Studies on Dissolved Metalloenzymes in Lake Water. III. Correlation between Dissolved Alkaline Phosphatase and Orthophosphate in Lake Water

Kensei KOBAYASHI,[†1] Kimikazu IWASE,[†2] Shinya HASHIMOTO,[†3] Hirotaka UEDA,[†4] Kitao FUJIWARA, Akira OTSUKI,[†] Keiichiro FUWA,[†] and Hiroki HARAGUCHI*
Department of Chemistry, Faculty of Science, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113
[†]Division of Chemistry and Physics, National Institute for Environmental Studies, Yatabe, Ibaraki 305
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Alkaline phosphatase activity (both dissolved and total) in Lake Kasumigaura was determined periodically together with concentrations of phosphorus compounds therein. Distinct negative correlation was found between the dissolved alkaline phosphatase activity and the orthophosphate concentration during the phosphorus-limited season. The decrease of the concentration of dissolved alkaline phosphatase was observed at the level of 1 ng ml^{-1} of orthophosphate. The present results suggest that both alkaline phosphatase in vivo and dissolved alkaline phosphatase in the lake water play important roles in phosphorus cycles in natural water.

Alkaline phosphatase in natural water was first studied in the context of the phosphorus cycle. In 1938, Steiner first suggested the possibility of the ecological participation of phosphatases in hydrolyzing phosphate esters to orthophosphate for phytoplankton growth in lakes.[1] Since then, several investigations on intercellular alkaline phosphatase have been carried out.[2-4] Alkaline phosphatase activity remaining in filtered water was first reported by Overbeck et al.[5] Not only total (cell-associated plus dissolved) phosphatase activity but also dissolved phosphatase activity per se, have been discussed in relation to their roles in the hydrolysis of phosphatase esters in sea water[6-8] and freshwater.[9-14] The correlation between phosphatase activity and other biological variables such as plankton biomass and chlorophyll *a* were reported as being positive.[15] However, the correlation between phosphatase activity and phosphate concentration has not been clarified. Stevens and Parr concluded that phosphatase activity could not be used as a reliable indicator of algal growth under conditions of phosphorus starvation.[16] Kobori and Taga reported a positive relationship between phosphatase activity and the inorganic phosphorus content of ocean sediment.[17] Berman reported that no correlation was found between them,[10] in spite of the fact that the production of alkaline phosphatase in bacteria is induced by phosphate-deficient media, and suppressed by phosphate-sufficient media.[18]

It has been confirmed in the previous papers[19,20] that dissolved phosphatase activity in sea and lake water is caused by zinc-containing metalloenzyme *alkaline phosphatase*. The seasonal changes in alkaline phosphatases and their sources (algae and bacteria) in Lake Kasumigaura have also been discussed previously.[21] In the present paper, the correlation between phosphatase activity and phosphorus concentration has been studied by using the data obtained through periodic analysis for two years in Lake Kasumigaura. The role of alkaline phosphatase in the phosphorus cycle in the lake is also discussed.

Experimental

Chemicals and Instruments. All the reagents used in the experiments were of analytical reagent grade.

Glassfilter (Whatman GF/C, pore size: 1.0 μm; diameter: 47 mm) was sterilized by heating at 450 °C for 3 h. The membrane filter was purchased from Toyo Kagaku Sangyo Co. (Type TM-4, pore size: 0.20 μm; diameter 47 mm). A Millipore filter holder (Type XX15-046-00) and a Millipore miniature vacuum-press pump Type XX61-000-00) were used for filtration, under mild suction (such that the suction flask pressure was no less than 250 Torr (1 Torr=133.322 Pa)). A thermostatic bath (Hirayama Manufacturing Co., Model TR-900) was used to incubate the sample-substrate mixture.

