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High potential activity of alkaline phosphatase in the benthic nepheloid layer of a large mesotrophic lake: implications for phosphorus regeneration in oxygenated hypolimnion

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ABSTRACT: Although the benthic nepheloid layer (BNL) in freshwater and marine systems is known to be an active site of material transformations, information is limited regarding the extent of and mechanisms underlying phosphorus regeneration in the BNL. We found that potential activity of particle-bound alkaline phosphatase (APase) was remarkably high in the BNL of a large (surface area: 674 km^2; maximum depth: 104 m), mesotrophic lake (Lake Biwa, Japan) during periods of thermal stratification. The enhancement in the BNL of other ectoenzyme activities was not as pronounced as that of the APase; the ratios of the particle-bound potential activities in the BNL relative to those in the upper hypolimnion were 2.6 ± 1.0 (mean ± SE, n = 22) and 2.0 ± 0.6 for β-glucosidase and leucine aminopeptidase, respectively, whereas the corresponding ratio for APase was 4.5 ± 2.3. The APase activity increased in the BNL with increasing concentrations of soluble reactive phosphorus, inconsistent with a general notion that phosphate represses microbial synthesis of APase. Incubation experiments revealed that the net regeneration of P proceeded in waters collected in the BNL at rates comparable to the *in situ* accumulation rate of soluble reactive phosphorus in the hypolimnion of the basin. Our data suggest that the enzymatic cleavage of the P moiety of organic phosphorus contributes to the active regeneration of P in the oxic BNL of Lake Biwa.

KEY WORDS: Phosphorus cycle · Alkaline phosphatase · Benthic nepheloid layer · Lake Biwa

INTRODUCTION

Enzymatic cleavage of organic phosphorus is of central importance in the regulation of P cycles in P-limited lakes and oceans (Ammerman 1991, Chróst & Siuda 2002), enabling phytoplankton and bacteria to utilize the P moiety of organic compounds that account for a substantial fraction of P inventories in the water column (Karl & Björkman 2002, Kim et al. 2006). Alkaline phosphatase (APase) is widespread among planktonic osmotrophs and hydrolyzes various phosphomonoesters (Berman 1969, Chróst & Siuda 2002). Generally, APase is known to be inducible, being actively synthesized by microbes especially when P limitation is severe (Chróst & Siuda 2002).

Although several studies have examined variations in APase activity as an indicator of P limitation in the euphotic zone (Chróst & Siuda 2002), few studies have examined the distribution of APase in deeper, aphotic layers. Among the few studies are those of Koike & Nagata (1997) and Hoppe & Ullrich (1999), who found high APase activity in meso- and bathypelagic water columns of the oceans. These results are somewhat paradoxical given the high concentrations of phosphate in deep oceans, a condition that is generally believed to be in favor of the repression of APase syn-

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thesis (Chróst & Siuda 2002). Although the mechanisms underlying the occurrence of high APase in deep oceanic waters are not entirely clear (Hoppe et al. 2002), the role of APase in P regeneration in aphotic layers clearly deserves closer scrutiny. This is particularly true in chronically P-limited large lakes, where hypolimnetic mineralization of organic matter represents a critical step in the internal loading of P that substantially affects basin-scale patterns of nutrient stoichiometry (Wetzel 2001).

The present study aimed at examining the basin-scale variation in the activity of APase and other ectoenzymes in the north basin of Lake Biwa, Japan, a large (surface area: 674 km^2; maximum depth: 104 m), P-limited, mesotrophic lake with an oxygenated hypolimnion (Kim et al. 2006). One notable feature of the physical settings of this basin is the development of the benthic nepheloid layer (BNL) during periods of thermal stratification (Kim et al. 2006). The BNL is a zone of high particle concentrations, which is formed by resuspension of particulate matter owing to the turbulence that is induced by the bottom current and internal waves (McCave 1986, Kalff 2002). Previous studies have found significant enhancement of bacterial production (Wainright 1987, Cotner et al. 2000) and ectoenzymatic activities (Chróst & Riemann 1994) in resuspended sediments, suggesting that the BNL can be an active site of material transformations, although decreased microbial activities in the BNL have been also noted (Giesenhagen & Hoppe 1991). However, data are scarce regarding the seasonal variations in APase and P regeneration in the BNL.

We conducted extensive measurements of basin-scale distributions of APase and other biogeochemical variables, with sufficient spatial resolutions to capture the zone of high particle concentrations near the bottom. Our purposes were (1) to analyze how APase activity in the hypolimnion relates to variations in other hydrolytic enzyme activities and biogeochemical variables and (2) to conduct incubation experiments in order to examine the extent of P transformations in the BNL of Lake Biwa.

MATERIALS AND METHODS

Study site. In the north basin of Lake Biwa, Japan, thermal stratification is established between May and August, followed by partial mixing between September and December. The entire water column is circulated due to convective mixing in February (Kim et al. 2006). During the thermal stratification period, the nutrient regime in the surface layer is characterized by severe P limitation, as indicated by depletion in P relative to nitrogen (N) and carbon (C) of dissolved and particulate constituents (Kim et al. 2006), as well as by results of nutrient addition bioassays (Nishimura et al. 2005). Hypolimnetic mineralization of organic carbon has been estimated to be 6.67 mol m^{-2} yr^{-1}, accounting for 18% of primary production (Kim et al. 2006), a value close to the lower range of the general estimates of the annual loss by sedimentation of primary production in large lakes (20 to 30%; Tilzer 1990). Hypolimnetic concentrations of dissolved oxygen decrease during the stratification period to reach a minimum level of 4 mg l^{-1} (34% saturation) in December (Kim et al. 2006).

