



# Simulated bioavailability of phosphorus from aquatic macrophytes and phytoplankton by aqueous suspension and incubation with alkaline phosphatase

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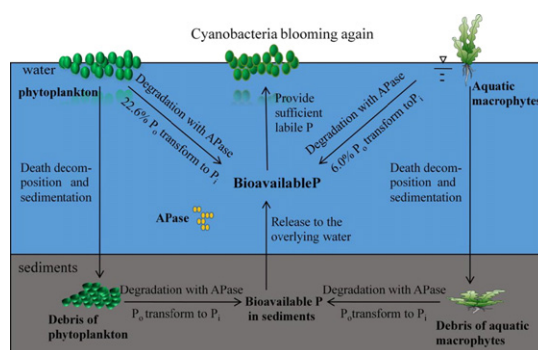
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## HIGHLIGHTS

- Simulation of natural release of P from aquatic macrophytes and phytoplankton in aqueous suspensions.
- P released in the presence or absence of the APase characterized by solution <sup>31</sup>P NMR.
- APase increased bioavailable orthophosphate through hydrolysis of P<sub>o</sub>.
- Release of P<sub>o</sub> from phytoplankton debris is the fastest way to supply phosphorus for repeated cyanobacterial blooming.
- After cessation of allochthonous inputs, removal of macrophyte biomass would be the most effective means to control eutrophication.

## GRAPHICAL ABSTRACT



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## ABSTRACT

Bioavailability of phosphorus (P) in biomass of aquatic macrophytes and phytoplankton and its possible relationship with eutrophication were explored by evaluation of forms and quantities of P in aqueous extracts of dried macrophytes. Specifically, effects of hydrolysis of organically-bound P by the enzyme alkaline phosphatase were studied by use of solution <sup>31</sup>P-nuclear magnetic resonance (NMR) spectroscopy. Laboratory suspensions and incubations with enzymes were used to simulate natural releases of P from plant debris. Three aquatic macrophytes and three phytoplankters were collected from Tai Lake, China, for use in this simulation study. The trend of hydrolysis of organic P (P<sub>o</sub>) by alkaline phosphatase was similar for aquatic macrophytes and phytoplankton. Most monoester P (15.3% of total dissolved P) and pyrophosphate (1.8%) and polyphosphate (0.4%) and DNA (3.2%) were transformed into orthophosphate (14.3%). The major forms of monoester P were glycerophosphate (8.8%), nucleotide (2.5%), phytate (0.4%) and other monoesters P (3.6%). Proportions of P<sub>o</sub> including condensed P hydrolyzed in phytoplankton and aquatic macrophytes were different, with the percentage of 22.6% and 6.0%, respectively. Proportion of P<sub>o</sub> hydrolyzed in debris from phytoplankton was approximately four times

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greater than that of  $P_o$  from aquatic macrophytes, and could be approximately twenty-five times greater than that of  $P_o$  in sediments. Thus, release and hydrolysis of  $P_o$ , derived from phytoplankton debris would be an important and fast way to provide bioavailable P to support cyanobacterial blooming in eutrophic lakes.

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## 1. Introduction

Phosphorus (P) is generally the most limiting nutrient of productivity, and thus excessive P is the key factor in controlling cyanobacterial blooming in many lakes (Kagaloua et al., 2008; Schindler et al., 2016). Many relevant studies, conducted previously, were focused primarily on the geochemical cycling of P from water and sediments in lakes and wetland environments (Cade-Menun et al., 2006; Ding et al., 2015; Han et al., 2015; Pant and Huang, 2015). Few studies have investigated P derived from aquatic macrophytes and phytoplankton, especially organic P ( $P_o$ ), which was an important component in aquatic macrophytes and phytoplankton, and could account for 46%–70% of total P in aquatic macrophytes and phytoplankton (Feng et al., 2016a). Mineralization of  $P_o$  into inorganic P had a direct effect on bioavailability of P in freshwater systems (Pant et al., 2002; Wang and Pant, 2010; Lehman et al., 2017). Forms and labilities of  $P_o$  of aquatic macrophytes and phytoplankton collected from Tai Lake, have been studied previously (Feng et al., 2016b). Results of that study have demonstrated that monoester P and pyrophosphate could be hydrolyzed to bioavailable orthophosphate by enzymes such as alkaline phosphatase. However, compositions and bioavailabilities of  $P_o$  in aqueous suspensions of aquatic macrophytes and phytoplankton had not been investigated.  $P_o$  in aqueous suspensions of aquatic macrophytes had been assumed to be easily released by enzymes to water columns of lakes (Zhu et al., 2016). Due to their rapid migration and transformation when released from plant litter, aqueous suspensions of organic matter, derived from aquatic macrophytes, has been shown to be the most dynamic component in biogeochemical cycling (Qu et al., 2013). Therefore, characterization of  $P_o$  in aqueous suspensions of aquatic macrophytes and phytoplankton was deemed to be of significance during degradation and recycling of nutrients in lakes.

