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Studies on Dissolved Metalloenzymes in Lake Water. II. Seasonal Variations in Phosphatase Activity in Lake Kasumigaura

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Alkaline phosphatase activity in Lake Kasumigaura was determined periodically and characterized by high-performance liquid chromatography and isoelectric focusing electrophoresis. There are two peaks in dissolved alkaline phosphatase activity; the bigger one in summer and the smaller one in winter. Molecular weight of ca. 89000 and isoelectric points 5—6 were found for most phosphatases in samples collected during the summer. A considerable part of the winter alkaline phosphatases, however, have much higher molecular weights, and major isoelectric points for these were ca. 4. These alkaline phosphatases were metalloenzymes activated by zinc. The observations above suggest that there are seasonal changes in the dominant microbial species producing dissolved alkaline phosphatase; i.e., blue-green algae, a procaryote, in summer and diatoms and flagellates, eucaryotes, in winter. The peak of alkaline phosphatase activity in lake water was predictive of a following increase in algal species therein.

Phosphorus is recognized as a key to fertilization in natural waters, i.e., orthophosphate is one essential nutrient for microorganisms such as algae and bacteria. When studying the cycle of phosphorus and the regulation of microorganisms, alkaline phosphatase activity should be considered in both its intracellular and extracellular (dissolved) forms.

Alkaline phosphatase (orthophosphoric monoester phosphohydrolyase with activity optimum in alkaline pH) is a typical metalloenzyme, containing several zinc ions at its active sites, and these zinc ions are essentially related to the appearance of phosphatase activity.[1] In most organisms, from primitive procarytes such as bacteria to eucaryotes such as mammals, alkaline phosphatase is found mostly in their periplasmic spaces.[2,3]

Alkaline phosphatase activity in lake waters[4] and seawater[5-7] has been reported by several researchers: Reichardt et al. first determined phosphatase activity not only in unfiltered freshwater but also in water which had been filtered through a 0.6 μm membrane. This suggested the existence of extracellular (dissolved) phosphatase in lake waters.[8] Since then, several researchers have found activity attributed to dissolved phosphatase in lake waters[9-11] and seawaters,[12] and have considered its role in the phosphorus cycle in natural waters.[13,14] In the previous paper,[15] it was confirmed that dissolved phosphatase activity in natural waters was due to the metalloenzymes (zinc-containing alkaline phosphatase).

In the present paper, seasonal variations in alkaline phosphatase in Lake Kasumigaura are examined by high performance liquid chromatography and isoelectric focusing electrophoresis.

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Field Description[16]

Lake Kasumigaura, the second largest lake in Japan, is situated in the eastern part of the Kanto Plain, about 50 km north-east of Tokyo. The lake basin is smooth and shallow, with a surface area of 171 km^2, a mean depth of 4 m, and a maximum depth of 7.4 m near the mouth of Takahamairi Bay. Due to the shallowness of the water and its constant agitation by wind, distinct thermal stratifications do not develop even in summer, except for a few calm days. Heavy blooms of blue-green algae are to be seen over the whole lake in summer. The bloom usually starts at the innermost parts of both Takahamairi and Tsuchiurairi Bays, which receive nutrient-rich inflowing waters, and gradually spreads to the center of the lake. By August, the bloom covers the whole lake.

Experimental

Chemicals. *E. coli* alkaline phosphatase purchased from Boehringer-Mannheim Co. (No. 15429) was used as a standard; its specific activity is 20 U mg^{-1} and molecular weight (M. W.) 89000.[12] In addition to *E. coli* alkaline phosphatase, the following enzymes purchased from Boehringer-Mannheim Co. were used as molecular weight standards for high performance liquid chromatography (HPLC): Urease (Jack bean; M. W. 480000); ferritin (indicator protein; M. W. 450000); Bovine carbonic anhydrase (M. W. 31000); and cytochrome c (indicator protein; M. W. 12500). To determine alkaline phosphatase activity, 1 mM (M=mol dm^{-3}) *p*-nitrophenyl dihydrogen phosphate (*p*-NPP) dissolved in 0.6 M tris(hydroxymethyl)methanamine (Tris) hydrochloride buffer (pH 8.0) was used as a substrate. LBK Ampholine Carrier Ampholite (pH 3.5—10) was used as an isoelectric focusing solution.