Water sampling. Details of the locations and methods of sampling are reported in Kim et al. (2006). Briefly, we collected water samples once every 2 mo between June 2001 and June 2002, principally at 6 transect stations deployed along the major axis of the lake (Fig. 1). Additional samplings were carried out at 2 stations (Stns 4 and 6) until February 2003. The hydrographic structure was determined with a CTD probe (SBE-911plus, Sea Bird Electronics Sealogger). Vertical sampling (Niskin-X bottles, General Oceanics Miami) was conducted at predetermined depths. We collected water samples near the bottom (1, 3, and 5 m

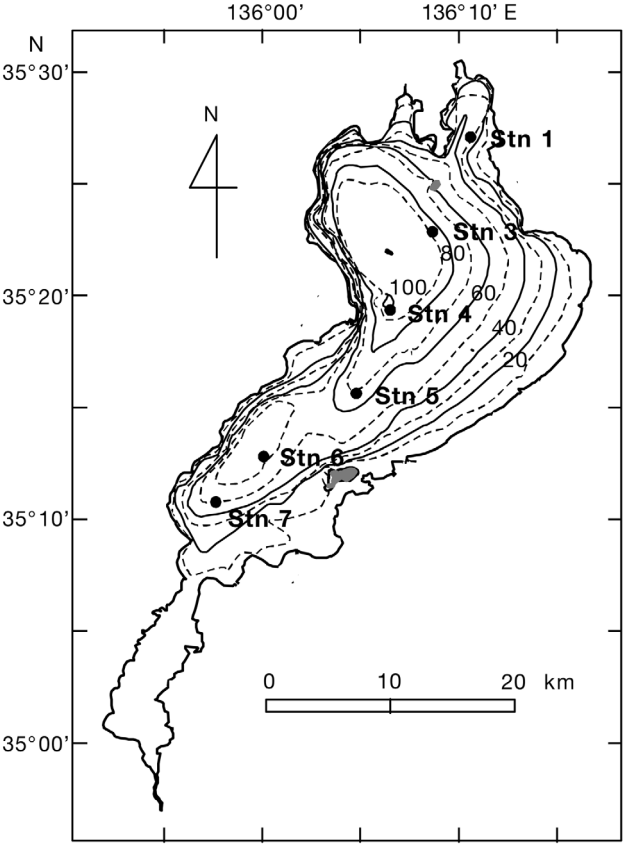

Fig. 1. Map of Lake Biwa, Japan, and the 6 stations sampled. Depth contours are shown in meters

above the bottom) using 3 sampling bottles sequentially attached to a wire. A sonar was used to help determine the position of the bottle for sampling at 1 m above the bottom. Actual sampling depths were ascertained with depth loggers attached to the sampling bottles. Sample waters for measurement of enzyme activities were contained in detergent- and acid-washed polyethylene bottles and stored in a cooler box until analysis. Assays were conducted within 2 h after sampling. Water samples were also collected for measurement of biogeochemical variables, including bacterial abundance, chlorophyll *a* (chl *a*) concentration, concentrations of particulate forms of phosphorus (PP), of organic nitrogen (PON), and of organic carbon (POC), concentrations of dissolved organic phosphorus (DOP), nitrogen (DON), and carbon (DOC), and concentrations of soluble reactive phosphorus (SRP), as described elsewhere (Nishimura et al. 2005, Kim et al. 2006).

