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Characterization of Organic Phosphorus in Lake Sediments by Sequential Fractionation and Enzymatic Hydrolysis

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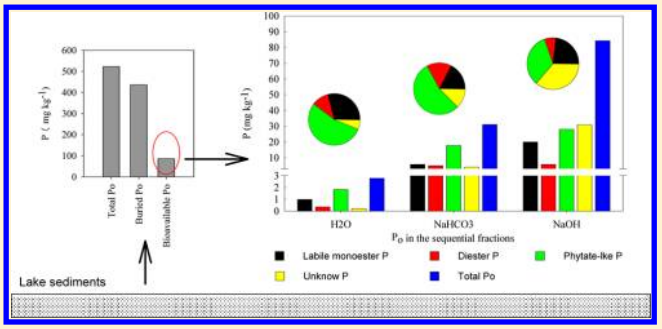
Supporting Information

ABSTRACT: The role of sediment-bound organic phosphorus (P_o) on lake eutrophication was studied using sequential extraction and enzymatic hydrolysis by collecting sediments from Dianchi Lake, China. Bioavailable P_o species including labile monoester P, diester P, and phytate-like P were identified in the sequential extractions by H_2O, $NaHCO_3$, and NaOH. For the H_2O-P_o, 36.7% (average) was labile monoester P, 14.8% was diester P, and 69.9% was phytate-like P. In $NaHCO_3-P_o$, 19.9% was labile monoester P, 17.5% was diester P, and 58.8% was phytate-like P. For $NaOH-P_o$, 25.6% was labile monoester P, 7.9% was diester P, and 35.9% was phytate-like P. Labile monoester P was active to support growth of algae to form blooms. Diester P mainly distributed in labile H_2O and $NaHCO_3$ fractions was readily available to cyanobacteria. Phytate-like P represents a major portion of the P_o in the NaOH fractions, also in the more labile H_2O and $NaHCO_3$ fractions. Based on results of sequential extraction of P_o and enzymatic hydrolysis, lability and bioavailability was in decreasing order as follows: H_2O-P_o > $NaHCO_3-P_o$ > $NaOH-P_o$, and bioavailable P_o accounted for only 12.1−27.2% of total P_o in sediments. These results suggest that the biogeochemical cycle of bioavailable P_o might play an important role in maintaining the eutrophic status of lakes.



INTRODUCTION

Phosphorus (P) is the limiting nutrient in most lake ecosystems, so excessive P is the critical factor that causes eutrophication.[1] As external inputs of P have been gradually reduced over the last few decades, release of internal P accumulated in sediments of lakes has become a significant source of P.[2,3] Therefore, transformation of P among various chemical forms, bioavailability, and exchange between sediments and overlying water have been extensively investigated.[2−4] Inorganic forms of phosphate, such as HPO_4^{2-} and $H_2PO_4^-$ are the predominant biologically available forms in sediments. Therefore most previous studies have focused on the role of inorganic P (P_i) in eutrophication of lakes. However, to lake sediments, organic P (P_o), including but not limited to nucleic acids, phospholipids, inositol phosphates, sugar phosphates, and condensed P, is also a potential source of P that is comparable in magnitude with P_i.[5] For example, concentrations of P_o accounted for 12−42.0% of total P (TP) in sediments from 43 lakes in China.[6] Although P_o is abundant in sediments, the composition and bioavailability of P_o in sediments remain poorly understood.[4] Thus, greater knowledge of the composition and bioavailability of P_o in sediments is needed for understanding dynamics of P in eutrophic lakes.

Sequential extraction schemes, based on the assumption that chemical extractants selectively dissolve discrete groups of P compounds, have been used to estimate relative concentrations and forms of constituents. These methods were first developed

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for soils and then extended to sediments.[7] They were then applied and developed to elucidate the chemical nature of P in sediments.[4,8,9] Several operationally defined schemes are available, but most of them focus on forms of P_i in sediments.[8−10] In recent years, the P_o pool in sediments has also been studied by sequential extraction.[4,11] A sequential extraction procedure was adapted by Hedley et al.[12] to separate P_i and P_o in environmental samples into several fractions associated with plant availability and various physicochemical associations. The fractions of P separated by the Hedley et al. procedure included labile adsorbed P, Fe- and Al-associated P, Ca-bound P, and recalcitrant insoluble P. The Hedley sequential procedure has been modified and used to characterize P in manure and soil.[13] To our knowledge, the Hedley et al. procedure has not been applied to investigate P_i and P_o fractions in sediments of freshwater lakes. This sequential procedure allows simple and rapid analysis of both P_i and P_o fractions in environmental samples, and also produces P_o fractions appropriate for subsequent enzymatic hydrolysis.