A Shimadzu UV-210A spectrophotometer with an SFU-6 semi-auto flow cell unit (optical path of the absorption cell: 10 mm) was used to determine phosphatase activity. Concentrations of orthophosphate were determined with a Technicon Autoanalyzer Type II, using the method of Murphy and Riley.[22]

Sampling and Pre-Treatment of Lake Water Samples. Lake water samples were collected with plastic Van Dorn bottles at 10 sampling stations on Lake Kasumigaura (shown in Figs. 2 and 3) once a month from April, 1980 through March, 1982. The sampling depths were: 0.5 m at every station (April, 1980-April, 1981); 1.5 m and 0.5 m at Stations 4 and 9 (April, 1980-April, 1981); and 1.0 m at every station (May, 1981-March, 1982).

[†1] Present address: Laboratory of Chemical Evolution, University of Maryland, College Park, Maryland 20742, U.S.A.

[†2] Present address: Science and Technology Agency, Kasumigaseki, Chiyoda-ku, Tokyo 101.

[†3] Present address: National Research Institute for Metals, Nakameguro, Meguro-ku, Tokyo 153.

[†4] Present address: Sumitomo Metal Mining Co., Ltd., Suehiro-cho, Ome, Tokyo 188.

The samples were cooled in an ice box immediately after being taken. Most of them were then filtered as soon as possible, as shown in Fig. 1. A portion of each sample, to which 2 v/v% of chloroform was added, was kept unfiltered to determine *total phosphatase activity* and *total phosphorus concentration*. A water sample filtered through the 1.0 μm pore size glassfiber membrane filter was used to determine *total dissolved phosphorus concentration* and *orthophosphate concentration*. A sample filtered through the 0.2 μm-membrane filter was used for the determination of *dissolved phosphatase activity*. All the filtered samples were stored at a constant temperature of 0 °C until analyzed.

Determination of Phosphatase Activity and Phosphorus Concentration. Procedures for determination of phosphorus concentration and phosphatase activity are shown in Fig. 1. Total phosphatase activity (TPA) and dissolved phosphatase activity (DPA) were determined by using *p*-nitrophenyl dihydrogen phosphate as a substrate, as described in the previous paper.[21]

Total phosphorus concentration (TP) and total dissolved phosphorus concentration (TDP) were determined with a Technicon Autoanalyzer after peroxodisulfate digestion in an autoclave at 120 °C for 45 min using a culture bottle (50 ml) with a polycarbonate screwcap.[23] Orthophosphate concentration (Pi) was determined with the Autoanalyzer using an unautoclaved filtered sample. The particulate phosphorus concentration (PP) was obtained by subtracting TDP from TP. The dissolved organic phosphorus concentration (DOP) was obtained by subtracting Pi from TDP.

Results and Discussion

Horizontal and Vertical Distribution of Phosphatase Activity and Phosphorus Concentration. Table 1 shows data for phosphatase activity and phosphorus concentrations in Lake Kasumigaura in July, 1980 and January, 1981. TP in Lake Kasumigaura was around 100 μg-P l^{-1} or more, much higher than the eutrophication level of 20 μg-P l^{-1}. No significant change in phosphatase activity and phosphorus concentration vs. vertical position was found, except for the phosphorus concentration at Station 4 in summer, which was higher at a depth of 5 m than at shallower levels. The vertical homogeneity of Lake Kasumigaura is due to the shallowness of the water and the constant agitation of the water by the wind. The increase in phosphorus at the 5.0 m depth at Station 4 in summer may be linked to the release of phosphate into the lake water from the sediments.[24]

Figures 2 and 3 show the horizontal distributions of phosphorus concentration and phosphatase activity,

Table 1. Phosphatase Activity and Phosphate Concentrations in Lake Kasumigaura in July, 1980 and January, 1981