Determination of potential enzyme activity. We used fluorescent substrates 4-methylumbelliferyl phosphate (MUF-P, Sigma), 4-methylumbelliferyl-β-D-glucose (MUF-G, Sigma), and L-leucine-7-amido-4-methyl coumarin hydrochloride (Leu-AMC, Sigma) to assay activities of APase, β-glucosidase (BGase), and leucine aminopeptidase (LAPase), respectively (Hoppe 1983). The substrates were dissolved in methoxyethanol. Water samples (1 ml for APase and BGase and 3 ml for LAPase) were dispensed into acid-washed polymethyl methacrylate cuvettes, amended with the substrate, and incubated at *in situ* temperature in the dark. The final concentrations of substrate were 500, 300, and 150 µM for APase, BGase, and LAPase, respectively. The substrate concentration that we used for the measurement of the potential APase activity is close to the substrate concentrations used in other studies conducted in lakes and marine systems (200 to 500 µM; Chróst et al. 1989, Hoppe & Ullrich 1999, Davey et al. 2001). Kinetic analyses have indicated that the APase in Lake Biwa displayed multiphasic kinetics, suggesting that the potential APase activities determined in the present study represent those of APase with a high half-saturation constant (data not shown). Incubation periods were 1 to 15 h, which varied depending on the activity. After incubation, the samples for the measurement of APase and BGase were mixed with 2 ml of borate buffer (0.5 M, pH 10.3), followed by the measurement of fluorescence (excitation and emission wavelengths were 365 and 445 nm, respectively) with a spectrofluorometer (FP-750, Jasco). For LAPase, the fluorescence was measured (excitation and emission wavelengths were 380 and 440 nm, respectively) without buffer. Methylumbelliferon (Sigma) was used as a calibration standard for APase and BGase assays, whereas LAPase activity was calibrated with 7-amino-4-methyl coumarin (Sigma). The slopes of the calibration curve obtained using heat-killed sample waters collected in the BNL generally differed little (<1%) from those obtained using heat-killed surface lake waters, suggesting that the matrix effect due to suspended particles (Hoppe 1983) in the BNL was minimal. Heat-treated sample waters were used as blanks. We also measured the enzyme activities in the dissolved fraction of lake water using sample waters that had been passed through syringe filters (pore size: 0.2 µm, Acrodisc, Pall). Gentle pressure was applied and care was taken to avoid exposing cells to the air at the end of filtration to minimize the breaking of fragile cells (Nagata & Kirchman 1990). The particle-bound enzyme activity was calculated as the difference in the activities of the total and dissolved fractions. The dependency of enzyme activity on temperature (range: 7 to 25°C) was examined on several occasions for different depths. The average Q_{10} values for APase, BGase, and LAPase were 2.33 (range: 1.86 to 3.07; SD = 0.40; n = 10), 2.09 (range: 1.57 to 2.81; SD = 0.45; n = 10), and 2.11 (range: 1.80 to 2.85; SD = 0.32; n = 10), respectively. These Q_{10} values were used to normalize the enzyme activity to the hydrolysis rate at 25°C. Temperature-normalized hydrolytic activities obtained by the above procedures are the potential activities (V_{max}), rather than activities *in situ*. Our intention of deriving the V_{max} was to facilitate the comparison of total enzyme present in the lake water sample (E), which is approximated to be proportional to V_{max}; V_{sat} ($\fallingdotseq V_{max}$) = $k_2 \times E$, where V_{sat} and k_2 are the hydrolysis velocity at the saturation level of the substrate and the rate constant, respectively (Christian & Karl 1995).

Determination of the net release of SRP in sample waters collected in the BNL. We examined the net release of P in sample waters collected in the BNL (1 m above the bottom) at Stn 4 (Fig. 1). Experiments were conducted during a thermal stratification period (3 September and 11 November 2003). Sample waters were collected using acid-washed 5 l Niskin bottles and filtered through 150 µm mesh net. The filtrate (2 l) was contained in acid-washed polycarbonate bottles (Nalgene) and incubated at *in situ* temperature (7.2°C) in the dark. Duplicate bottles were prepared. We collected subsamples at intervals of 1 to 2 wk for 35 d (September experiment) or 70 d (November experiment) to measure SRP concentrations (see above). To examine whether the increase in particle concentration would result in increasing regeneration, 10 l of sample water (prefiltered through a 150 µm mesh) was filtered through 0.8 µm pore size Nuclepore filters (Whatman). Particles on the filters were resuspended in 2 l of sample water that had been prefiltered through 0.2 µm pore size Nuclepore filters (Whatman).

With this treatment, we expected that particles >0.8 µm were enriched by a factor of 5 compared to the concentrations of particles in the original lake water. Subsampling and analyses were conducted as described above.

RESULTS

Spatio-temporal variations of particle-bound and dissolved APase activities

The BNL, near the bottom layer containing high concentrations (>15 µM) of POC, was widely distributed in the water basin during periods of thermal stratification (between June and December) (Figs. 2A & 3A); the BNL was detected in 67% (22 of 33) of depth profiles collected during the study period. For these data, we divided the water column into 3 layers (epilimnion + metalimnion, upper hypolimnion, and BNL) to compare average enzyme activities and biogeochemical variables among different layers (Figs. 4 & 5, Table 1). The BNL was accompanied by higher concentrations of chl *a*, PP, and SRP relative to the upper hypolimnion, but such a trend was less evident for concentrations of DOC, DON, and DOP (Fig. 4, Table 1). Vertical patterns of enzymatic activities varied depending on enzymes (APase, BGase, and LAPase) and size fractions (particle-bound vs. dissolved) (Fig. 5, Table 1). The most outstanding feature was the remarkably high particle-bound APase activity (APase-p) in the BNL, which was equivalent to or even higher than that in the epilimnion (Fig. 5A, Table 1). This pattern was consistently found during periods of thermal stratification throughout the basin (Figs. 2B & 3B). High APase activities in the BNL were generally associated with higher concentrations of SRP (Figs. 2C & 3C). On average, the APase-p activity in the BNL was 4.5 times higher than that in the upper hypolimnion (Table 1). In contrast, dissolved APase activity (APase-d) displayed rather homogeneous distribution throughout the hypolimnetic water column, with no clear maximum in the BNL (Fig. 5A, Table 1). APase-d accounted for 23 ± 2% (mean ± SE, n = 20) of total APase activity in the BNL, whereas the corresponding value in the upper hypolimnion was 48 ± 3% (n = 20).