Enzymatic hydrolysis has been used as a novel approach to characterize P_o compounds and their bioavailability in various environmental samples.[14] When ^{31}P NMR and enzymatic hydrolysis methods were compared,[15,16] both were suitable for characterization of P in environmental samples, but enzymatic hydrolysis was preferable for determining labile P_o. Most P_o is first hydrolyzed to P_i prior to uptake by algae or other organisms. Thus, enzymatic hydrolysis can provide an estimate of hydrolyzable, and thus bioavailable P_o in lake sediments.[5,17] Phosphatase enzymes with different specificities have been used, alone and in combination, to assess bioavailability of P_o, even the P_o species.[18−21] Sequential fractionation, coupled with phosphatase hydrolysis, was a powerful tool to identify and quantify individual P_o species, and to monitor transformation and bioavailability of P_o species in the environment.[21,22] Most studies that have applied these approaches involved water and extracts of soils or manure, while few studies have been conducted on sediments.[14]

Dianchi Lake, the sixth largest freshwater lake in China, has an area of approximately 306 km^2, with mean depth of 5 m, a maximum depth of 9.7 m, and total volume of 12.9×10^8 m^3. This lake, which is situated in Southwest China and is designated as the backup water supply for Kunming City, has become severely eutrophic. The watershed of Dianchi Lake, which receives less water per capita than Israel or Jordan, is an area of severe water shortage. Because there are several rivers flowing into the lake, and only one outlet, the lake has a relatively long residence period of 3−8 years, which would exacerbate retention and accumulation of nutrients. In recent years, external inputs of P have been controlled to a certain extent. However, quality of water in the lake has not improved significantly and algal blooms still occur annually. The load of internal nutrients is a major factor that continues to influence the trophic status of the lake. Since P is the most critical nutrient limiting primary productivity in Dianchi Lake, it is important to know the relative sources of P before effective control measures can be applied. However, few studies have reported contributions or internal cycling of P from sediment, especially P_o, to the water column.[23] Therefore, the objectives of this study were to (1) characterize P_i and P_o fractions by sequential extraction in sediments collected from different locations in Dianchi Lake, (2) identify functional classes of P_o in these sequentially extracted fractions by phosphatase hydrolysis, and (3) assess the bioavailability of P_o, and discuss the biogeochemical cycle of P_o in sediments.

MATERIALS AND METHODS

Study Area and Sediment Sampling. In May 2010, surface sediments (approximately 10 cm deep) were collected from 18 locations in Dianchi Lake by use of a Peterson grab sampler (Supporting Information (SI) Figure S1). Of these sites (identified as S1−S24), S1, S2, S3, and S4 were in the southwest of the lake, near the Kunyang and Kunming Phosphate Fertilizer Factory. S13 was in the northern lake where, due to both nutrients and wind conditions, algal blooms aggregate. S19 and S20 were in the deepest part of the lake, near the center where P has been accumulated in recent years. The other sampling sites, including S6, S9, S10, S17, S18, S22, and S24−S28 were near the lakeshore, and are influenced by nonpoint inputs from agriculture. Sediments were transported to the laboratory in air-sealed plastic bags and cold storage with ice. Sediments were lyophilized and ground to powder with a pestle and mortar and stored at −20 °C before analysis. Sediment characteristics, including TP, P_i, P_o, total organic carbon (TOC), and total nitrogen (TN) were measured. Details of analytical methods and results are in SI Text S1.1 and S2.1.

Sequential Fractionation of Sediment Phosphorus. Fractions containing different forms of P were sequentially extracted from sediments by use of a modified sequential extraction procedure.[22] Briefly, duplicate extractions of each dry sediment (1.0 g, dry weight, d.w.) were extracted with 25 mL of deionized water at room temperature (H_2O fraction). After 2 h, extracts were centrifuged at 6470 rpm for 15 min and the supernatant was passed through a 0.45-μm filter. Using the same procedure, residues were then sequentially extracted with 0.5 mol L^{-1} $NaHCO_3$ (pH 8.5), 0.1 mol L^{-1} NaOH, and 1 mol L^{-1} HCl for 16 h each to separate the $NaHCO_3$, NaOH, and HCl fractions. To reduce contamination of subsequent extracts by residual extractant and P in the pellet, after $NaHCO_3$ and NaOH extraction, residues were washed by hand with 5 mL of water, and the supernatant was discarded after centrifugation. Concentrations of TP, P_i, P_o, and TOC were determined, and the pH of the $NaHCO_3$ and NaOH fractions was adjusted to 7.0. The pH was adjusted to 5.15 in order to prevent HCl fractions from precipitating after pH adjustment (SI Text S1.2).

Enzymes, Buffers, and Assay Procedure. Alkaline phosphatase (APase) from bovine intestinal mucosa (EC 3.1.3.1), phosphodiesterase (PDEase) from *Crotalus atrox* (EC 3.1.4.1), and crude phytase from wheat (EC 3.1.3.26) were purchased from Sigma-Aldrich Chemicals (A China Branch, or St. Louis, MO, USA). APase and PDEase were prepared in Tris-HCl buffer (0.1 mol L^{-1}, pH 9.0) at concentrations of 1 and 0.02 unit mL^{-1}, respectively. Crude phytase was purified to remove phosphates. One hundred twenty mg of enzyme in 10 mL of 10 mmol L^{-1} NaAc-HAc buffer (pH 5.15) was dialyzed five times using a Spectra/Por Float-A-Lyzer (MWCO: 3500−5000, Spectrum Laboratories, Inc.) for about 12 h with 2 L of buffer. Dialyzed enzyme solution was then centrifuged at 9150 rpm for 10 min. Crude phytase was purified immediately before use. Purified phytase was prepared in Tris-HCl buffer (0.1 mol L^{-1}, pH 7.0) or NaAc-HAc buffer (0.1 mol L^{-1}, pH 5.15) at concentration of 0.06 unit mL^{-1}. APase was used alone, but PDEase was used in combination with APase to achieve complete hydrolysis of diester phosphates. Phytase prepared in NaAc-HAc buffer (0.1