Date	Sta.	depth m	DPA nM min^{-1}	TP ng ml^{-1}	PP ng ml^{-1}	DTP ng ml^{-1}	Pi ng ml^{-1}	DPA/PP mmol/g/min
Jun. 21, 1980	1	0.5	3.9	147	121	26	2	0.032
	2	0.5	9.0	155	127	28	2	0.071
	3	0.5	4.9	118	95	23	2	0.052
	4	0.5	1.1	87	68	19	< 2	0.016
		1.5	1.3	81	65	16	< 2	0.020
		5.0	1.1	109	81	28	14	0.014
	6	0.5	2.6	149	128	21	3	0.020
	7	0.5	1.9	97	78	19	2	0.024
	8	0.5	0.9	73	56	17	< 2	0.016
	9	0.5	0.4	64	51	13	< 2	0.008
		1.5	1.5	70	55	15	< 2	0.027
		5.0	1.1	66	53	13	< 2	0.021
Jan. 26, 1981	1	0.5	3.4	77	57	20	6	0.060
	2	0.5	4.7	67	53	14	2	0.089
	3	0.5	3.8	57	47	10	< 2	0.081
	4	0.5	2.2	46	38	8	< 2	0.057
		1.5	2.4	47	38	9	< 2	0.063
		5.0	2.5	47	39	8	< 2	0.065
	6	0.5	0.6	92	80	12	7	0.008
	7	0.5	2.4	39	33	6	2	0.073
	8	0.5	2.5	36	30	6	2	0.085
	9	0.5	2.4	39	33	6	< 2	0.073
		1.5	2.7	38	32	6	< 2	0.083
		5.0	2.4	38	32	6	< 2	0.075

DPA: dissolved phosphatase activity, TP: total phosphorus-P concentration, PP: particulate phosphorus-P concentration, DTP: dissolved total phosphorus-P concentration, Pi: orthophosphate-P concentration.

respectively. Both the concentrations of phosphorus and the phosphatase activity decrease with the horizontal decline of the water stream from the inner part of the two bays, (e.g., Stations 1 and 6), to the center (Station 9) or outlet (Station 12) of the lake. This trend is particularly noticeable in summer. There is known to be a linear relationship between particulate phosphorus concentration (PP) and chlorophyll *a* concentration (which is proportionate to the biomass of photoautotrophs) over the whole area of Lake Kasumigaura.[25] Waters highly mixed with waste-water near urban area (for example, Stations 1 and 6) seem to have a large biomass (which gives high particulate phosphate concentration), and consequently have a high level of phosphatase activity produced by algae and bacteria.[21]

Dissolved phosphatase activity (DPA) was around 10% of total phosphatase activity (TPA), which was about the same ratio as that found in Tokyo Bay[8] and in the Pluss-See.[9]

The correlation factors for phosphatase activity and phosphorus concentration are shown in Table 2. There were positive correlations between the various

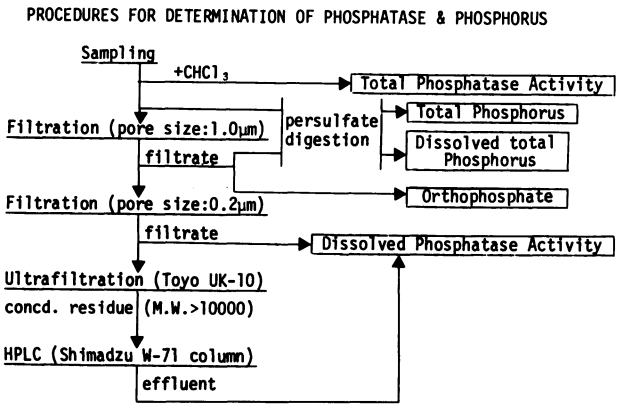

Fig. 1. Procedures for determination of phosphatase activity and phosphorus concentration.