Particle-bound activities of BGase (BGase-p) and LAPase (LAPase-p) tended to be higher in the BNL than in the upper hypolimnion, but the magnitude of the enhancement in the BNL was not as pronounced as that for APase-p (Fig. 5B,C, Table 1). Because of the difference in vertical distributions among activities of different enzymes, activity ratios of APase-p to BGase-p and those of APase-p to LAPase-p in the BNL (31 ± 5 and 5.1 ± 4.2 [mean ± SE] for APase-p/BGase-p and APase-p/LAPase-p, respectively) were significantly ($p < 0.05$) greater than those in the upper hypolimnion (20 ± 4 and 2.4 ± 2 for APase-p/BGase-p and APase-p/LAPase-p, respectively) (Fig. 5D,E). For BGase and LAPase, contributions of dissolved enzyme activities to total activities were generally low (<25 and <10% for BGase and LAPase, respectively) and exhibited no clear vertical trend (Fig. 5B,C). Our data also revealed that the aver-

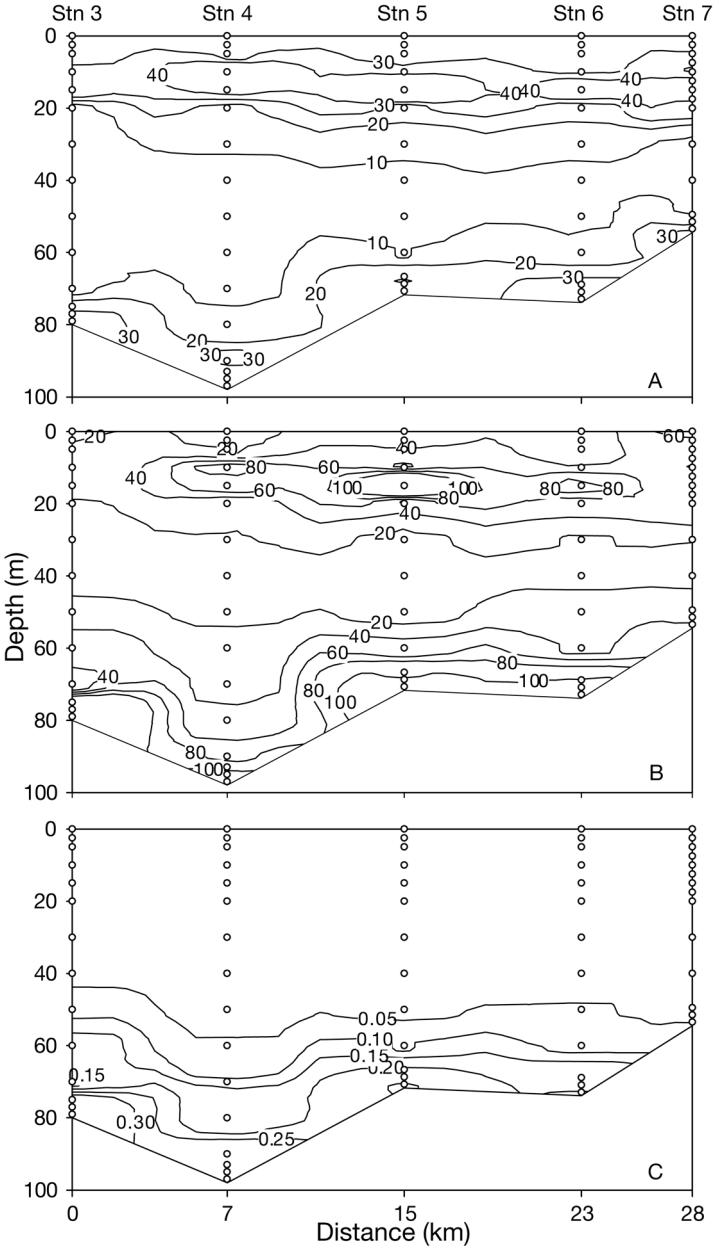

Fig. 2. Basin-scale distribution patterns of (A) particulate organic carbon (POC; µM), (B) particle-bound alkaline phosphatase activities (APase-p; nM h^{-1}), and (C) soluble reactive phosphorus (SRP; µM). Data are from the survey conducted in August 2001. *x*-axis indicates distance from Stn 3

age bacterial abundance in the BNL was only marginally higher (1.2-fold) than that in the upper hypolimnion (Table 1, Fig. 5F), although our enumeration of bacteria using flow cytometry (Nishimura et al. 2005) could have resulted in underestimation of the abundance of bacteria attached to particles in the BNL.

We conducted Pearson's correlation analyses to examine relationships between APase activity and biogeochemical variables in the hypolimnion (upper hypolimnion and BNL). The activity of APase-p was positively correlated with the concentrations of SRP ($r = 0.674$, $p < 0.001$, $n = 142$) and with particulate constituents including POC ($r = 0.771$, $p < 0.001$, $n = 145$) and PP ($r = 0.838$, $p < 0.001$, $n = 143$), whereas the correlations between APase-p activity and the concentrations of dissolved organic constituents were weak (DOC: $r = 0.224$, $p = 0.009$, $n = 137$) or insignificant (DON: $p = 0.643$, $n = 142$; DOP: $p = 0.077$, $n = 135$) (Table 2). There were only weak ($r < 0.35$) or insignificant ($p > 0.05$) correlations between APase-d activity and other biogeochemical variables (Table 2).