Table 1. Enzymatic Hydrolysis (%) of Model Compounds by Phosphatase Enzymes (Values are Mean of Triplicate Samples)

	recovery									
model P compounds[a]	APase pH 9 37 °C	PDEase+ APase pH 9 37 °C	buffer pH 9 37 °C (0 h)	buffer pH 9 37 °C (16 h)	phytase pH 5.15 55 °C	buffer pH 5.15 55 °C (0 h)	buffer pH 5.15 55 °C (16 h)	phytase + PDEase+ APase pH 7 37 °C	buffer pH 7.0 37 °C (0 h)	buffer pH 7.0 37 °C (16 h)
*p*NPP	100.1	100.0	1.8	1.8	105.3	0.7	30.7	100.7	2.0	12.0
Glu6P	84.9	85.0	1.6	1.8	29.2	0.1	8.3	83.6	1.4	11.9
AMP	90.5	89.3	2.0	2.0	95.8	0.6	27.3	85.7	0.7	9.8
IP6	0.9	2.5	1.0	0.6	65.5	1.3	0.0	84.8	0.0	0.0
bis-*p*NPP	3.2	56.5	1.6	1.0	85.2	0.2	0.0	96.0	0.1	0.0
DNA	4.9	98.2	1.5	1.1	39.7	0.4	0.0	82.1	0.0	0.2
ATP	98.4	94.3	1.9	3.6	96.4	1.3	14.2	96.8	0.3	13.6
TPP	90.1	91.4	1.5	6.2	99.5	2.0	29.4	95.7	0.1	13.3

[a] *p*NPP, *p*-nitrophenyl phosphate; Glu6P, glucose-6-phosphate; AMP, adenosine 5′ monophosphate; IP6, inositol hexakisphosphate; bis-*p*NPP, bis-(para-nitrophenyl) phosphate; DNA, DNA; ATP, adenosine 5′ triphosphate; TPP, tetra-sodium pyrophosphate.

mol L^{-1}, pH 5.15) was used alone. Phytase prepared in Tris-HCl buffer (0.1 mol L^{-1}, pH 7.0) was used in combination with APase and PDEase (0.1 mol L^{-1}, pH 7.0) to ensure that hydrolysis of dissolved organic phosphorus (DOP) was as complete as possible.

All buffers contained 0.002 mol L^{-1} $MgCl_2$ as an activator for the enzymes.[17] Tris-sodium citrate ($Na_3C_6H_5O_7{\cdot}2H_2O$) was added to prevent adsorption of released P_i by metal-hydroxides during assay.[18] The assay preparation consisted of 5 mL of the H_2O fraction or pH-adjusted extracts including $NaHCO_3$, NaOH, and HCl fraction, 0.44 mL of each different enzyme mixture (APase at pH 9.0, APase + PDEase at pH 9.0, APase + PDEase + phytase at pH 7.0, or phytase at pH 5.15) in appropriate buffer and 0.05 mL of 0.68 mol L^{-1} trisodium citrate. Mixtures were incubated for 16 h at 37 °C in the colorimetric tube.[18,19] But the HCl fraction was only hydrolyzed by use of phytase at pH 5.15 and 55 °C to prevent precipitation. Organic P hydrolyzed by each enzyme preparation was calculated as the difference between P_i concentrations determined before and after incubation. A duplicate sample, containing enzyme-free buffer, was incubated simultaneously to monitor and correct for any nonenzymatic hydrolysis and/or matrix blank. Phosphate was analyzed by the molybdenum blue/ascorbic acid method,[24] 2% SDS was added (accounted in the final volume 10 mL) prior to analysis to prevent enzyme precipitation.[25] To correct the interference induced by matrix, calibration curves were developed simultaneously with each procedure.[14]

Substrate Specificity of Phosphatase Preparations. The substrate specificity of the enzyme preparations was investigated for a range of model P compounds, including orthophosphate monoesters (*para*-nitrophenyl phosphate, adenosine 5′ monophosphate, glucose-6-phosphate, inositol hexakisphosphate), orthophosphate diesters (bis-*para*-nitrophenyl phosphate, DNA), and condensed-P compounds (tetra-sodium pyrophosphate, adenosine 5′-triphosphate) purchased from Sigma or Amresco Chemicals. Model compounds were prepared accurately in ultrapure water at a concentration of 8 mg P L^{-1} and diluted to 400 μg L^{-1} then incubated in triplicate as described in the above assay procedure, and concentrations of P_i were quantified. Controls containing enzyme-free buffer were incubated simultaneously before and after incubation, which represented chemical (nonenzymatic) degradation. The acid-induced slight breakdown of organic and condensed P compounds caused by acid possibly occurs during analysis by molybdenum blue/ascorbic acid method (Table 1).

Data Analysis. Data were checked for deviations from normality of variance before analysis. To check whether there was a significant linear relationship between bioavailable P_o and TOC, Pearson correlation coefficients (r values, two-tailed) at $p \leq 0.01$ and $p \leq 0.05$ were determined using SPSS 11.5. The gradient distributions of P_o species, bioavailable P_o, and TOC in sediments of Dianchi Lake were calculated using inverse-distance weighting (IDW) by ArcGis 10.0 Spatial Analyst. The box plot and histogram were performed using Sigma Plot 10.0.

