:* A practical handbook of seawater analysis, rev. ed. pp 181-184. Ottawa: Fisheries Research Board of Canada 1972. (Bull. Fish. Res. Bd Can. No. 167)

Taga, N.: Some ecological aspects of marine bacteria in the Kuroshio Current. Bull. Misaki mar. biol. Inst. (Kyoto Univ.) *12*, 65-76 (1968)

Torriani, A.: Influence of inorganic phosphate in the formation of phosphatases by *Escherichia coli*. Biochim. biophys. Acta *38*, 460-479 (1959)

Wai, N., T.C. Hung and Y.H. Lu: Alkaline phosphatase in Taiwan sea water. Bull. Inst. Chem. Acad. sin. *3*, 1-10 (1960)

Yentsch, C.S. and D.W. Menzel: A method for the determination of phytoplankton chlorophyll and phaeophytin by fluorescence. Deep-Sea Res. *10*, 221-231 (1963)

Dr. N. Taga
Ocean Research Institute
University of Tokyo
1-15-1, Minamidai, Nakano-ku
Tokyo 164
Japan

Date of final manuscript acceptance: July 14, 1978. Communicated by M. Anraku, Nagasaki