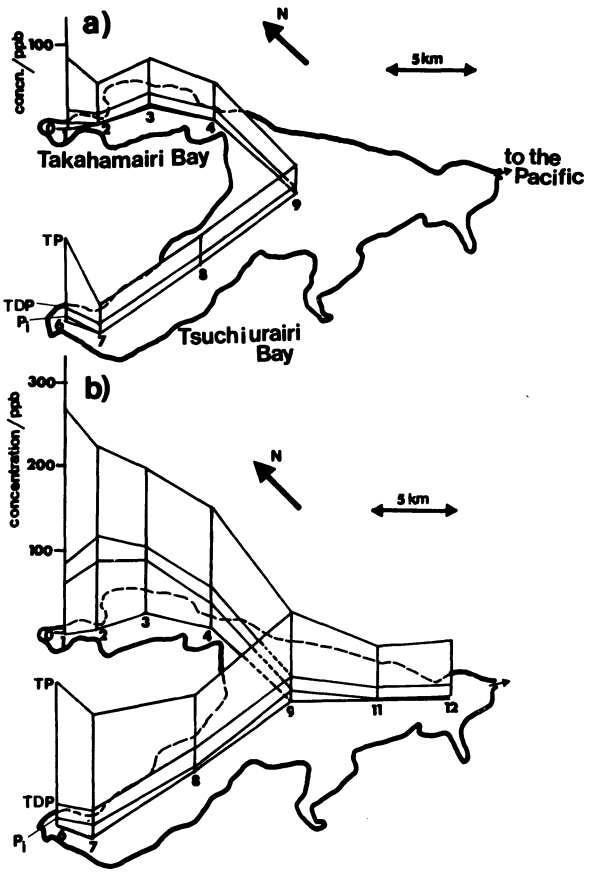

Fig. 2. Horizontal distribution of phosphorus concentration in Lake Kasumigaura.
a) February, 1981; b) August, 1981.
TP: total phosphorus-P concentration; TDP: total dissolved phosphorus-P concentration; Pi: orthophosphate-P concentration.

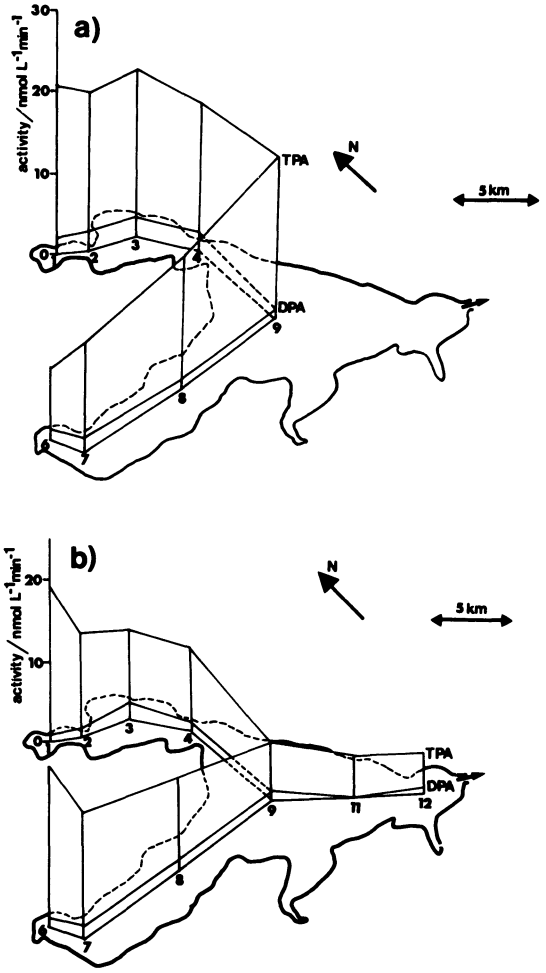

Fig. 3. Horizontal distribution of phosphatase activity in Lake Kasumigaura.
a) February, 1981; b) August, 1981.
TPA: total phosphatase activity; DPA: dissolved phosphatase activity.

phosphorus concentrations (such as TP vs. PP; PP vs. DOP), but there was no apparent correlation between the phosphorus concentrations and phosphatase activity (TPA or DPA). At this point, the value of DPA/PP was seen as being a possible indication of phosphatase activity in lake water normalized by the biomass. As shown in Fig. 4, there was an apparent negative correlation between DPA/PP and Pi (orthophosphate concentration) in May, 1981 (correlation coefficient (r) is −0.72), but no correlation in August, 1981 (r is 0.00).