RESULTS AND DISCUSSION

Distribution of P Fractions in Sequentially Extracted Fractions. Concentrations of P_i and P_o varied among the four sequentially extracted fractions (SI Figure S2). Recoveries of P_i in all four fractions ranged from 93.9% to 114.0% of the total P_i determined in sediments. Inorganic P extracted by H_2O was loosely adsorbed to sediment particles or in the interstitial water of sediments, which was transferred more easily in the interface of water−sediment than other forms of P. Contents of P_i in the H_2O fraction, which represent P in sediments that is immediately available for accumulation by phytoplankton,[2] ranged from 0 to 1.4 mg kg^{-1} d.w., and accounted for only 0.6−49.8% of total P in the H_2O fraction. Inorganic P in $NaHCO_3$ extracts, which represents slightly less labile adsorbed P, can be regarded as a quantitative index of available P for algae in sediments.[2,4,26,27] Concentrations of P_i in the $NaHCO_3$ fraction ranged from 50.9 to 213.4 mg kg^{-1} d.w, and accounted for 68.4−83.8% of total P in this fraction. Inorganic P soluble in NaOH represents less labile P_i that is associated with Fe and Al oxides,[13,26] which could be used to estimate available P to algae from sediments for both short-term (2-d) and long-term (14-d) incubations.[28,29] Concentrations of P_i extracted from sediments by NaOH, ranged from 232.2 to 635.7 mg kg^{-1} d.w, and accounted for 74.3−87.3% of total P in this fraction. Inorganic P extracted by HCl accounted for the greatest portion of total P_i in sediments from Dianchi Lake, with concentrations of 574.0−1067.5 mg kg^{-1} d.w. However, HCl−P_i was P primarily bound to calcium, which was released from sediments with difficulty, and therefore would not be readily available to phytoplankton.[30] Based on the sequential extraction schemes, the lability of P_i fractions was in decreasing order of H_2O-P_i > $NaHCO_3-P_i$ > $NaOH-P_i$ > $HCl-P_i$ in sediments.

The mechanism of adsorption of P_o to sediments or minerals was similar to that of P_i. This is because most naturally

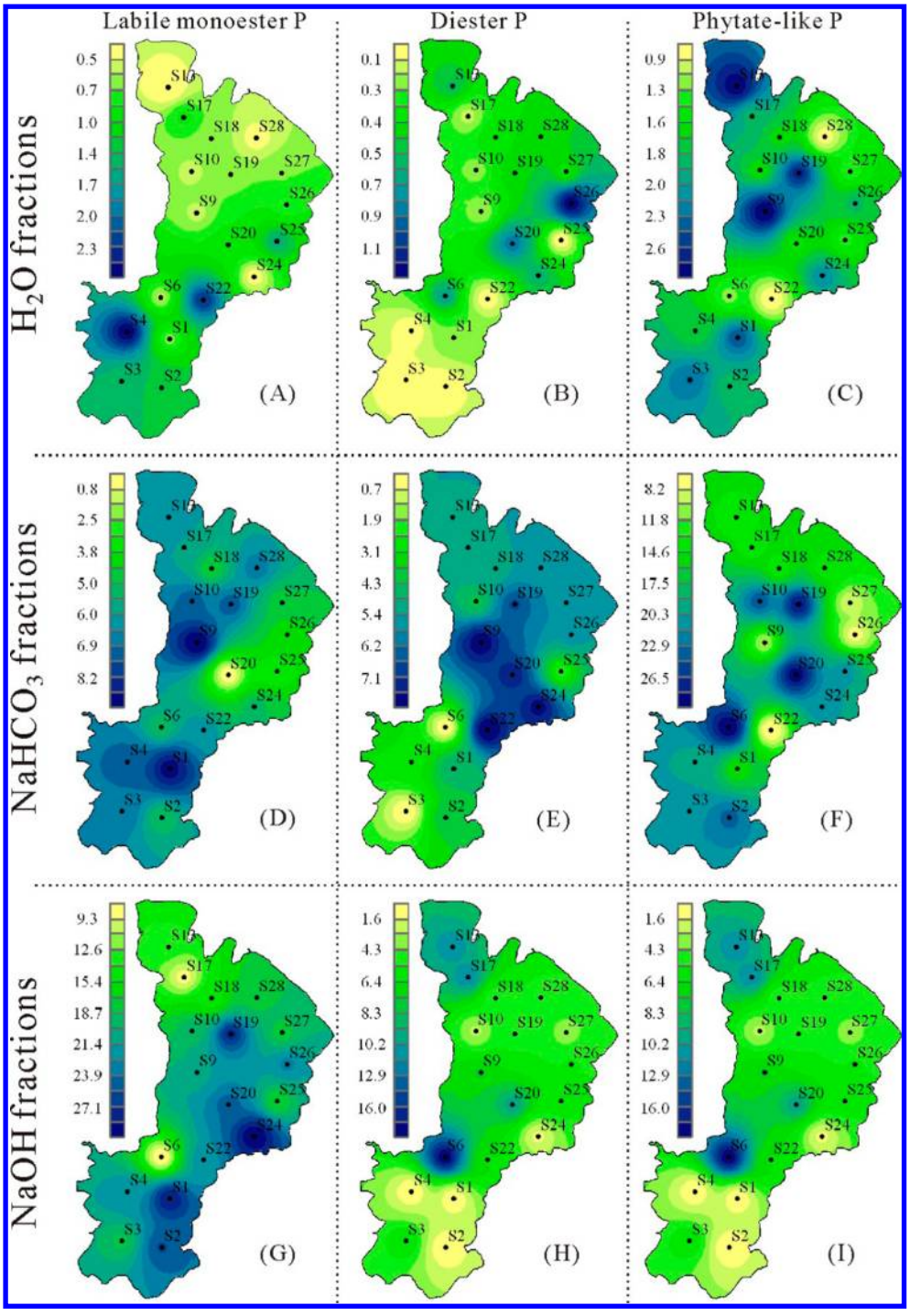

Figure 1. Spatial distribution of contents of P_o species (mg kg^{-1} d.w.) characterized by phosphatase in the H_2O, $NaHCO_3$, and NaOH fractions.