Solution  $^{31}\text{P}$  nuclear magnetic resonance (NMR) spectroscopy has been used to characterize  $P_o$  from aquatic macrophytes and phytoplankton in eutrophic lakes (Feng et al., 2016a; Liu et al., 2016). Enzymatic hydrolysis with commercially available phosphatase has also been used to characterize forms of P and evaluate their bioavailability in environmental samples (He and Honeycutt, 2001; Pant et al., 2002; Young et al., 2013; Jarosch et al., 2015). Alkaline phosphatase (APase), an important enzyme for internal cycling of P in lakes, widely exists in water and sediments (Jansson et al., 1988; Zhang et al., 2007; Zhou et al., 2008; Zhu et al., 2016). Phosphatase activities in lakes can be due to enzymes localized on surfaces of algal and bacterial cells, dissolved in water or sediments, released during autolysis or excreted from phytoplankton, bacteria or zooplankton (Jansson et al., 1988). While  $P_o$  can be hydrolyzed either spontaneously or enzymatically (He et al., 2006a; Jaisi et al., 2014), simulation of releases of  $P_o$  in aqueous suspensions with enzymatic incubation provides a rapid, controlled method of evaluating bioavailability of  $P_o$  in relevant environmental samples (He et al., 2003, 2006a). Therefore, characterization of  $P_o$  forms and their lability by  $^{31}\text{P}$  NMR and enzymatic hydrolysis in aqueous suspension of aquatic macrophytes and phytoplankton would be environmentally meaningful as it could help elucidate mechanisms of recycling of P that result in repeated cyanobacterial blooming, even when loadings of P to eutrophic lakes were curtailed.

Thus, in this work, three representative aquatic macrophytes and three representative phytoplankton isolated from Tai Lake were studied as examples of materials that could contribute to the biogeochemical cycling of  $P_o$  in eutrophic lakes. To simulate natural releases of  $P_o$ , dry powders of the six samples were suspended in water and incubated in

the absence and presence of alkaline phosphatase. Changes in the P forms of quantities were evaluated by solution  $^{31}\text{P}$  NMR spectroscopy. With these data, the bioavailability of P in biomass of aquatic macrophytes and phytoplankton and its possible relationship with blooming of cyanobacteria were discussed. Results of this study shed light on more effective control of eutrophication of freshwater lakes.

## 2. Materials and methods

### 2.1. Sample collection and preparation

Three representative aquatic macrophytes, Foxtail algae (*Myriophyllum spicatum* Linn.), Common reed (*Phragmites australis trinngramieae*) and Black algae (*Hydrilla verticillata*) were collected from Tai Lake (Ch: Taihu) (33°55′–31°32′N, 119°52′–120°47′E), which was a large shallow eutrophic lake, located in Jiangsu Province, China. Tai Lake has a surface area of 2338 km<sup>2</sup> with mean and maximum depths of 1.9 m and 3.3 m, respectively (Yu et al., 2013). Biomass in the euphotic zone of Tai Lake is dominated by cyanobacteria, typically *Microcystis* (Zhou et al., 2008). Aquatic macrophytes were collected by a plant collector, sealed in plastic bags and taken to the laboratory as soon as possible and kept at 4 °C. Three species of phytoplankton, including a coccoid, green alga (*Chlorella* sp.), and two genera of cyanobacteria (*Microcystis* and *Spirulina*), were provided by the Institute of Hydrobiology, Chinese Academy of Sciences. All samples were carefully washed with deionized water. Aquatic macrophytes and phytoplankton were dried at 60 °C, to a constant mass, ground, and sieved through a 2-mm screen (Feng et al., 2016b). The resulting powdered samples were stored at –20 °C until use.

### 2.2. Chemical analysis of aquatic macrophytes and phytoplankton samples

Total concentrations of P, calcium (Ca), magnesium (Mg), potassium (K), iron (Fe), manganese (Mn) and aluminum (Al) in powdered, aquatic macrophytes and phytoplankton samples were determined, in triplicate. Samples were digested in a microwave in the presence of concentrated HNO<sub>3</sub>, followed by quantification by inductively coupled plasma-optical emission spectroscopy (ICP-OES; Thermo Scientific ICAP 6300 Duo, Shanghai, China). The limit of quantification by ICP-OES was 0.1 mg·kg<sup>-1</sup>. Proportions (%) of carbon (C) and nitrogen (N) were determined by an elemental analyzer (Element arvarin macro EL, Berlin, Germany).

### 2.3. Simulation experiment with APase treatment

APase (EC 3.1.3.1) was purchased from Sigma-Aldrich Chemicals (St Louis, MO, USA). The experiment was conducted as two groups, the treatment group and a control group. Each sample (0.5 g of aquatic macrophytes or 0.2 g of phytoplankton) was placed into a 500-mL conical flask. The treatment group suspension containing 6.67 mg of APase was prepared by dissolution of the sample in 200 mL of Tris-HCl buffer (0.01 mol·L<sup>-1</sup>, pH 9.0) at the final activity concentration of 1.0 U·mL<sup>-1</sup> (Zhu et al., 2013b; Feng et al., 2016b). The control suspension was prepared in the same way without APase. To inhibit the growth of microbes, the biocide sodium azide (1 mL of 1 mol·L<sup>-1</sup> NaN<sub>3</sub>) was added to both suspensions.

The two suspensions were incubated at 37 °C for 18 h with gentle shaking (150 r min<sup>-1</sup>). The incubation experiment was conducted in triplicate. Suspensions were then centrifuged at 10,000 ×g for 30 min,

and filtered through a 0.45- $\mu\text{m}$  membrane (Zhu et al., 2016). Dissolved inorganic P was measured by the molybdenum blue/ascorbic acid method (Murphy and Riley, 1962). Total dissolved P (TDP) was determined with the same method after potassium persulfate ( $\text{K}_2\text{S}_2\text{O}_8$ ) digestion of the sample in an autoclave at 121 °C for 30 min. Sodium dodecyl sulfate (SDS) was added at 2% (v/v) into the assay solution of the incubated samples to prevent enzyme precipitation (He and Honeycutt, 2005; Zhu et al., 2013b). Remaining supernatants were freeze-dried and kept at  $-20$  °C until  $^{31}\text{P}$  NMR analysis.