Net release of SRP in the BNL

We conducted incubation experiments to examine whether the net release of SRP occurs in waters in the BNL. The SRP concentrations increased linearly over the incubation period of 35 d (September experiment) or 70 d (November experiment), with net release rates (V_p) of 0.85 nM d^{-1} (September experiment) or 0.50 nM d^{-1} (November experiment) (Table 3). The increase in the particle concentrations resulted in the proportional enhancement of SRP release, yielding V_p values of 4.3 and 2.6 nM d^{-1} for September and November experiments, respectively (Table 3). Factors of the enhancement of V_p, i.e. V_p in concentrate/V_p in raw water, were 5.1 and 5.2 for September and November experiments, respectively, which are close to the factor of concentrating particulate matter (5-fold). This result suggests that SRP was mostly released from particulate rather than dissolved matter. The V_p (0.50 to 0.85 nM d^{-1}) determined experimentally accounted for 45 to 76% of the seasonal accumulation of SRP in the hypolimnion (1.1 ± 0.6 nM d^{-1}, the apparent accumulation rate of SRP in the BNL during a period between June 2001 and December 2002 at 5 stations), which indicates that net release of SRP in the BNL affects the hypolimnetic accumulation of P during stratification periods.

DISCUSSION

Formation of the BNL is a wide-spread phenomenon in large to medium-sized lakes (Kalff 2002) and marine systems (McCave 1986). In these environments, investigators have reported that resuspension of sediments results in increases in hydrolytic enzyme activities (glucosidase and peptidase) (Chróst & Rieman 1994) and bacterial production (Wainright 1987, Cotner et al. 2000). However, to our knowledge, this study is the first to demonstrate remarkably high APase activity in the BNL. Notably, the enhanced APase activity in the BNL was much more pronounced than that of other

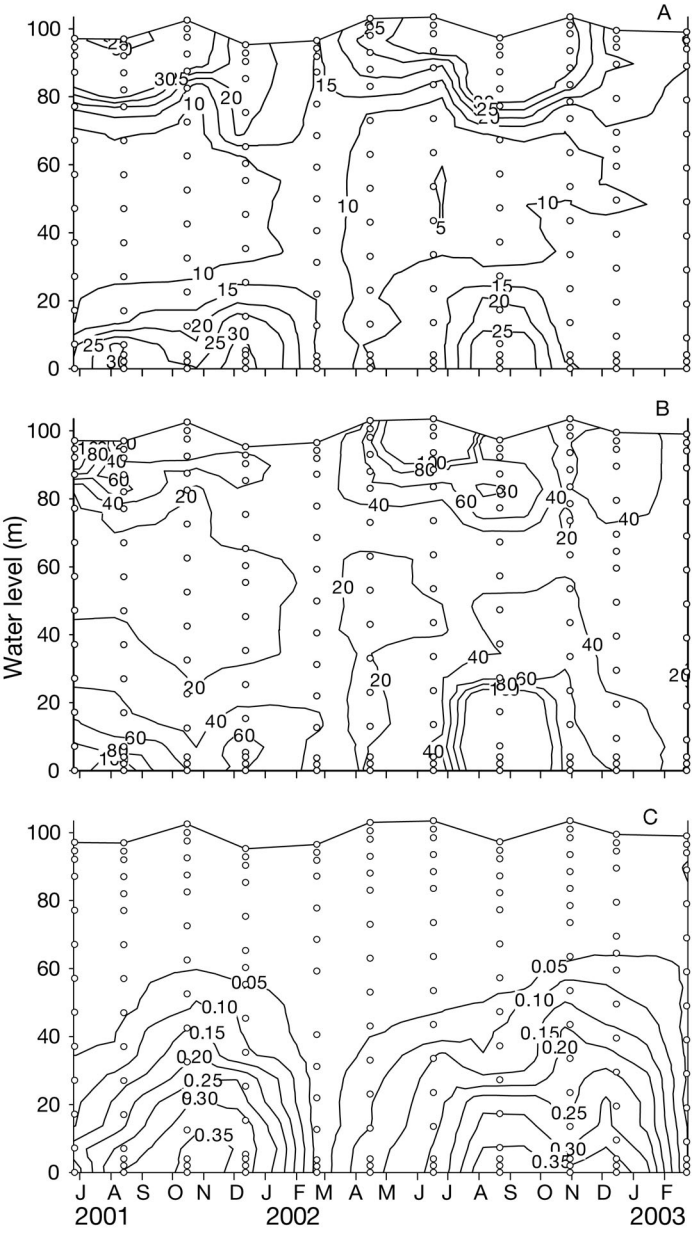

Fig. 3. Seasonal and vertical distributions of (A) particulate organic carbon (POC; µM), (B) particle-bound alkaline phosphatase activities (APase-p; nM h^{-1}), and (C) soluble reactive phosphorus (SRP; µM). Data collected at Stn 4 are presented. *y*-axis indicates height (m) above the bottom

Table 1. Summary of potential activities of particle-bound (denoted by -p after the enzyme names) and dissolved (-d) forms of ectoenzymes (APase, BGase and LAPase; nM h^{-1}). The data of 22 depth profiles obtained during stratification periods at various regions of the north basin of Lake Biwa (Fig. 1) were used to compare the means (±SE, n = 22) and ranges (in parentheses) of activities among different depth layers (Epi + Meta, Hypo-u, and BNL, see Fig. 4 for definitions). In order to assess the magnitude of the enhancement of activities in the BNL relative to the Hypo-u, the ratios of the values between BNL and Hypo-u are shown (BNL/Hypo-u). Also compiled are the data of biogeochemical variables, including bacterial abundance (Bacteria; $\times 10^6$ cells ml^{-1}), chl *a* concentrations (µg l^{-1}), and concentrations of different forms of C, N, and P (µM)