occurring P_o compounds are present in either the mono- or diester form.[31] The one or two nonester hydroxyl groups imparted some inorganic P-like (orthophosphate bond) properties to these organic P compounds.[32−34] Therefore, lability of P_o released to overlying water in sequential fractions was also similar to that of P_i. Concentrations of P_o fractions of sediments from Dianchi Lake decreased in the following order: NaOH−P_o > $NaHCO_3$−P_o > HCl−P_o > H_2O−P_o. Concentrations of P_o in the H_2O fraction of sediments ranged from 1.4 to 5.1 mg kg^{-1} d.w (2.8 mg kg^{-1} d.w., average), and were the primary constituent of total P in the H_2O fraction of sediments. This was likely due to the hyper eutrophication of Dianchi Lake and the requirement of phosphate for growth of algae.[20] Concentrations of P_o in the $NaHCO_3$ fraction ranged from 16.6 to 45.8 mg kg^{-1} d.w. (31.1 mg kg^{-1} d.w., average). The greatest portion of P_o was extracted by NaOH, with concentrations ranging from 45.8 to 220.4 mg kg^{-1} d.w. (84.3 mg kg^{-1} d.w., average). NaOH−P_o could be separated into two parts: moderately labile organic P that was considered to be combined with fulvic acid, and nonlabile organic P that is combined with humic acid.[4] There were small portions of P_o compared with P_i detected in the HCl fraction of sediments, with concentrations ranging from 0 to 25.7 mg kg^{-1} d.w. (6.8 mg kg^{-1} d.w., average), only accounting for 0−2.4% of total P in this fraction.

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Concentrations of HCl−P_o in sediments were less than those observed in soil and litter.[16] The sum of P_o in the four fractions ranged from 69 to 267 mg kg^{-1} d.w., and accounted for 15.4−49.4% of total P_o in sediments. The unextractable P_o could be refractory P_o, which might not be bioavailable.[30]

The sum of concentrations of potentially bioavailable P_i,[30] including H_2O−P_i, $NaHCO_3$−P_i, and NaOH−P_i, ranged from 283 to 850 mg kg^{-1} d.w. and accounted for 17.2% to 32.4% of TP in sediments. Compared with potentially bioavailable P_i, concentrations of P_o in these fractions ranged from 69 to 265 mg kg^{-1} d.w. and accounted for 4.2−10.1% of TP in sediments. Bioavailability of P_o in these fractions is unknown, and thus still needs to be clarified by enzymatic hydrolysis.

Substrate Specificity of Phosphatase Preparations. APase hydrolyzed 84.9−100.1% of the model phosphate monoester, including *p*NPP, Glu6P, and AMP, and condensed phosphates, including ATP and TPP (Table 1). However, APase did not decompose phosphate diesters, such as bis-*p*NPP or DNA, and the phosphate monoester inositol hexakisphosphate (IP6). PDEase combined with APase preparation gave a similar yield of hydrolyzed condensed phosphates, the phosphate monoester and phosphate diester (DNA). Only 56.5% of the diester bis-*p*NPP was hydrolyzed by this enzyme preparation. The monoester inositol hexakisphosphate (IP6) was not hydrolyzed by PDEase. Phytase could act on all model P_o compounds. However, phosphate monoester (Glu6P) and the phosphate diester (DNA) were only partially hydrolyzed (29.2% and 39.7%, respectively). The most hydrolyzed substrate was IP6 (65.5%). The mixture of enzymes (phytase + PDEase + APase) resulted in the greatest recovery of all model P_o compounds. This enzyme preparation could act on all model P_o compounds well, with the greatest release of 84.8% for IP6.

Spontaneous hydrolysis before incubation was usually small (approximately 1%) in samples containing enzyme-free buffer with quantification by the molybdenum blue/ascorbic acid method. However, there was partial spontaneous hydrolysis of P_o after incubation in enzyme-free buffer, especially of phosphate monoester and condensed phosphate in the NaAc-HAc buffer at pH 5.15.

Results of the substrate specificity tests were similar to those of previous studies.[18,19,21] Recovery of IP6 in this assay procedure was greater with phytase than what had been reported previously, 38%[21] and 52%,[18] respectively, but slightly less than the yield reported by Turner et al. (95.7%).[19] However, the procedure used here still allowed classification of enzymatically hydrolyzed P into the following groups: (1) labile monoester P (hydrolyzed by APase), (2) diester P (hydrolyzed by APase + PDEase minus labile monoester P), (3) phytate-like P (hydrolyzed by APase + PDEase + phytase minus labile monoester P and diester P).