#### 2.4. $^{31}\text{P}$ NMR experiments

Freeze-dried supernatants were re-dissolved in 1.0 mL of  $1 \text{ mol}\cdot\text{L}^{-1}$  NaOH +  $0.1 \text{ mol}\cdot\text{L}^{-1}$  EDTA and  $0.1 \text{ mL}$  of deuterium oxide ( $\text{D}_2\text{O}$ ). Samples were then centrifuged at  $10000 \times g$  for 30 min, transferred to NMR tubes, and stored at 4 °C before analysis within 24 h (Turner et al., 2003; Doolette et al., 2009). Solution  $^{31}\text{P}$  NMR spectra were acquired at 161.98 MHz on a Bruker AVANCE 400 MHz spectrometer (Germany) equipped with a 5-mm broadband probe, using a 90° pulse, 0.21 s acquisition time, 5 s relaxation delay, and 5 Hz spinning (Feng et al., 2016a). For each sample, total duration of acquisition of NMR data lasted approximately 15 h, with 24,000 scans accumulated.

#### 2.5. Data analysis

Phosphorus compounds were identified by their chemical shifts (ppm) relative to an internal standard of orthophosphate ( $\text{PO}_4$ , ortho-P). Solution  $^{31}\text{P}$  NMR spectra were analyzed by MestReNova software 9.0 (Feng et al., 2016b), using automated peak analysis and spectral integration, based on total peak area (Cade-Menun, 2005). Peak assignments were made using  $^{31}\text{P}$  NMR chemical shifts of orthophosphate (Ortho-P, 6.0 ppm), pyrophosphate (Pyro-P,  $-4$  to  $-5$  ppm), polyphosphate (Poly-P,  $-17$  to  $-22$  ppm), monoester P (Mono-P, 5.9 to 3.6 ppm) and diester P (Di-P, 2.5 to  $-1.0$  ppm), which have been established previously (He et al., 2011; Cade-Menun, 2015; Feng et al., 2016b). There were thirty-six treatments in the simulation experiment with APase. Data were checked for deviations from normality and homogeneity of variance before performing statistical analyses. Pearson, pair-wise, correlation coefficients between metal element concentrations and detected P classes were determined in SPSS (IBM, SPSS, Statistics 21).

### 3. Results and discussion

#### 3.1. Properties of aquatic macrophytes and phytoplankton

Nutrients carbon (C), nitrogen (N) and P were important elements that contribute to eutrophication (Liu et al., 2016). Mean percentages of C and N in aquatic macrophytes were 40.04% and 3.29%, respectively, while percentages of them in phytoplankton were 44.94% and 9.28%, respectively (Table 1). Contents of C and N of phytoplankton were greater than those of aquatic macrophytes. However, contents of C and N in aquatic macrophytes and phytoplankton were greater than those of sediments (Qu et al., 2013). This indicated that debris derived from

aquatic macrophytes and phytoplankton, but especially phytoplankton, were likely to contribute to endogenous nutrients and support eutrophication of lakes.

The ratio of C/N ranged from 8.95 to 17.39 with a mean of 13.05 in the aquatic macrophytes. The ratio of C/N ranged from 4.01 to 5.57 with a mean of 4.87 in phytoplankton. The ratio of C/N of phytoplankton was less than that of aquatic macrophytes. Generally, the ratio of C/N was an effective predictor of the degradation of plants (Atkinson and Smith, 1983). The lower ratio of C/N indicated a greater potential for degradation of debris derived from phytoplankton, which was consistent with results of previous studies (Feng et al., 2016a).

Contents of total P and selected metals of the six samples were also measured (Table 1). These data were showed that P ranged from 0.97 to 7.80, Ca from 0.85 to 45.88, Mg from 1.13 to 3.23, K from 6.37 to 31.02, Fe from 0.18 to 2.25, Mn from 0.01 to 0.37 and Al from 0.45 to  $3.52 \text{ g}\cdot\text{kg}^{-1}$ , on the dry mass (dm) basis. Contents of Ca and K of aquatic macrophytes were greater than those of phytoplankton. This result was related to several factors, such as the species, biomass, primary productivity of aquatic macrophytes and phytoplankton and other environmental factors.