	Epi + Meta	Hypo-u	BNL	BNL/Hypo-u
APase-p	68 ± 63 (26–229)	22 ± 10 (6–46)	87 ± 37 (27–159)	4.5 ± 2.3 (1.6–12)
APase-d	31 ± 12 (8–49)	18 ± 8 (5–35)	23 ± 12 (10–53)	1.4 ± 0.5 (0.6–2.2)
BGase-p	4.6 ± 1.7 (2.4–10)	1.4 ± 0.62 (0.59–3.3)	3.4 ± 1.1 (0.89–5.3)	2.6 ± 1.0 (1.2–4.3)
BGase-d	3.7 ± 2.6 (0.17–9.4)	0.70 ± 0.85 (0.20–3.5)	0.87 ± 0.54 (0.03–2.3)	1.8 ± 0.7 (0.5–3.1)
LAPase-p	97 ± 48 (29–216)	12 ± 6 (5–26)	24 ± 18 (8–50)	2.0 ± 0.6 (0.9–3.1)
LAPase-d	14 ± 11 (1.8–38)	1.2 ± 0.79 (0.3–3.6)	2.5 ± 1.2 (0.7–5.2)	2.3 ± 1.1 (1.1–5.8)
Bacteria	3.6 ± 1.4 (1.7–6.0)	1.1 ± 0.2 (0.9–1.9)	1.4 ± 0.2 (1.1–1.8)	1.2 ± 0.2 (0.9–1.5)
Chl *a*	3.2 ± 0.9 (2.4–5.7)	0.4 ± 0.2 (0.2–1.2)	0.7 ± 0.4 (0.2–1.7)	1.8 ± 1.1 (0.7–5.4)
POC	40.0 ± 4.2 (22.0–41.8)	9.6 ± 2.0 (6.6–14.1)	26.6 ± 8.1 (15.3–48.5)	2.8 ± 0.9 (1.4–4.7)
PON	3.54 ± 0.75 (2.30–5.36)	1.08 ± 0.40 (0.52–1.93)	2.17 ± 0.68 (0.98–3.54)	2.2 ± 0.8 (0.8–3.8)
PP	0.131 ± 0.028 (0.052–0.177)	0.055 ± 0.010 (0.040–0.075)	0.110 ± 0.035 (0.045–0.208)	2.1 ± 0.6 (0.8–3.7)
DOC	109 ± 6.6 (101–122)	84 ± 4.6 (75–90)	86 ± 5.9 (79–101)	1.0 ± 0.1 (0.9–1.2)
DON	8.03 ± 1.16 (4.52–10.5)	5.47 ± 1.10 (3.61–7.91)	5.54 ± 1.04 (3.87–7.21)	1.0 ± 0.1 (0.9–1.3)
DOP	0.073 ± 0.030 (0.022–0.144)	0.036 ± 0.019 (<0.010–0.076)	0.055 ± 0.073 (<0.010–0.339)	1.2 ± 1.1 (0.1–4.4)

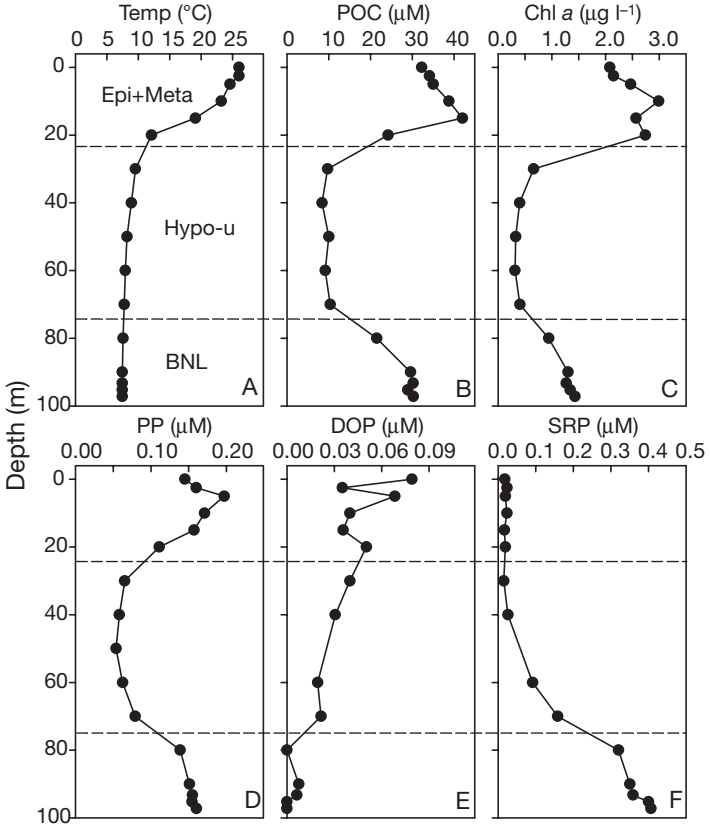

Fig. 4. Vertical distributions of (A) temperature (Temp) and (B–F) biogeochemical variables at Stn 4 on 22 August 2002. The water column was divided into 3 layers based on the temperature and particulate organic carbon (POC) distributions: the epilimnion and metalimnion (Epi + Meta), upper hypolimnion (Hypo-u), and the benthic nepheloid layer (BNL). The BNL was defined as the layer with POC > 15 µM

enzymes (BGase and LAPase), suggesting that the BNL is a zone of active P transformation. The increase in APase activity with a concomitant increase in SRP concentrations in the hypolimnion of Lake Biwa appears to contradict the conventional notion that APase synthesis by microbes is generally induced when P is deficient. In fact, for surface communities, previous data have demonstrated a general tendency of the suppression of APase synthesis with increasing ambient concentrations of phosphate (Chróst & Siuda 2002). These results question the mechanisms underlying the high APase activity in the BNL of Lake Biwa.