P_o Species and Bioavailability in the Sequential Fractions. Based on specificity of substrates for phosphatase preparations and the sequential P_o fractions hydrolyzed by these enzymes (SI Text S2.3), several P_o species were identified in the H_2O, $NaHCO_3$, and NaOH fractions of sediments from Dianchi Lake (Figures 1 and 2). Several ^{31}P NMR studies have demonstrated that phosphate monoesters are the primary species of P_o in sediments of Dianchi Lake[6] and some other lakes.[35,36] Although it was possible to hydrolyze diester P (e.g., RNA) and phospholipids to phosphate monoesters associated with ^{31}P NMR measurement,[37] enzymatic hydrolysis provided a relatively mild approach for characterizing labile P_o.[38]

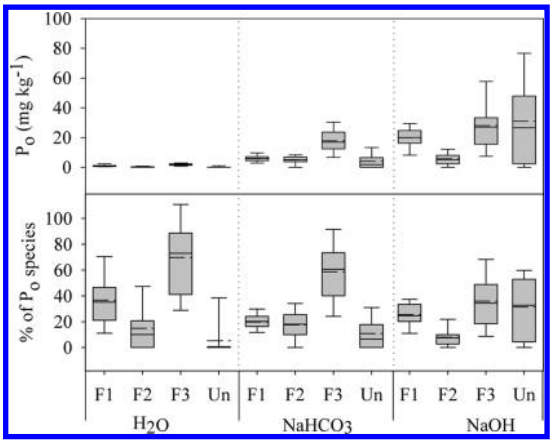

Figure 2. Contents and relative abundance of labile monoester P (F1), diester P (F2) and phytate-like P (F3), and unknown P_o (Un) in the H_2O, $NaHCO_3$, and NaOH fractions, respectively. The lower boundary of each box indicates the 25th percentile, the full line within the box shows the median, the dashed line represents the mean, and the upper boundary shows the 75th percentile. Error bars indicate the 95th and 5th percentiles.

Phosphate monoesters, including labile monoester P and phytate-like P were all present in the H_2O, $NaHCO_3$,and NaOH P_o fractions. Labile monoester P, the P_o released by APase, includes mononucleotides, sugar phosphates, by-products of phospholipids decomposition, and pyrophosphates in sediments of lakes.[36] Amounts of labile monoester P were 0.3−2.7 mg kg^{-1} d.w., and accounted for 7.8−85.3% in H_2O−P_o, and with an average concentration of 1.0 mg kg^{-1} d.w. (36.7%) (Figure 2). For $NaHCO_3$−P_o, concentrations of labile monoester P were 0−10.3 mg kg^{-1} d.w. (0−33.4%), with an average value of 5.9 mg kg^{-1} d.w. (19.9%). Contents of labile monoester P in NaOH−P_o were 7.0−31.7 mg kg^{-1} d.w., with an average value of 20.0 mg kg^{-1} d.w. Labile monoester P was also a significant portion of NaOH−P_o, and accounted for 8.9−40.2%, with an average value of 25.6% of NaOH−P_o. Overall, the percentage of labile monoester P was greater in the H_2O fractions than that in the $NaHCO_3$ and NaOH fractions. There were no relationships between the spatial distribution of labile monoester P and TOC in the sediments (Figure 1A, D, G and Figure 4B). Especially, the contents of labile monoester P in the H_2O fractions were low in the north and central part of Dianchi Lake (Figure 1A). The percentage also varied in a large scale among locations (Figure 2) which suggests that labile monoester P was an important bioavailable P_o and readily bioavailable in this fraction. The percentage of labile monoester P was only 11.4% in the H_2O−P_o from S13, which was possibly hydrolyzed and released to the overlying water for supporting the algae bloom.

Phytate-like P, mainly consisting of inositol phosphates as well as inositol phosphates bound to proteins and fulvic acids,[20] can be a significant proportion of phosphate monoester in sediments of lakes.[36,39] Inositol phosphates were considered to be relatively refractory in sediments, because they were likely sorbed onto iron oxides to form an insoluble Fe_4−phytate that resists enzymatic hydrolysis.[36,40] However, it was not only present in the NaOH fraction and residual P_o in sediments,[39,41] but also a major P_o species in the more labile H_2O and $NaHCO_3$ fractions (Figure 2). The amounts of phytate-like P were 0.7−3.0 mg kg^{-1} d.w. (average, 1.8 mg kg^{-1} d.w.), and accounted for 24.4−116.2% (average, 69.9%) in the total P_o of

H_2O fraction. For $NaHCO_3$–P_o, contents of phytate-like P were 5.2–31.3 mg kg^{-1} d.w. (average, 17.9 mg kg^{-1} d.w.), and accounted for 22–120.1% (average, 58.8%) of total P_o in the $NaHCO_3$ fraction. Concentrations of phytate-like P were 6.5–71.4 mg kg^{-1} d.w. (average, 28.1 mg kg^{-1} d.w.), and accounted for 6.6–71.2% (average, 35.9%) of total P_o in the NaOH fraction. The spatial distribution of phytate-like P in the labile H_2O and $NaHCO_3$ fractions was similar to that of TOC in the sediments (Figure 1C, F and Figure 4B). Phytate-like P was significantly positively correlated with TOC in the H_2O and $NaHCO_3$ fractions (p = 0.039 and 0.032, respectively, SI Figure S7), which showed that the contents of phytate-like P in the labile H_2O and $NaHCO_3$ fractions were possibly closely related with organic matter accumulated in sediments of Dianchi Lake. However, there was no correlation between phytate-like P and organic matter in the NaOH fraction (Figure 1I and SI Figure S7 C). Due to the large concentrations of metals and humic in the NaOH fraction, it was postulated that the phytate-like P was in more complex forms, such as being associated with humic material or metals.[18,22,39] Therefore, the percentages of unknown P_o were the greatest in the NaOH fraction (0–60.2%, average, 31.5%).