#### 3.2. Characteristics of P in supernatants with or without alkaline phosphatase

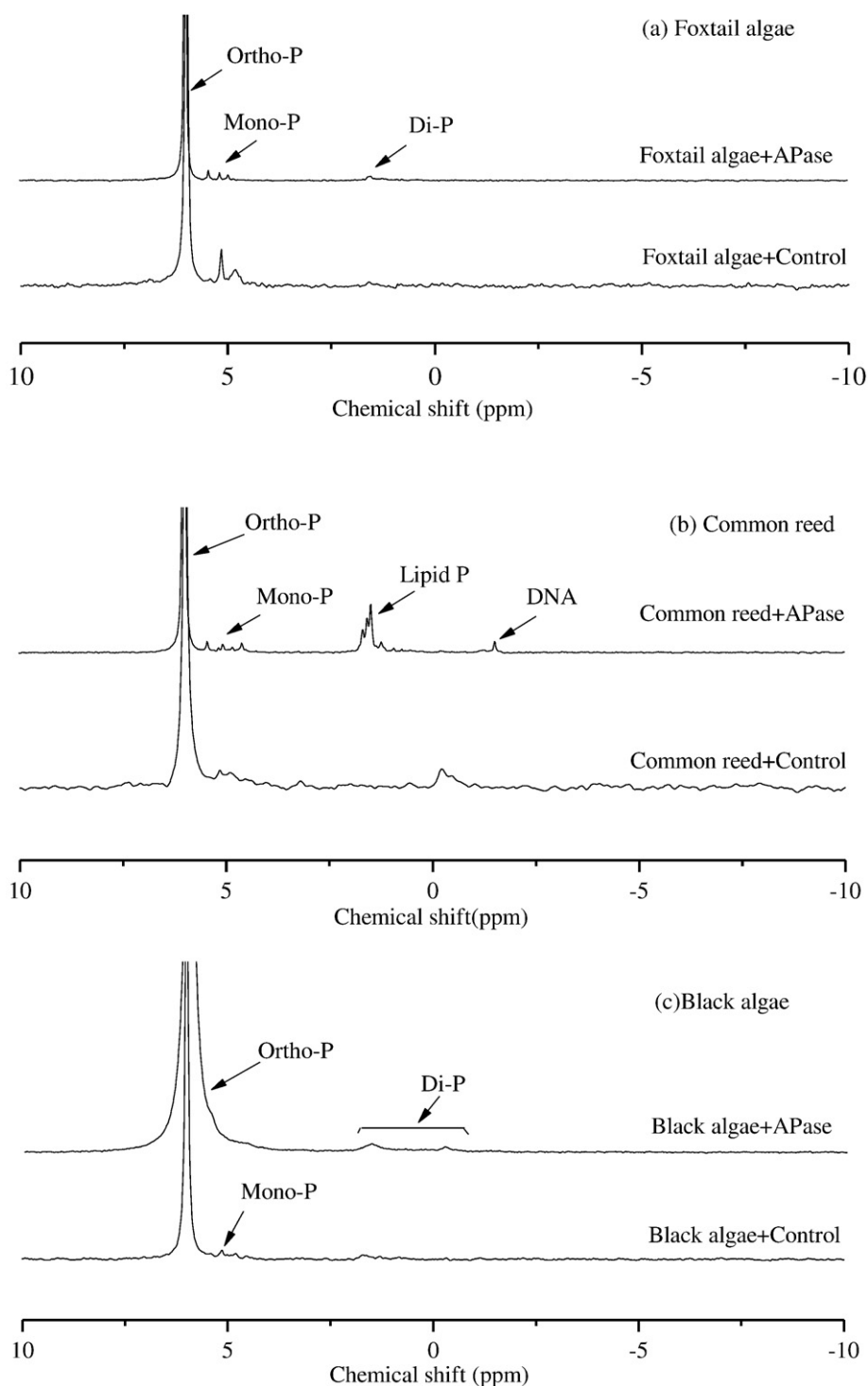
Solution  $^{31}\text{P}$  NMR spectra of supernatants from the suspensions treated with APase or buffer control could be found in Figs. 1 and 2. Inorganic P ( $\text{P}_i$ ) species including Ortho-P, Pyro-P, and Poly-P were detected. Mean concentration of Ortho-P was  $1209 \text{ mg}\cdot\text{kg}^{-1} \text{ dm}$ , and accounted for 82.5% of total dissolved P in aquatic macrophytes (Table 2). Mean concentration of Ortho-P was  $4272 \text{ mg}\cdot\text{kg}^{-1} \text{ dm}$  which accounted for 58.8% of total dissolved P in phytoplankton (Table 3). These data indicated that Ortho-P was the primary component of P in both aquatic macrophytes and phytoplankton. As Ortho-P was a biologically available form of P (Zhu et al., 2013a; Lin et al., 2016; Bai et al., 2017), this observation suggested that the Ortho-P in debris of aquatic macrophytes and phytoplankton could significantly contribute to the growth and reproduction of aquatic organisms in lakes. The difference in Ortho-P between aquatic macrophytes and phytoplankton implied their distinct effects on the ecosystem nutrient cycling, which was consistent with previous reports (Hobbie, 1992; Knops et al., 2002).

Organic P ( $\text{P}_o$ ), including both Di-P and Mono-P were also observed in these  $^{31}\text{P}$  NMR spectra (Figs. 1, 2). Di-P could be further divided into DNA ( $-0.36 \pm 0.19$  ppm), phospholipids (lipid-P), RNA ( $1.9$  to  $-0.2$  ppm) and other unidentified Di-P (Table 2, Table 3). Other unidentified Di-P accounted for 0.13% (mean) of TP in supernatants. Generally, stability of Di-P was less than that of Mono-P. They were easily degraded and assimilated by microorganisms (Turner et al., 2012; Zhu et al., 2013b). Mono-P ( $3.6$ – $5.9$  ppm) were detected, such as phytate, D-glucose 6-phosphate (Glu-6,  $5.14 \pm 0.02$  ppm) and a number of other Mono-P components (Figs. 1, 2, Table 2, Table 3). For example, the peak at  $4.73 \pm 0.07$  ppm was assigned to  $\alpha$ -glycerophosphate, the peak at  $4.56 \pm 0.07$  ppm to  $\beta$ -glycerophosphate, and peak at  $4.44 \pm 0.03$  ppm to nucleotide (Figs. 1, 2, Table 2, Table 3) (He et al., 2011).