Instruments. A Shimadzu UV-210A spectrophotometer with a SFU-6 semiauto flow cell unit (optical path of absorption cell: 10mm) was used to determine enzymatic activity. A Shimadzu LC-3A high performance liquid chromatograph was used for HPLC separation, using Shimadzu aqueous

gel column Type W-71; detection was accomplished by using an SPD-2A spectrophotometric detector. An LBK Ampholine 8100-1 Electrofocusing Equipment (column volume: 110 mL) with a power supply (Atto Co., V-C Stabilizer SJ-1061; maximum voltage: 1000 V) was used for isoelectric focusing. Ultrafiltration was performed with an Amicon Diaflo Cell Type 202. A Thermostatic Bath Circulator (Thomas Scientific Co., Model TRL-111SP), a fraction collector (Toyo Kagaku Sangyo Co., Signal Fracon SF-60L), and a peristaltic pump (Toyo Kagaku Sangyo Co., Signal Pump TMP-6L) were also used in HPLC and electrophoresis fractionation.

Pretreatment of Lake Water Samples. Lake water was sampled at 10 points in Lake Kasumigaura (Ibaraki Prefecture), once a month as from April 1980 through March 1982 (see Fig. 1). The collected water was filtered with glass fiber membrane filters (pore size: 1.0 μm), and then filtered with Toyo Kagaku UK-2 membrane filters (pore size: 0.45 μm) or UK-4 (pore size: 0.2 μm) to exclude bioorganisms. All the filtrations were performed immediately after sampling, and the samples were then stored in a cold room (4 C).

An unfiltered sample, to which 2 v/v% of chloroform was added, was also kept in a cold room to determine total phosphatase activity.

Before separation by HPLC or electrophoresis, the 0.45 μm-filtered sample was concentrated ca. 1000 times by ultrafiltration, using a Toyo Kagaku UK-10 type ultrafilter, with which only molecules larger than M. W. 10000 can be concentrated. Samples were kept under constant temperature at 4 C throughout ultrafiltration and storage. Experimental vessels and containers were cleaned and sterilized by heating to 110 C, by soaking in 40% ethanol aqueous solution and/or by soaking in 7 M nitric acid.

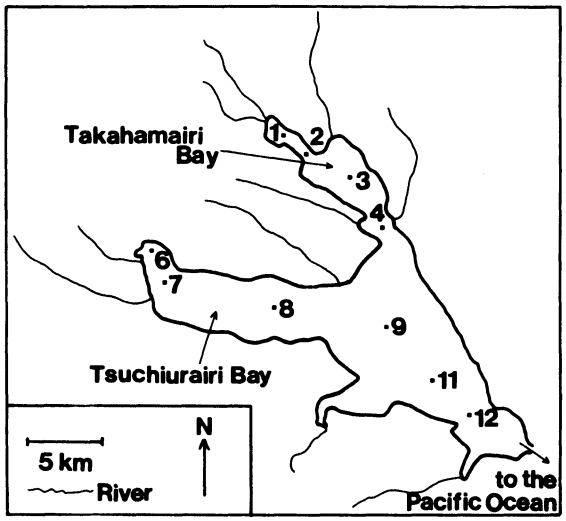

Fig. 1. Sampling stations in Lake Kasumigaura.

Determination of Phosphatase Activity. Alkaline phosphatase catalyzes the hydrolysis of *p*-NPP to *p*-nitrophenol:

$$O_2N\text{-}C_6H_4\text{-}OPO_3H_2 + H_2O \rightarrow O_2N\text{-}C_6H_4\text{-}OH + H_3PO_4 \quad (1)$$

The rate of production of the *p*-nitrophenol is followed spectrophotometrically and provides a measure of the concentration of the catalyst. Dissolved alkaline phosphatase activity was determined by using 0.2 μm-filtered lake water as a sample, as described in our previous paper.[15] Total alkaline phosphatase activity was determined in the same way as dissolved alkaline phosphatase activity, using an unfiltered sample; the *p*-NPP substrate was saturated with chloroform for its bacteriostatic effect.[17]

HPLC Chromatograms. For HPLC separation, two aqueous porous gel columns (both Type W-71) were available, one column 7.9 mm i.d.×30 cm and the other 7.9 mm i.d.×50 cm. Either column was used or both were joined to make an 80-cm length. Instead of the phosphate buffer, which is generally used as a carrier for the W-71 column, 0.1 M Tris-HCl buffer (pH 8.0) was used for determining the phosphatase activity of the untreated effluent, because phosphate ion inhibits phosphatase activity. The effluent was fractionated every 0.5—1.0 min (0.5—1.0 cm^3) with a fraction collector, and the phosphatase activity of the thirty or so individual fractions was then measured.