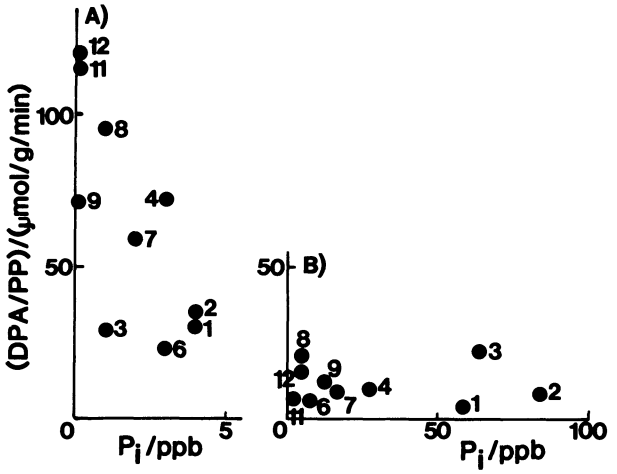

Fig. 4. Correlation between phosphatase activity and orthophosphate concentration.
A) May, 1981; B) August, 1981.
DPA: dissolved phosphatase activity: PP; particulate phosphorus-P concentration; Pi: orthophosphate-P concentration.

Seasonal Variations in Phosphatase Activity and Phosphorus Concentration. The seasonal variations in phosphorus concentrations at Stations 2 and 9 are shown in Fig. 5. In most of the stations, phosphorus increased in summer and decreased in winter, as is seen more markedly in the changes at Station 2. Such phenomena are also characteristic of algal blooms in eutrophic lakes.[26] In Fig. 5, the seasonal changes of phosphorus compounds at Station 9 were quite smaller than those at Station 2. These facts may be ascribed to less eutrophication near the lake center at Station 9 compared to the Takahamairi area at Station 2. In 1980, the summer was cooler than usual, so that the phosphorus peak for summer in 1980 shown in Fig. 5 was somewhat distored. The phenomenal increase in orthophosphate concentration (Pi) during mid-summer is probably caused by the chemical and biological release of phosphate from the sediment.[27]

Figure 6 shows the seasonal variations in dissolved phosphatase activity (DPA) and orthophosphate (Pi) at Station 2. DPA has two peaks a year, one of which would appear to be caused by the heavy blooms of blue-green algae (*Microcystis aeruginosa*) in summer, and the other by blooms of diatoms and flagellates in winter or spring.[28] Figure 6 also shows a negative correlation between DPA and Pi.

Seasonal variations in the correlation coefficients (r) of Pi and DPA/PP at the different stations and in the TP/TPA values are shown in Table 3. TP/TPA, whose dimension is $[\text{time}]^{-1}$, may indicate the

Table 2. Correlation Coefficients among Phosphatase Activity and Phosphorus Concentration

May 25, 1981							
	DPA	TPA	TP	TDP	PP	DOP	Pi
DPA	×	0.67	−0.36	−0.24	−0.38	−0.37	−0.39
TPA		×	−0.10	−0.20	−0.01	0.00	−0.45
TP			×	0.90	1.00	0.99	0.50
TDP				×	0.85	0.83	0.79
PP					×	1.00	0.37
DOP						×	0.73
Pi							×
August 17, 1981							
	DPA	TPA	TP	TDP	PP	DOP	Pi
DPA	×	−0.08	0.01	0.25	−0.20	−0.19	0.29
TPA		×	0.45	0.37	0.91	0.74	0.33
TP			×	0.83	0.89	0.35	0.81
TDP				×	0.50	−0.22	1.00
PP					×	0.73	0.47
DOP						×	−0.26
Pi							×

DPA: dissolved phosphatase activity, TPA: total phosphatase activity, TP: total phosphorus-P concentration, TDP: total dissolved phosphorus-P concentration, PP: particulate phosphorus-P concentration, DOP: dissolved organic phosphorus-P concentration, Pi: orthophosphate-P concentration.

turnover time of phosphorus in lake water. In May, TP/TPA was at its minimum value, and r was at its maximum value. These results suggest that microorganisms produce more alkaline phosphatase than usual in vivo, and that they are forced to use phosphorus in lake water more efficiently than usual in order to bloom in the phosphate-deficient season of May.