**Table 1**  
Contents of selected elements in aquatic macrophytes and phytoplankton.

Samples	C (%)	N (%)	C/N	P	Ca	Mg	K	Fe	Mn	Al
				$\text{g}\cdot\text{kg}^{-1}$						
Foxtail algae	$34.43 \pm 2.6^a$	$2.69 \pm 0.6$	12.80	$1.30 \pm 0.1$	$45.88 \pm 2.4$	$2.38 \pm 0.1$	$10.40 \pm 1.2$	$2.11 \pm 0.3$	$0.37 \pm 0.1$	$3.49 \pm 0.2$
Common reed	$44.17 \pm 3.4$	$2.54 \pm 0.5$	17.39	$0.97 \pm 0.2$	$2.21 \pm 0.2$	$1.13 \pm 0.6$	$11.42 \pm 0.6$	$0.18 \pm 0.1$	$0.09 \pm 0.1$	$0.45 \pm 0.2$
Black algae	$41.52 \pm 2.1$	$4.64 \pm 1.0$	8.95	$1.98 \pm 0.5$	$26.98 \pm 0.2$	$3.09 \pm 0.1$	$31.02 \pm 2.1$	$2.25 \pm 0.5$	$0.01 \pm 0.01$	$3.52 \pm 0.4$
Microcystis	$44.32 \pm 5.0$	$8.82 \pm 0.2$	5.02	$4.04 \pm 0.5$	$7.88 \pm 0.6$	$2.69 \pm 0.2$	$6.37 \pm 0.3$	$0.69 \pm 0.1$	$0.03 \pm 0.01$	$1.20 \pm 0.1$
Chlorella	$51.09 \pm 4.8$	$9.18 \pm 0.9$	5.57	$6.46 \pm 0.3$	$0.85 \pm 0.2$	$2.49 \pm 0.1$	$8.07 \pm 0.2$	$0.91 \pm 0.3$	$0.03 \pm 0.02$	$0.92 \pm 0.2$
Spirulina	$39.40 \pm 3.5$	$9.83 \pm 0.5$	4.01	$7.80 \pm 0.1$	$0.96 \pm 0.6$	$3.23 \pm 0.6$	$11.98 \pm 0.9$	$0.47 \pm 0.1$	$0.02 \pm 0.01$	$0.93 \pm 0.3$

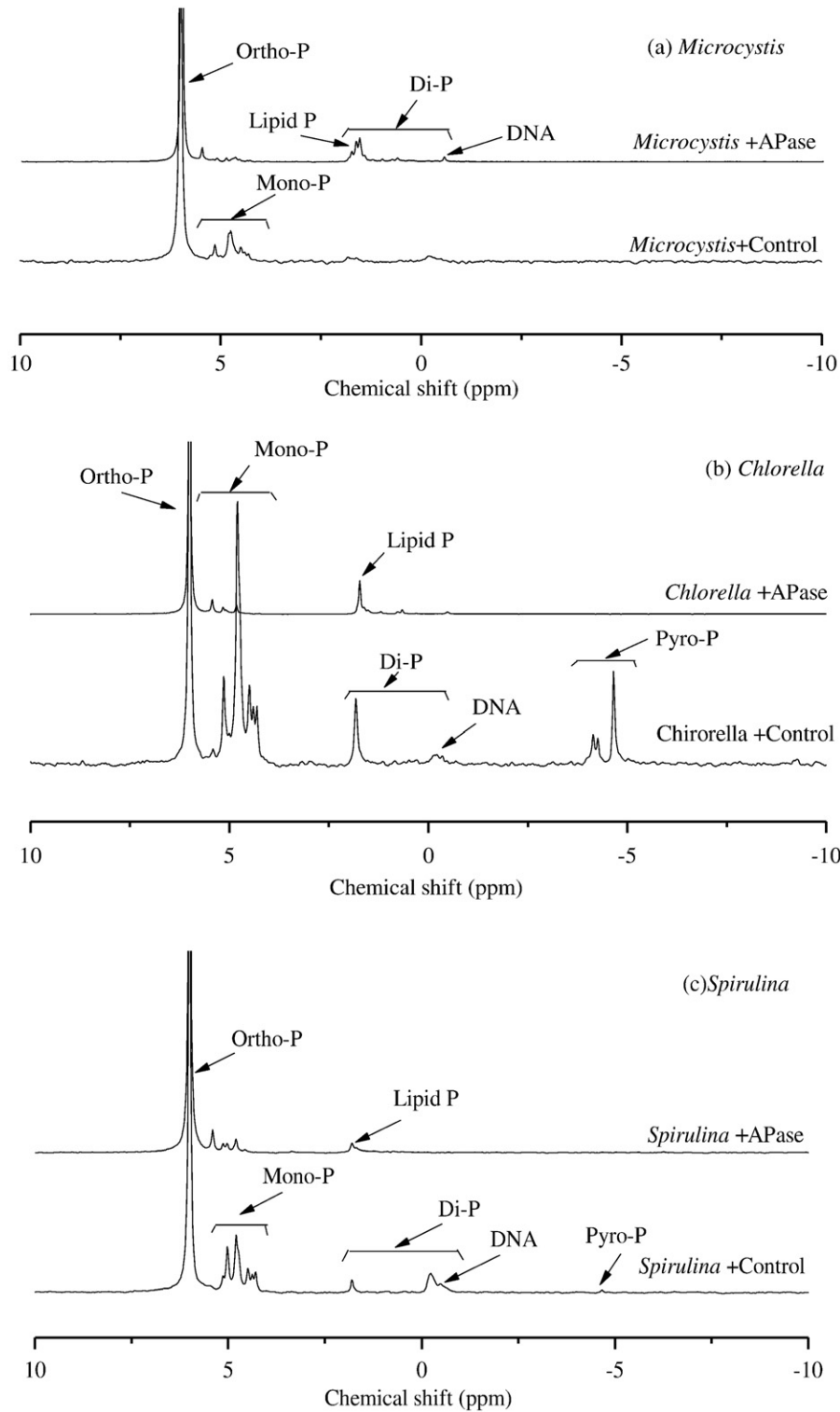
<sup>a</sup> Means  $\pm$  standard deviation of 3 analytical replicates.