Isoelectric Focusing Chromatograms. Isoelectric focusing was carried out in accordance with the instruction manual of LKB Ampholine 8100 electrofocusing equipment. The solutions used in the present experiment are shown in Table 1. The system was thermostated, and electrofocusing was carried out at 4 C for 48 h. After the electrode solutions had been removed from the column, the electrofocused solution was fractionated every 2.5 cm^3 with a peristaltic pump and a fraction collector. The phosphatase activities of the individual fractions were determined by the method described earlier.

Results

Distribution of Phosphatase Activity in Lake Kasumigaura. Total phosphatase activity (TPA) and dissolved phosphatase activity (DPA) in Lake Kasumigaura from April, 1981 to March, 1982

Table 1. Solutions Used for Isoelectric Focusing

Solution	Content		Total Volume
Dense gradient solution	Sucrose	27 g	54 cm^3
	Ampholine	2 cm^3	
	concd. sample plus water	35 cm^3	
Light gradient solution	Sucrose	2.7 g	54 cm^3
	Ampholine	0.7 cm^3	
	concd. sample plus water	50.3 cm^3	
Cathode solution	0.25 M NaOH	10 cm^3	10 cm^3
Anode solution	Sucrose	15 g	25 cm^3
	0.25 M H_2SO_4	16 cm^3	

Table 2. Phosphatase Activities in Lake Kasumigaura from April, 1981 to March, 1982 (activity: nmol dm^{-3} min^{-1})

Sta.		Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar
1	DPA[a]	5.1	2.0	3.1	1.3	0.9	0.8	1.0	0.6	0.8	1.2	1.2	1.5
	TPA[b]	27.1	18.0	20.7	14.2	18.9	16.0	17.5	11.5	14.6	26.0	18.9	19.6
2	DPA	1.5	3.2	3.4	4.3	1.0	1.7	0.4	2.3	1.0	1.3	1.0	2.2
	TPA	14.7	41.2	56.2	17.6	12.8	18.5	10.5	26.5	37.3	24.5	24.3	21.4
3	DPA	1.7	2.3	0.9	3.7	2.1	0.4	<0.4	1.1	0.9	1.6	1.4	5.1
	TPA	19.8	29.2	14.5	12.4	11.0	7.0	9.7	18.9	34.4	17.3	32.1	23.0
4	DPA	3.1	4.8	0.4	1.0	1.0	0.4	0.4	<0.4	1.0	1.2	1.4	3.1
	TPA	20.3	40.0	12.7	11.3	10.3	10.4	11.8	12.6	37.5	12.7	19.3	14.3
6	DPA	1.7	1.9	1.3	0.7	0.9	0.5	0.4	0.6	0.9	1.2	0.5	1.4
	TPA	9.1	23.2	23.0	12.1	19.6	13.7	5.5	3.7	14.6	8.7	6.9	5.6
7	DPA	2.9	4.1	0.7	1.9	1.0	<0.4	0.4	0.9	1.9	1.1	0.6	1.2
	TPA	13.1	82.2	18.3	16.6	15.3	13.7	8.9	14.2	35.4	11.4	15.0	8.4
8	DPA	1.9	5.4	0.7	1.1	1.5	0.5	0.6	1.0	1.4	1.5	0.9	1.7
	TPA	18.3	61.2	18.6	9.1	11.5	9.6	11.8	20.2	37.5	13.0	18.5	10.8
9	DPA	2.4	3.2	1.7	1.2	0.9	<0.4	<0.4	0.5	1.5	1.2	0.8	1.7
	TPA	18.4	42.8	45.9	4.6	6.9	12.0	9.7	11.0	42.9	17.8	22.4	14.5
11	DPA	—	4.3	1.2	2.7	<0.4	<0.4	<0.4	<0.4	1.5	0.9	1.1	1.6
	TPA	—	38.2	33.0	6.8	5.0	6.5	8.7	13.9	37.1	11.9	17.0	10.8
12	DPA	—	3.6	1.3	1.1	0.8	<0.4	<0.4	1.4	1.5	0.9	0.5	1.8
	TPA	—	32.0	32.7	10.5	5.0	7.2	10.1	19.7	52.0	15.1	17.5	15.3

a) DPA=Dissolved phosphatase activity. b) TPA=Total phosphatase activity.