Table 3. Seasonal Variations in Correlation between Phosphatase Activity and Phosphorus Concentration

Year	Month	Pi	r(Pi: DPA/PP)	TP/TPA
1980	December	3	−0.72	—
1981	January	2	−0.71	—
	February	< 2	−0.78	2.0±1.7
	March	6	−0.59	5.9±3.2
	April	6	−0.31	3.4±1.4
	May	4	−0.72	1.2±0.7
	June	6	+0.32	2.4±1.3
	July	78	+0.51	4.8±1.7
	August	85	0.00	7.0±1.7
	September	3	+0.26	5.3±1.4
	October	3	+0.14	4.6±1.4
	November	1	−0.48	3.5±2.5
	December	3	−0.57	2.4±1.4
1982	January	3	−0.68	2.8±1.5
	February	4	−0.73	2.7±2.7
	March	11	−0.80	6.4±5.7

Pi: orthophosphate-P concentration at Station 2 [ng/ml], r(Pi: DPA/PP): correlation coefficient between orthophosphate and (dissolved phosphatase activity/particulate phosphorus-P concentration) at all the stations, TP/TPA: (total phosphorus-P concentration/total phosphatase activity.

The Role of Alkaline Phosphatase in the Phosphorus Cycle in Lake Water. Phosphorus is one of the most important nutrient elements. Microorganisms in natural water use phosphorus in the form of orthophosphate. It is recognised that the synthesis of an enzyme in vivo is suppressed by the presence of the

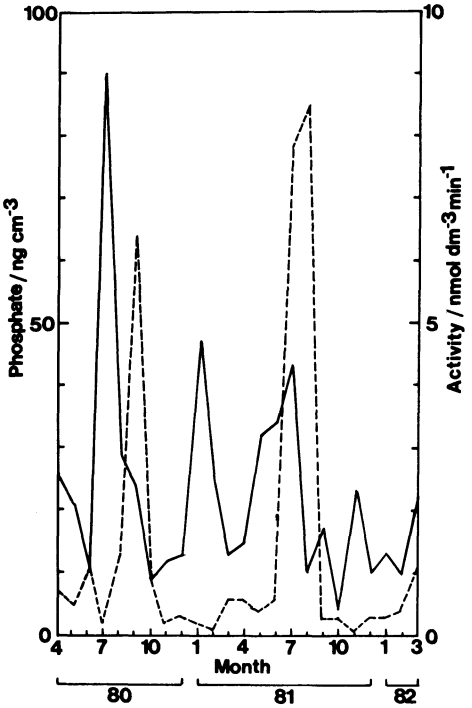

Fig. 6. Seasonal variations in dissolved phosphatase activity and orthophosphate concentration in Lake Kasumigaura (Station 2).
——: Dissolved phosphatase activity; —·—: orthophosphate-P concentration.

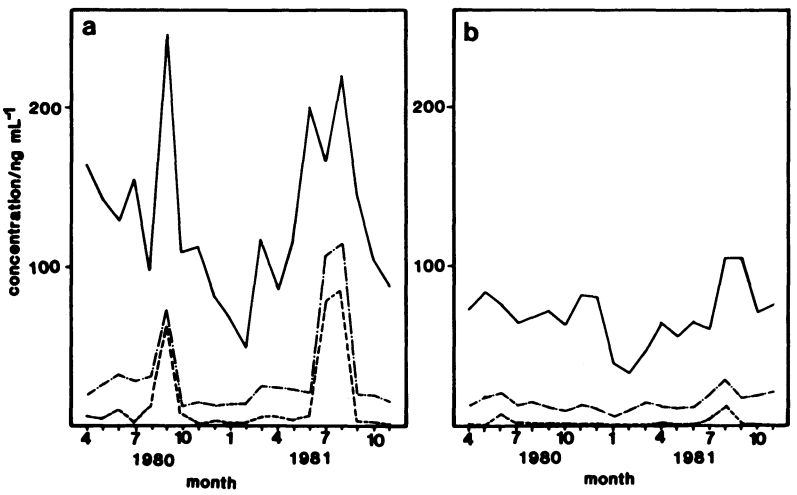

Fig. 5. Seasonal variations in phosphorus concentration in Lake Kasumigaura.
a) Station 2; b) Station 9.
——: Total phosphorus-P concentration; —·—: total dissolved phosphorus-P concentration; ----: orthophosphate-P concentration.