**Fig. 1.** Solution  $^{31}\text{P}$  NMR spectra of the supernatants of aqueous suspensions of aquatic macrophytes with APase treatment and control. Ortho-P, orthophosphate; Mono-P, monoester P; Diester P, Di-P; Lipid P, phospholipids; DNA, deoxyribonucleic acid.

Other unidentified Mono-P peaks accounted for 2.88% (mean) of TP in supernatants of these samples. Peaks in the Mono-P region were significant in reference samples, especially *Chlorella vulgaris* and *Sprulina* (Fig. 2b, c). The percentages of Mono-P were 43.8% and 25.1% in TP of supernatants, respectively (Table 3). Although phytate could be abundant  $\text{P}_o$  compound found widely in the environment (Suzumura and Kamatani, 1993; He et al., 2006b; Turner et al., 2012), phytate accounted for only 0.2% to 3.3% of supernatant TP in these samples. Phytate forms stable compounds with metal ions (Turner et al., 2002; He et al., 2006b; Lin et al., 2016). Phytate has been thought to be derived from exogenous

sources, such as soils and terrestrial plants (Turner et al., 2012; Giles and Cade-Menun, 2014; Zhang et al., 2017). The limited proportion of P in the form of phytate, observed in the macrophytes and phytoplankton studied here might have been due to the fact that phytate was mainly a storage form of P in fruit and seed parts with lesser concentrations in other parts of plants (Lott et al., 2000; He et al., 2013; Noack et al., 2012). Alternatively, these results indicated that aquatic macrophytes could also be sources of phytate in sediments of lakes. This conclusion was supported by observations of phytate-like P in and aquatic macrophytes from Tai Lake (Ding et al., 2013; Zhu et al., 2015; Liu et al., 2016).



**Fig. 2.** Solution  $^{31}\text{P}$  NMR spectra of the supernatants of aqueous suspensions of phytoplankton with APase treatment and control. Ortho-P, orthophosphate; Pyro-P, pyrophosphate; Lipid P, phospholipids; DNA, deoxyribonucleic acid.

### 3.3. Changes in P compounds in supernatants with alkaline phosphatase treatment

Differences in both absolute and relative concentrations of P compounds between samples treated with APase and the buffer control were observed (Table 4). The proportions of Ortho-P after treatment with APase were increased in a range from 1.4% to 45.9%. Especially,

the increase reached to 78.8% and 85.5%, respectively, for the samples of *Chlorella vulgaris* and *Spirulina*. Concurrently, percentages of most Mono-P compounds, including glycerophosphate, nucleotide and other unidentified Mono-P compounds decreased. On average, proportions of glycerophosphate, nucleotide, and other unidentified Mono-P compounds decreased by 8.8%, 2.5% and 3.6%, respectively (Table 4). Mean proportions of DNA were decreased by 3.2%. Furthermore, all of

**Table 2**  
Concentrations (mg/kg) and percentage (%) of P compounds in supernatants of aquatic macrophytes with or without APase treatment.

Samples	Treatment	P <sub>i</sub> <sup>a</sup>			P <sub>o</sub>										Total P <sub>o</sub>
					Monoester P					Diester P					
					Phytate	Other monoester P									
		Ortho-P	Poly-P	Total P <sub>i</sub>		Glu-6	α-Gly	β-Gly	Re-Mono-P	Total Mono-P	Lipid P + RNA	DNA	Re-Di-P	Total Di-P	
Foxtail algae	APase	1101 (92.8) <sup>b</sup>	– <sup>c</sup>	1101 (92.8)	4 (0.3)	14 (1.2)	–	–	36 (3.0)	53 (4.5)	32 (2.7)	–	–	32 (2.7)	85 (7.2)
	Buffer (control)	970 (85.7)	–	970 (85.72)	–	–	63 (5.6)	–	83 (7.4)	147 (13.0)	15 (1.3)	–	–	15 (1.3)	162 (14.3)
Common reed	APase	845 (77.5)	–	845 (77.5)	5 (0.5)	–	–	19 (1.7)	34 (3.2)	59 (5.4)	160 (14.7)	10 (1.0)	17 (1.5)	187 (17.1)	245 (22.5)
	Buffer (control)	742 (71.5)	–	742 (71.5)	34 (3.3)	–	50 (4.9)	–	61 (5.9)	179 (17.2)	26 (2.5)	92 (8.8)	–	117 (11.3)	296 (28.5)
Black algae	APase	2006 (95.2)	7 (0.2)	2010 (95.4)	–	–	–	–	–	–	66 (3.1)	31 (1.5)	–	97 (4.6)	97 (4.6)
	Buffer (control)	1915 (90.3)	–	1915 (90.3)	14 (0.7)	–	33 (1.6)	–	106 (5.0)	186 (8.8)	20 (0.9)	–	–	20 (0.9)	206 (9.7)

<sup>a</sup> P<sub>i</sub>, inorganic P; P<sub>o</sub>, organic P; Ortho-P, orthophosphate; Poly-P, polyphosphate; Total P<sub>i</sub>, the sum of Ortho-P and Poly-P; Glu-6, D-glucose 6-phosphate; α-gly, α-glycerophosphate; β-gly, β-glycerophosphate; Re-Mono-P, remaining Mono-P; Total Mono-P, the sum of phytate and other monoester P; Lipid P + RNA, the sum of lipids phosphate and ribonucleic acid; DNA, deoxyribonucleic acid; Re-Di-P, remaining Di-P; Total Di-P, the sum of Lipid P and RNA and DNA and re-Di-P; Total P<sub>o</sub>, the sum of Mono-P and Di-P.

<sup>b</sup> Values in parentheses are percentages (relative abundance) of individual P compound in supernatant TP of samples.