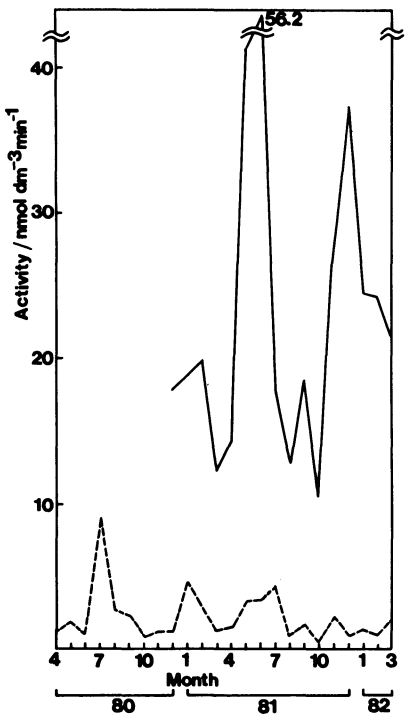

Fig. 2. Seasonal variations in total and dissolved phosphatase activities at Sta. 2 in Lake Kasumigaura. ——: Total alkaline phosphatase activity; -----: dissolved alkaline phosphatase activity.

are shown in Table 2. Average DPA levels in Lake Kasumigaura were ca. 1 nmol dm^{-3} min^{-1}, about the same as those determined in the Shinobazu pond[15] and in Lake Kinneret.[6] At each location, average TPA was about ten times higher than DPA, but phosphatase activity near the edge of the lake ((e.g., Stations (Stas.) 1, 2, 6, and 7)) was greater than in the center of the lake (e.g., Sta. 9). This trend was more obvious in the summer than in spring.

Seasonal Variations in Phosphatase Activity. Seasonal variations in DPA and TPA at Sta. 2 in Lake Kasumigaura are shown in Fig. 2. Very distinct peaks were observed in summer, and other smaller peaks were observed in winter. The patterns of the two curves are almost the same.

Alkaline phosphatase activity in lake water in all seasons was diminished when samples were treated with chelating agents, such as 2,6-pyridinedicarboxylic acid; the activity was restored on addition of zinc ion (see Table 3.)

HPLC Chromatograms. HPLC chromatograms of concentrated lake water sample in August and December, 1980, are shown in Figs. 3 and 4. In summer (Fig. 3), a very distinct single peak was seen at the elution time of *E. coli* alkaline phosphatase. The chromatograms for winter (Fig. 4) through spring had more complicated features, showing the presence

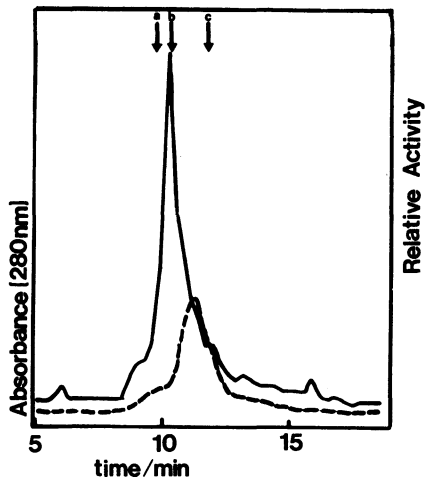

Fig. 3. HPLC chromatograms of concentrated lake water collected in August, 1980.
Sample: concentrated (×2300) water sample collected in Lake Kasumigaura (Sta. 2) on August 16, 1980; sample volume: 100 mm^3; column: Shimadzu aqueous porous gel column W-71 (7.9 mm i.d.×50 cm); carrier: 0.01 M Tris-HCl buffer (pH 8.0); flow rate: 1.0 $cm^3 min^{-1}$.
——: Phosphatase activity; -----: UV absorbance at 280 nm. Elution time of standard proteins are as follows:
a: Urease (M.W.: 480000); b: *E. coli* alkaline phosphatase (M.W.: 89000); c: Bovine carbonic anhydrase (M.W.: 31000).