Concentrations of diester P were less than the pool of monoester P (the sum of labile monoester and phytate-like P), extracted from sediments of Dianchi Lake by NaOH-EDTA when characterized by ^{31}P NMR.[6] This result is consistent with the distribution of diester P among the H_2O, $NaHCO_3$, and NaOH P_o fractions (Figure 2). Concentrations of diester P were 0–1.3 mg kg^{-1} d.w. (average, 0.4 mg kg^{-1} d.w.), and accounted for 0–64.3% (average, 14.8%) in the total P_o of H_2O fraction; 0–8.6 mg kg^{-1} d.w. (0–36.7%), with an average concentration of 5.0 mg kg^{-1} d.w. (17.5%) in the $NaHCO_3$ fraction; and 0–19.8 mg kg^{-1} d.w. (average, 5.8 mg kg^{-1} d.w.), accounting for 0–24.5% (average, 7.9%) in the NaOH fractions. Compared with the NaOH fraction, the percentages of diester P were greater in H_2O and $NaHCO_3$ fractions. More than 55.0% (average), and as much as 100% in some samples, of the diester P sequentially extracted by H_2O, $NaHCO_3$, and NaOH was distributed in the more labile H_2O and $NaHCO_3$ fractions. There were no relationships between the spatial distributions of diester P and TOC in the sediments (Figures 1B, E, H, and 4B), which possibly showed that diester was active P_o species in the sediments. These results implied that diester P was also weakly adsorbed to sediments, as in the soil,[19] and was readily available to blue–green algae in the lake.

There was no hydrolyzable P_o in the HCl fraction of sediments from Dianchi Lake (SI Figure S6). Compared with the contents of P_i in the HCl fraction, there might have been a small amount of P_o hydrolyzed after dilution which was undetectable using the analytical method used in this research. In addition, P_o in the HCl fraction was also possibly hydrolyzed during the process of acid extraction.

Concentrations of total P_o hydrolyzed by enzymes were in the increasing order NaOH–P_o > $NaHCO_3$–P_o > H_2O–P_o. These concentrations were correlated with TOC and P_o in the according extractants (Figure 3A, SI Text S2.2). However, the order of relative percentages of P_o hydrolyzed by enzymes to total P_o in the H_2O, $NaHCO_3$, and NaOH fractions, respectively, was reversed (Figure 3B). Therefore, P_o fractions characterized by sequential extraction and enzymatic hydrolysis in sediments from Dianchi Lake could be classified based on their decreasing lability and bioavailability in the order H_2O–P_o > $NaHCO_3$–P_o > NaOH–P_o. In the traditional sequential P_o fractionation, $NaHCO_3$ P_o fractions have been considered to be labile P_o and NaOH P_o fractions including moderately labile P_o and nonlabile P_o in the sediments.[4,27] However, P_o in these fractions was not completely hydrolyzed by APase, PDEase, and phytase. The traditional sequential fractionation of P_o was crude, which would overestimate the labile P_o in sediments.

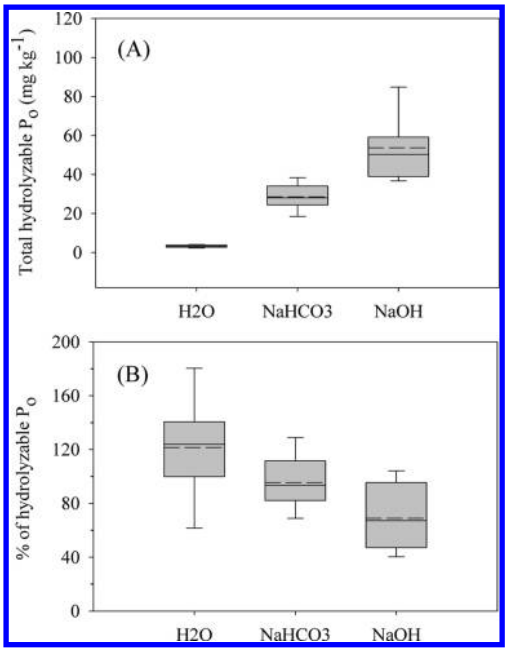

Figure 3. Summary of total hydrolyzable P_o in H_2O, $NaHCO_3$, and NaOH fractions of sediments from Dianchi Lake: (A) the contents, (B) the percentage of P_o released in according fractions. The lower boundary of each box indicates the 25th percentile, the full line within the box is the median, the dashed line shows the mean, and the upper boundary is the 75th percentile. Error bars indicate the 95th and 5th percentiles.