<sup>c</sup> “–”: not detected.

the pyrophosphate was hydrolyzed by APase. However, concentrations of phytate, glucose 6-phosphate, polyphosphate and other unidentified Di-P only changed slightly (<3%). After treatment with APase, proportions of P<sub>o</sub> compounds hydrolyzed ranged from 1.4% to 30.4%, with a mean of 12.2%. with 8.5% to 40.3% (mean, 19.0%) of Mono-P hydrolyzed. Whereas the trends of hydrolysis of P<sub>o</sub> compounds in suspensions of dried aquatic macrophytes and phytoplankton were similar (Table 4), percentages of P<sub>o</sub> transformed to bioavailable P from phytoplankton and aquatic macrophytes were different. For example, in the suspensions of macrophytes after the enzymatic hydrolysis, Mono-P (glycerophosphate-4.0%, phytate-1.1%, remaining Mono-P-4.0%) and DNA (2.1%) were transformed into Ortho-P. In contrast, for suspensions of dried phytoplankton, most of Mono-P (e.g., glycerophosphate (13.6%), nucleotide (4.9%), remaining Mono-P (3.2%), DNA (4.3%), pyrophosphate (3.5%) and polyphosphate (0.8%) were transformed into Ortho-P (22.6%). Approximate 21% of P<sub>o</sub> in aquatic macrophytes was hydrolyzed, while it was 79% for phytoplankton. It was predicted that phytoplankton would be a major contributor of ortho-P from

internally-loaded P<sub>o</sub> in eutrophic lakes. Results of this study were consistent with those of a previous study (Li et al., 2014). Per Li et al. (2014), when concentrations of Ortho-P were less than 5% of TP in the water column of lakes, 80% of organic matter in debris of phytoplankton would be mineralized rapidly, and thus biologically available P components be released, thus maintained growth and reproduction of phytoplankton.

APase was a widespread enzyme in water columns of lakes, which has been shown to play an important role in the biological geochemical cycle of P (Gage and Gorham, 1985). Results of this study showed that the majority of P<sub>o</sub> in aquatic macrophytes and phytoplankton could be transformed to Ortho-P that organisms could use directly. Newly-released Ortho-P entered into water columns of lakes so that Ortho-P in the water was replenished quickly. With this mechanism, internal recycling of Ortho-P was assumed to be the key factor of supporting continued cyanobacterial blooming, thus explaining the repeated phytoplankton outbreaks in eutrophic lakes even though exogenous sources of P have been controlled.

**Table 3**  
Concentrations (mg/kg) and percentages (%) of P compounds in supernatants of phytoplankton with or without APase treatment.

Samples	Treatment	P <sub>i</sub> <sup>a</sup>				P <sub>o</sub>										Total P <sub>o</sub>	
						Monoester P					Diester P						
						Phytate	Other monoester P										
		Ortho-P	Pyro-P	Poly-P	Total P <sub>i</sub>		Glu-6	α-Gly	β-Gly	Nuc	Re-Mono-P	Total Mono-P	Lipid P + RNA	DNA	Re-Di-P		Total Di-P
Microcystis	APase	3476 (79.9) <sup>b</sup>	–	– <sup>c</sup>	3476 (79.9)	21 (0.5)	20 (0.5)	27 (0.6)	59 (1.4)	–	172 (4.0)	300 (6.9)	530 (12.2)	–	44 (1.0)	574 (13.2)	874 (20.1)
	Buffer (control)	3418 (78.6)	–	–	3418 (78.6)	–	–	372 (8.6)	–	134 (3.1)	226 (5.2)	733 (16.8)	77 (1.8)	123 (2.8)	201 (4.6)	933 (21.5)	
Chlorella	APase	7208 (78.8)	–	–	7208 (78.8)	17 (0.2)	137 (1.5)	201 (2.2)	–	495 (5.4)	851 (9.3)	1040 (11.4)	–	49 (0.5)	1090 (11.9)	1941 (21.2)	
	Buffer (control)	2980 (32.9)	924 (10.2)	477 (5.3)	4381 (48.4)	–	–	2205 (24.3)	–	656 (7.2)	1102 (12.2)	3963 (43.8)	506 (5.6)	208 (2.3)	715 (7.9)	4679 (51.7)	
Spirulina	APase	8459 (85.5)	–	275 (2.8)	8734 (88.3)	–	–	–	–	804 (8.1)	1160 (11.7)	275 (2.8)	–	–	–	1160 (11.7)	
	Buffer (control)	6419 (64.8)	37 (0.4)	–	6456 (65.2)	–	–	1073 (10.8)	–	437 (4.4)	970 (9.8)	5155 (25.1)	189 (1.9)	778 (7.9)	968 (9.8)	3448 (34.8)	

<sup>a</sup> P<sub>i</sub>, inorganic P; P<sub>o</sub>, organic P; Ortho-P, orthophosphate; Poly-P, polyphosphate; Total P<sub>i</sub>, the sum of Ortho-P and Poly-P; Glu-6, D-glucose 6-phosphate; α-gly, α-glycerophosphate; β-gly, β-glycerophosphate; Nuc, nucleotide; Re-Mono-P, remaining Mono-P; Total Mono-P, the sum of phytate and other Mono-P; Lipid P + RNA, the sum of lipids phosphate and ribonucleic acid; DNA, deoxyribonucleic acid; Re-Di-P, remaining Di-P; Total Di-P, the sum of Lipid P and RNA and DNA and re-Di-P; Total P<sub>o</sub>, the sum of Mono-P and Di-P.

<sup>b</sup> Values in parentheses are percentages (relative abundance) of individual P compound in supernatant TP of samples.

<sup>c</sup> “–”: not detected.