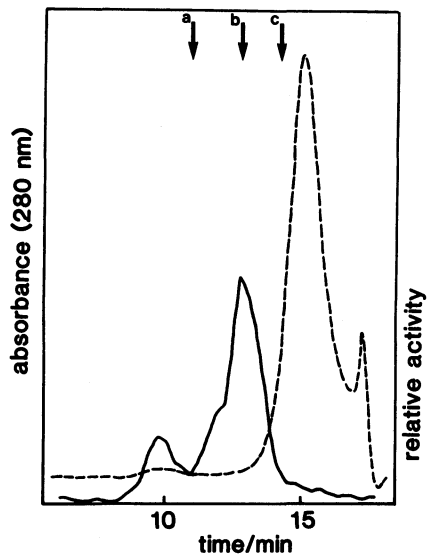

Fig. 4. HPLC chromatograms of concentrated lake water collected in December, 1980.
Sample: concentrated (×1400) water sample collected in Lake Kasumigaura (Sta. 2) on December 22, 1980; sample volume: 200 mm^3, column: Shimadzu W-71 (7.9 mm×50 cm); carrier: 0.1 M Tris-HCl buffer (pH 8.0); flow rate: 1.0 $cm^3 min^{-1}$.
——: Phosphatase activity; -----: absorbance at 280 nm. a: Ferritin (M.W.: 450000); b: *E. coli* alkaline phosphatase (M.W.: 89000); c: Bovine carbonic anhydrase (M.W.: 31000).

Table 3. Recovery Ratios of Phosphatase Activity when Various Metal Ions Added to Lake Water Treated with Chelating Agent

Metal ion	Relative activity	
	August, 1980[a)]	May, 1981[b)]
None	0%	0%
Manganese(II)	41	25
Cobalt(II)	32	27
Nickel(II)	4	18
Copper(II)	9	0
Zinc(II)	100	100
Cadmium(II)	4	0

a) Cited from Ref. 15. b) A mixture of 33 mm^3 of concentrated (×440) lake water (Sta. 2) and 17 mm^3 of 10 mM 2,6-pyridinedicarboxylic acid (pH 7.0) was used as an "apo-sample." Fifty μg of each metal ion(II) was added to the apo-sample.

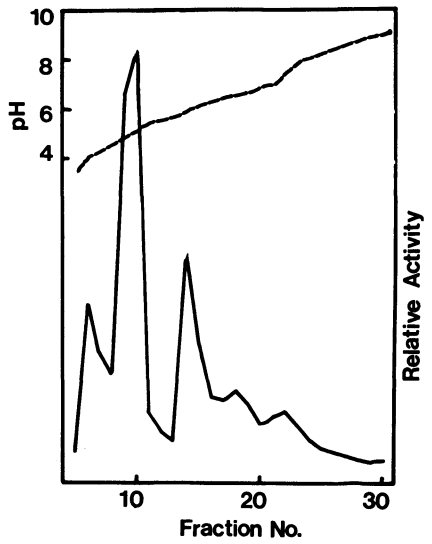

Fig. 5. Isoelectric focusing chromatogram of concentrated lake water for October, 1980.
Sample: concentrated water sample collected in Lake Kasumigaura (Sta. 2) on October 22, 1980.

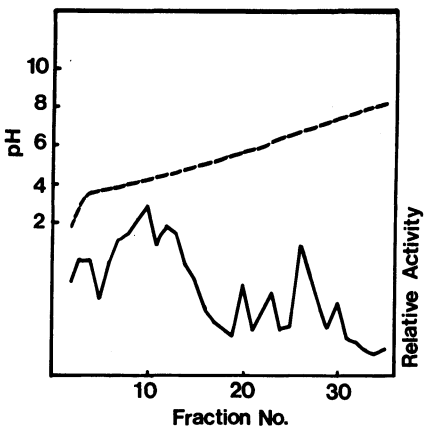

Fig. 6. Isoelectric focusing chromatogram of concentrated lake water for April, 1981.
Sample: concentrated water sample collected in Lake Kasumigaura (Sta. 2) on April 21, 1981.

of alkaline phosphatase with a greater molecular weight than those of *E. coli*.

Isoelectric Focusing Chromatograms. Figures 5 and 6 show the isoelectric focusing (IEF) chromatograms of concentrated lake water in October, 1980 and April, 1981, individually. All the chromatograms had several peaks of phosphatase activity, but the isoelectric points (pI) of the peaks showing high phosphatase activity were not the same. In summer and fall (Fig. 5), the peaks of phosphatase activity at pI 5—6 were significant, but those at ca. pI 4 were dominant in other seasons (Fig. 6). These results show seasonal changes in the composition of the isozymes of alkaline phosphatase in Lake Kasumigaura.