Biogeochemical Cycle of P_o in the Lake Sediments. Phosphatase hydrolysis is involved in the biogeochemical process of P_o in lake sediments.[14,17,42] The sum of hydrolyzable P_o in the H_2O, $NaHCO_3$, and NaOH fractions ranged from 62.5 to 147.0 mg kg^{-1} d.w. (Figure 4A). Potential availability of P_o extracted by H_2O, $NaHCO_3$, or NaOH then hydrolyzed by enzymes accounted for 12.1–27.2% of total P_o and 3.1–6.3% of TP, respectively, in sediments from Dianchi Lake. It has been argued that the majority of P_o in surface sediments was likely stabilized and did not contribute to internal loading of P from lake sediments directly.[43] Aggregative results of this research demonstrate that the majority of P_o in the sediments from Dianchi Lake, such as the unextratable phytate-like P, would be buried in sediments. However, the labile and hydrolyzable P_o by enzymes would be active in the biogeochemical process of P cycle of the lake. APase and PDEase exist in water and sediments of lakes, and thus are important in the internal cycling of P in sediments.[42,44–47] Phytase present in soils can be transported into lake ecosystems.[48] The protein sequence identity also suggests that β-propeller phytase is widespread in aquatic environments.[49,50] Therefore, the P_o in the H_2O, $NaHCO_3$, and NaOH fractions that could be released then hydrolyzed by enzymes was a potentially bioavailable P_o for lake ecosystems.

There were statistically significant relationships between the sum of hydrolyzable P_o and TOC (%) in sediments (r = 0.527, p = 0.025, n = 18). Especially, contents of hydrolyzable P_o in

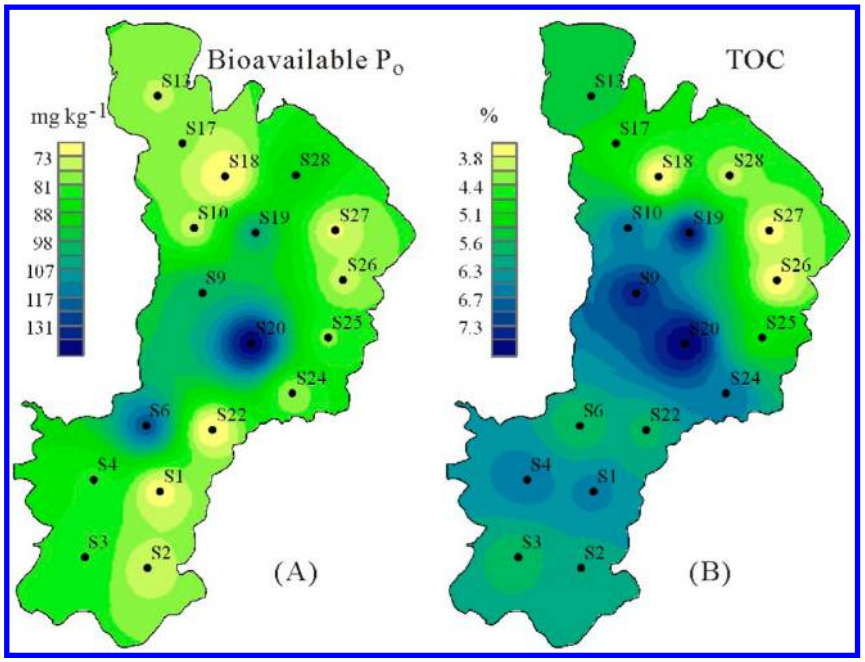

Figure 4. Spatial distributions of bioavailable P_o (A) and TOC (B) in surface sediments of Dianchi Lake. The sum of bioavailable P_o was calculated by P_o hydrolyzed in the H_2O, $NaHCO_3$, and NaOH fractions by the combination of APase, PDEase, and phytase.

labile H_2O-P_o and $NaHCO_3-P_o$ were significantly correlated with organic matter (SI Figure 8). Hydrolyzable P_o in the more labile H_2O-P_o and $NaHCO_3-P_o$ were active, which could lead to different accumulation and degradation rates of bioavailable P_o. Therefore, bioavailable P_o in some locations was outside the general trends. Though the $NaOH-P_o$ was significantly correlated with organic matter ($P \leq 0.003$, SI Text 2.2), there were no relationships between bioavailable P_o and organic matter in NaOH fractions, which indicates possible complexation with polyvalent cations and complex organic matter that could render the $NaOH-P_o$ less available for enzymatic hydrolysis.[18,51] For example, Creecchio and Stotzky[51] reported that DNA bound on humic acid was protected more against degradation by DNase than free DNA. Though the correlation was not strong, contents of bioavailable P_o were greater in sediments where organic matter had accumulated, such as in the central part of Dianchi Lake (Figure 4A, B). For different type of lakes, labile and moderately labile P_o (characterized by Ivanoff et al. scheme[27]) were also significantly positively correlated with organic matter reported in our previous research.[4] In sediments of Dianchi Lake, organic matter and P_o originated primarily from autochthonous sources (SI Text 2.1 and Text 2.2). Thus, although most P_o was stable, sediments of the lake would accumulate more bioavailable P_o in the process of eutrophication. Furthermore, phosphatase (e.g., APase) activity was proportional to organic matter and P_o in sediments.[46,52] With greater phytoplankton biomass in eutrophic lakes, activities of APase would be expected to be greater in sediments.[44] Therefore, the bioavailable P_o would be released and continue to support eutrophication during periods when less orthophosphate is available. The biogeochemical cycle of bioavailable P_o might be an important process to self-regulate the nutrient status for eutrophic lakes and maintain their eutrophic status longer even after external sources of P have been controlled.

■ ASSOCIATED CONTENT

Supporting Information

Methods, table of sediment characteristics, and figures of sampling map, distribution of phosphorus and TOC in the sequential fractions, hydrolysis of organic phosphorus by phosphatase enzymes in the sequential fractions, and others. This information is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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