One potential mechanism is that bacteria produce APase to utilize carbon rather than phosphorus moiety of organic phosphorus during the decomposition of organic matter (Hoppe & Ullrich 1999). In marine environments, Hoppe & Ullrich (1999) have found high APase activity in meso- and bathypelagic waters where P limitation is unlikely to occur due to high SRP concentration. They hypothesized that this phenomenon is ascribed to the APase production by bacteria in deep waters to cope with C limitation by facilitating the uptake of carbon moiety of organic phosphorus, although biochemical basis of this process is not entirely clear. In Lake Biwa, Y. Nishimura et al. (unpubl. data) examined seasonal changes in bacterial production (leucine incorporation rate) in the hypolimnion (includ-

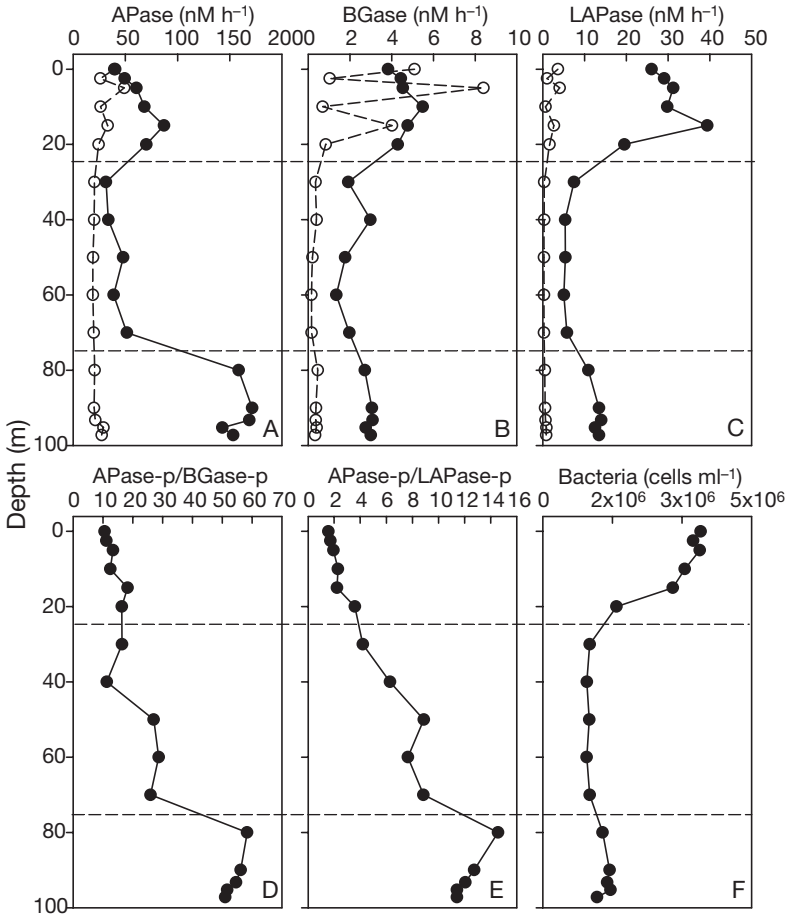

Fig. 5. Vertical distributions of (A–C) ectoenzyme activities (in dissolved [○] and particle-bound [●] fractions), (D,E) ectoenzyme activity ratios of particle-bound (-p) fractions, and (F) bacterial abundance at Stn 4 on 22 August 2002. APase: alkaline phosphatase; BGase: β-glucosidase; LAPase: leucine aminopeptidase

ing the BNL) to reveal that bacterial production was positively correlated with concentrations of POC, but not with those of SRP. This observation is consistent with the hypothesis that bacteria in the BNL are limited by C. However, the induction of APase synthesis by bacteria due to P limitation or P stress might occur in the BNL, despite the accumulation of SRP, because of the high C:P ratio of particulate organic matter (257 ± 83, n = 62) and because phosphorus (or substrate in general) requirements by bacteria may increase with decreasing temperature (Simon & Wunsch 1998, Pomeroy & Wiebe 2001). Obviously, further studies are needed to examine the interactive effects of low temperature and substrates (C and P) on bacterial synthesis of extracellular enzymes in the BNL of Lake Biwa.

An alternative hypothesis is that APase produced by phytoplankton and other organisms in surface waters sinks to deeper layers in the form of particles (aggregates or lake snow; Simon et al. 2002) to accumulate in the BNL as an active catalytic agent. In marine systems, previous studies have suggested that APase activity is high compared to other ectoenzymes in sinking particles (Smith et al. 1992), leading to the proposition that APase is actively transported to deep oceans to mediate P regeneration at depth (Koike & Nagata 1997). Although we lack information on APase activity associated with sinking particles in Lake Biwa, we speculate that the settling of organic aggregates that contain 'detrital APase' derived from surface communities could result in the transport of APase from the surface to the BNL. We also point out the possibility that APase is less easily degraded (inactivated) than other enzymes, which might explain the increase in ratios of APase-p/BGase-p and APase-p/LAPase-p with depth. The above scenario of the transport of 'detrital APase' differs from the notion that ectoenzyme activities reflect metabolic states of the living microbes present in the water in which enzyme activities are determined, but it is similar to the idea of Wetzel (1991), who proposed that detrital APase (APase bound to humic matter) produced in the littoral zone is