final product of its catalysis in the media.[18] It seems that the same phenomenon occurs in ecological systems, such as lake water. In algae's blooming season, the ecological system has a high requirement for nutrients such as inorganic phosphorus and nitrogen. If there is no sufficient orthophosphate in the lake water for the algae to grow, the algae have to produce more alkaline phosphatase in order to get a quick supply of orthophosphate. On the other hand, when the phosphate concentration is high enough to supply the algae's needs, the key element in the bloom of algae is not phosphorus but other nutrient elements such as nitrogen. When the latter conditions prevail, there is no correlation between DPA/PP and Pi. This hypothesis is supported not only by the clear negative correlation between DPA/PP and Pi, but also by the low apparent turnover time of phosphorus (TP/TPA) in phosphorus-deficient season (such as May, 1981).

In the laboratory incubation experiments of bacteria (*Escherichia coli*)[18] and algae,[29] the depression of the enzyme production can be observed under orthophosphate concentration of 7—10 μg-P ml^{-1}. On the other hand, the decrease in the dissolved alkaline phosphatase in the lake water appears at ca. 1 ng-P ml^{-1}, which means more than three order of magnitude in difference between the lake and experimental laboratory systems. When considering that the surface area of Lake Kasumigaura is 171 km^2, it is important to note that such negative feedback is also maintained between orthophosphate and the release of dissolved phosphatase on the scale of a large lake.

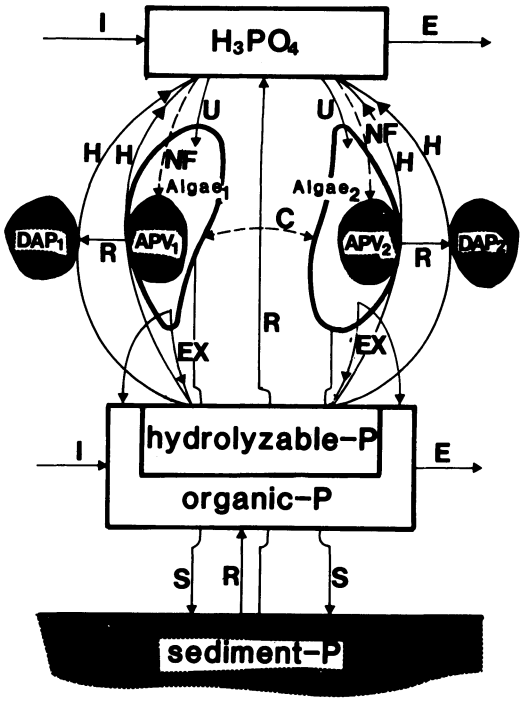

Fig. 7. A schematic diagram of phosphorus cycle in lake water.
DPA: dissolved alkaline phosphatase; APV: alkaline phosphatase in vivo; I: influx; E: efflux; U: uptake; R: release; S: sedimentation; H: hydrolysis; EX: excretion; NF: negative feedback; C: competition.

Conclusion

A simplified representation of the role of alkaline phosphatase in the phosphorus cycle in lake water is illustrated in Fig. 7. As described above, it is suggested that intercellular phosphatase controls the uptake of phosphorus by algae at times when there is a phosphate deficiency, and that dissolved alkaline phosphatase also plays a part in this process. Conversely, an excess of phosphate suppresses the biosynthesis of alkaline phosphatase in vivo, and is followed by a decrease in dissolved alkaline phosphatase.