Discussion

Horizontal and Vertical Distribution of Phosphatase Activity. Lake Kasumigaura is notable as one of the most fertilized lake in Japan. This can be attributed mainly to its shallowness and the large inflow of nutrient-rich waters. The shallowness of the lake and the constant agitation of the water by the wind mean that there is little vertical variation of phosphatase activity in the water. Thus vertical variations of phosphatase activity can be ignored.[15]

As shown in Table 2, phosphatase activity varied appreciably with sampling location. Coastal waters in Takahamairi Bay (Stas. 1 and 2) or Tsuchiurairi Bay (Sta. 6) generally gave high TPA and DPA values that seemed to relate to the degree of fertilization or the biomass of microorganisms.

Seasonal Changes in Phosphatase Activity. In Lake Kasumigaura, heavy blooms of *Microcystis aeruginosa*, a species of blue-green algae, are to be seen covering the whole lake in summer. Other algae such as diatoms and flagellates can not breed during this period. When the blue-green algae decrease in autumn, other algae can increase.[18] The correlation between the peaks of DPA and of the biomass can be seen; the peak of DPA in summer corresponded to that of blue-green algae; the smaller peaks of DPA in winter and spring might correspond to those of diatoms and flagellates. The peaks of DPA (or TPA) slightly preceded the bloom of algae. This suggests that algae produce more alkaline phosphatase before multiplying.

Bacteria may also put alkaline phosphatase into the lake water, although the biomass of bacteria is much less than that of algae.[19] Thus, while most of the DPA originated from algae, DPA originating from bacteria could not be ignored.

HPLC and IEF Chromatograms. The seasonal variations described above are also shown as the HPLC and IEF chromatograms. Figures 3—6 show that various isozymes of alkaline phosphatase were present as dissolved enzymes in the lake water. The very distinct single peak in the HPLC chromatograms for summer (Fig. 3) is believed to be *Microcystis aeruginosa* alkaline phosphatase, whose molecular weight is close to that of *E. coli* alkaline phosphatase (89000). Alkaline phosphatases of higher molecular weight (eluted earlier than *E. coli* alkaline phosphatase) are seen in Fig. 4. These appear to be alkaline phosphatase originating from eucaryotes such as diatoms and flagellates. The eucaryotic alkaline phosphatase, unlike *E. coli* alkaline phosphatase, are glycoproteins, whose amino acid compositions, however, are strikingly similar to that of *E. coli* alkaline phosphatase.[20] Consequently eucaryotic alkaline phosphatase seems to elute rather sooner than might be estimated from the calibration curve for simple proteins.

There were several peaks in each IEF chromatogram, but the dominant peaks differed according to the sample. Unlike the HPLC chromatogram, which gave only a single dominant peak, a number of major peaks appeared in the IEF chromatogram for summer and fall (Fig. 5). This suggests that the procaryotic alkaline phosphatases, which could not be separated using a method based on molecular weight, can be separated using electrophoresis. The positions of the dominant IEF peaks (ca. pI 4) for winter and spring were markedly different from those for summer and fall (pI 5—6), and this also reflected the seasonal changes in the algae in Lake Kasumigaura.

Conclusion

Phosphatase activity in Lake Kasumigaura varied seasonally. The peak of phosphatase activity seemed to predict the increase in a certain species of algae. In addition to the change in total activity, a change in isozymes was observed in the HPLC and IEF chromatograms. This was interpreted as being caused by seasonal changes in phosphatase in natural water in Lake Kasumigaura; i.e., procaryotes (blue-green algae) in summer and eucaryotes (such as diatoms and flagellate) in winter and spring.

The various alkaline phosphatases found in the different seasons were all zinc-containing metalloenzymes, and are therefore also of interest as a significant source of zinc in natural water.[15,21] This suggests that other heavy metals may similarly be found in biochemical substances in natural water.

The amount of phosphatase in natural water is influenced not only by the biomass of microorganisms producing enzymes, but also by orthophosphate.[22] The nature of the relationship between alkaline phosphatase activity and concentration of various phosphorus compounds and the role of alkaline phosphatase in phosphorus cycles in lake water will be considered in the following paper.

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