Table 2. Results of Pearson's correlation analyses of the relationships between APase activities (particle-bound and dissolved) and environmental variables in the hypolimnion (including the BNL). Bold type indicates $p < 0.001$. Further details as in Table 1

	Chl *a*	POC	PP	DOC	DON	DOP	SRP	Bacteria
APase-p								
r	**0.57**	**0.771**	**0.838**	0.224	0.039	−0.153	**0.674**	**0.388**
p	**<0.001**	**<0.001**	**<0.001**	0.009	0.643	0.077	**<0.001**	**<0.001**
n	143	145	143	137	142	135	142	140
APase-d								
r	−0.010	0.174	**0.319**	0.181	**0.331**	−0.184	**0.291**	0.064
p	0.902	0.038	**<0.001**	0.034	**<0.001**	0.033	**<0.001**	0.453
n	160	160	160	157	158	152	159	160

Table 3. Linear regression analyses of the temporal changes in soluble reactive phosphorus (SRP) concentrations during the incubation of lake waters collected from the benthic nepheloid layer (BNL) at Stn 4 (Fig. 1). Experiments were conducted using raw sample water (Non-treatment) and water samples amended with particulate materials (>0.8 µm) so that particle concentrations increased 5-fold (×5 concentrate)

Period of incubation (d)	Treatment	Linear regression parameters						
		Slope (nM d^{-1})	SE	*y*-intercept (nM)	SE	r^2	p	n
September								
35	Non-treatment	0.847	0.168	462	3.2	0.809	0.002	8
35	×5 concentrate	4.323	0.284	454	5.4	0.975	<0.001	8
November								
70	Non-treatment	0.498	0.086	288	3.7	0.829	<0.001	10
70	×5 concentrate	2.585	0.249	321	10.4	0.931	<0.001	10

transported by advection to the pelagic area to facilitate the P regeneration therein.

The results of our incubation experiments suggested that SRP is released from particulate matter in the BNL. Note that our approach using batch cultures may underestimate the rate of the P regeneration in the BNL because, during incubation, lake waters contained in tanks receive no delivery of 'fresh' organic matter, which, in a real basin, is continuously supplied by the sinking of particles. Despite this limitation, our results revealed that the rate of SRP release was significant, accounting for a large fraction (45 to 76%) of the *in situ* accumulation rate of SRP in the hypolimnion of this basin. This high release rate of SRP in oxic waters of the BNL of Lake Biwa has implications for modeling the P cycling in the lake. Previous studies have found that in lakes with an oxygenated hypolimnion, phosphorus is not regenerated during the decomposition of suspended particulate organic matter and sinking particles during transit through the water column (Gächter & Mares 1985, Tezuka 1986). In Lake Lucerne, Gächter & Mares (1985) suggested that bacterial communities attached to sinking particles take up P, rather than regenerate it, presumably because of the P-poor nature of the organic matter available for bacterial consumption. In Lake Biwa, Tezuka (1986) found no net regeneration of SRP during the incubation of particulate organic matter collected in surface waters. On the basis of the above results, investigators have suggested that the accumulation of SRP in the oxygenated hypolimnion of deep lakes during stratification periods is mainly due to diffusion from bottom sediments in which anoxic conditions favor P release governed by redox chemistry and microbial physiology (Mortimer 1941, Gächter et al. 1988, Kalff 2002). Our experimental results showing release, at significant rates, of SRP in the waters collected from the BNL of Lake Biwa suggest that future studies should examine in greater detail the relative importance of sediments and the BNL as the sites of P recycling in large lakes with an oxygenated hypolimnion.

Although evidence on the exact mechanisms underlying significant SRP release in the BNL waters of Lake Biwa is not conclusive, one potential process that mediates this flux of SRP is the enzymatic hydrolysis of particulate organic P by APase, which we found to be highly abundant in the BNL. We are aware that the APase activity determined by adding a high concentration of substrate (i.e. potential APase activity) does not necessarily indicate *in situ* regeneration of phosphate; the seasonal accumulation of SRP in the BNL accompanied with the increasing activities of APase in that layer is suggestive of an active involvement of APase in P recycling in the BNL. Other possible mechanisms of P release in the BNL include the enzymatic action of 5′-nucleotidase, an ectoenzyme known to be abundant in marine and freshwaters (Ammerman & Azam 1985, Cotner & Wetzel 1991), and desorption or dissociation of phosphate bound to resuspended sediments (Eadie et al. 1984), which might be accelerated by the development of anoxic microenvironments inside aggregates of suspended matter.

To summarize, our extensive measurements of APase and other biotic and biochemical variables in the whole water column of Lake Biwa revealed novel features in ectoenzymatic distributions in a large freshwater basin. A notable feature is that APase is highly abundant in the BNL, leading us to hypothesize that APase plays a significant role in hypolimnetic P regeneration of Lake Biwa. Given that a mechanistic understanding of the internal loading of P is fundamentally important for the improvement of deductive biogeochemical models of lakes, future studies should examine the microbial metabolism of C and P, the origin and type of APase, and the mode of biogeochemical controls of P fluxes in the BNL of large lakes.

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