Various kinds of microorganisms are present in natural water, and each of them produces its own type of alkaline phosphatase.[21] It is known that different types of algae are frequently in competition within any particular ecological systems; for example, blue-green algae inhibit the blooming of other algae such as diatoms in natural water.[28] In order to elucidate further the role of alkaline phosphatase (in vivo and dissolved forms), it will be necessary to determine alkaline phosphatase activity originating from each source.

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References

1) M. Steiner, *Naturwissenshaften*, **26**, 723 (1938).
2) R. Y. Morita and R. A. Howe, *Deep-Sea Research*, **4**, 254 (1957).
3) H. Goldschmiedt, M. W. Mettenleiter, and P. R. Borchardt, *Turtox News*, **36**, 269 (1958).
4) J. Overbeck, *Intern. Ver. Theoret. Angew. Limnol. Verhand.*, **14**, 226 (1959).
5) J. Overbeck and H.-D. Babenzien, *Arch. Hydrobiol.*, **60**, 107 (1964).
6) M. J. Perry, *Mar. Biol.*, **15**, 113 (1972).
7) J. Kim and C. E. ZoBell, *Eff. Ocean Environ. Microb. Act., Proc. U.S. Jpn. Conf., 2nd*, **1972**, 369 (Pub. 1974).
8) N. Taga and H. Kobori, *Mar. Biol.*, **49**, 223 (1978).
9) W. Reichardt, J. Overbeck, and L. Steubing, *Nature*, **216**, 1345 (1967).
10) T. Berman, *Limnol. Oceanogr.*, **15**, 663 (1970).
11) W. Reichardt, *Hydrobiologia*, **38**, 377 (1971).

12) T. Berman and G. Moses, *Hydrobiologia,* **40**, 487 (1972).
13) M. Janson, *Hydrobiologia,* **56**, 175 (1977).
14) J. Olah and E. O. Toth, *Aquaculture Hungaria (Szaarbas),* **1**, 15 (1978).
15) A. Miliuis and M. Pork, *Eesti NSV Tead. Akad. Toim., Biol.,* **26**, 128 (1977).
16) R. J. Stevens and M. P. Parr, *Freshwater Biol.,* **7**, 351 (1977).
17) H. Kobori and N. Taga, *Deep-Sea Res.,* **26A**, 799 (1979).
18) A. Torriani, *Biophys. Biochem. Acta,* **38**, 460 (1960).
19) K. Kobayashi, K. Iwase, M. Matsui, M. Watanabe, H. Ueda, K. Fujiwara, H. Haraguchi, and K. Fuwa, *Bull. Chem. Soc. Jpn.,* **55**, 3459 (1982).
20) K. Kobayashi, M. Matsui, H. Haraguchi, and K. Fuwa, *J. Inorg. Biochem.,* **18**, 41 (1983).
21) K. Kobayashi, S. Hashimoto, A. Otsuki, K. Fujiwara, K. Fuwa, and H. Haraguchi, *Bull. Chem. Soc. Jpn.,* **59**, 3067 (1986).
22) J. Murphy and J. P. Riley, *Anal. Chim. Acta,* **27**, 31 (1962).
23) D. W. Menzel and N. Corwin, *Limnol. Oceanogr.,* **10**, 280 (1965).
24) T. Kawai, A. Otsuki, M. Aizaki, and M. Nishikawa, *Res. Rep. Natl. Inst. Environ. Stud.,* No. 22, 23 (1981).
25) A. Otsuki, S. Kasuga, and T. Kawai, *Verh. Internal. Verein. Limnol.,* **21**, 602 (1981).
26) T. Okino, *Jpn. J. Bol.,* **20**, 381 (1973).
27) A. Otsuki, T. Kawai, and M. Aizaki, *Res. Rep. Natl. Inst. Environ. Stud.,* No. 22, 3 (1981).
28) N. Imamura and M. Yasuno, *Res. Rep. Natl. Inst. Environ. Stud.,* No. 22, 123 (1981).
29) G. P. Fitzgerald and T. C. Nelson, *J. Phycol.,* **11**, 32 (1966).