**Table 4**  
Change in P species concentrations and relative percentage as measured by <sup>31</sup>P NMR for incubations in the absence and presence of APase.

Samples	P <sub>i</sub> <sup>a</sup>				P <sub>o</sub>										
					Monoester P					Diester P				Total P <sub>o</sub>	
					Phytate		Other Monoester P								
	Ortho-P	Pyro-P	Poly-P	Total P <sub>i</sub>		Glu-6	Gly	Nuc	Re-Mono-P	Total Mono-P	Lipid P + RNA	DNA	Re-Di-P	Total Di-P	
Foxtail algae	131 (7.1) <sup>b</sup>	- <sup>d</sup>	-	131 (7.1)	4 (0.3)	14 (1.2)	-63 <sup>c</sup> (-5.6)	-	-47 (-4.3)	-94 (-8.5)	17 (1.4)	-	-	17 (1.4)	-77 (-7.1)
Common reed	103 (6.0)	-	-	103 (6.0)	-29 (-2.8)	-	-31 (-4.9)	-	-27 (-2.8)	120 (-11.8)	134 (12.2)	-82 (-7.9)	17 (1.5)	70 (5.8)	-51 (-6.0)
Black algae	91 (4.9)	-	7 (0.2)	95 (5.1)	-14 (-0.7)	-	-33 (-1.6)	-	-106 (-5.0)	-186 (-8.8)	46 (2.2)	31 (1.5)	-	77 (3.7)	-109 (-5.1)
<i>Microcystis</i>	58 (1.4)	-	-	58 (1.4)	21 (0.5)	20 (0.5)	-286 (-7.9)	-134 (-3.1)	-54 (-1.2)	-433 (-9.9)	453 (10.4)	-123 (-2.8)	44 (1.0)	373 (8.6)	-59 (-1.4)
<i>Chlorella</i>	4228 (45.9)	-924 (-10.2)	-477 (-5.3)	2827 (30.4)	17 (0.2)	137 (1.5)	-2004 (-22.1)	-656 (-7.2)	-607 (-6.8)	-3112 (-34.5)	534 (5.8)	-208 (-2.3)	49 (0.5)	375 (4.0)	-2738 (-30.4)
<i>Spirulina</i>	2040 (20.7)	-37 (-0.4)	275 (2.8)	2278 (23.1)	-	-	-1073 (-10.8)	-437 (-4.4)	-166 (-1.7)	-3995 (-40.3)	86 (0.9)	-778 (-7.9)	-	-968 (-9.8)	-2288 (-23.1)
Mean value	1109 (14.3)	-160 (-1.8)	-33 (-0.4)	915 (12.2)	0.2 (-0.4)	29 (0.5)	-582 (-8.8)	-205 (-2.5)	-168 (-3.6)	-1323 (-19.0)	212 (5.5)	-193 (-3.2)	18 (0.5)	-9 (2.3)	-887 (-12.2)

<sup>a</sup> P<sub>i</sub>, inorganic P; P<sub>o</sub>, organic P; Ortho-P, orthophosphate; Pyro-P, pyrophosphate; Poly-P, polyphosphate; Total P<sub>i</sub>, the sum of Ortho-P and Pyro-P and Poly-P; Glu-6, D-glucose 6-phosphate; Gly, the sum of α-glycerophosphate and β-glycerophosphate; Nuc, nucleotide; Re-Mono-P, remaining Mono-P; Total Mono-P, the sum of phytate and other Mono-P; Lipid P + RNA, the sum of phospholipids and ribonucleic acid. DNA, deoxyribonucleic acid; Re-Di-P, remaining Di-P; Total Di-P, the sum of DNA and re-Di-P; Total P<sub>o</sub>, the sum of Mono-P and Di-P.

<sup>b</sup> Values are the change of P concentration in mg·kg<sup>-1</sup>. Values in parentheses are the corresponding changes of P compounds in percentage.

<sup>c</sup> Plus and minus symbols indicate increase and decrease in P concentration after the enzyme treatment, respectively.

<sup>d</sup> “-”: not detected.

### 3.4. Correlations between P compounds and metal elements from aquatic macrophytes and phytoplankton

Metal elements in lake ecosystem could play an important role in the release of internal P loadings and lake eutrophication. They were easy to combine with P, such as Fe-bound P and Ca-bound P; therefore studies on the relationship between metal elements and P compounds would be helpful for understanding the biogeochemical cycling of internal P load in eutrophic lakes. Using solution <sup>31</sup>P NMR spectroscopy, P compounds derived from aquatic macrophytes and phytoplankton were classified into five categories: Ortho-P, Mono-P, Di-P, Pyro-P and Poly-P; then correlations between metal elements and different P compounds were analyzed (Table 5). Ortho-P, Mono-P and Di-P were significantly positively correlated with total P in aquatic macrophytes and phytoplankton (r = 0.909, p < 0.05; r = 0.958, p < 0.01; and r = 0.957, p < 0.01 respectively). Ortho-P mainly exists in aquatic macrophytes and phytoplankton in salt phosphate form, which was also the main nutrient for lake ecosystem (Tables 2, 3). When the indigenous P was insufficient in the overlying water body, these recycling Ortho-P would be the nutrient materials for the growth of aquatic macrophytes and phytoplankton.

No significant correlations were observed between Pyro-P, Poly-P and other P compounds (Ortho-P, Mono-P, Di-P) and metal elements. Based on previous studies (Ahlgren et al., 2005; Reizel et al., 2006), Pyro-P and Poly-P were more labile than other P compounds, because of their shorter half-lives. Thus, no significant correlations between Pyro-P, Poly-P and other P compounds were likely due to the hydrolysis of Pyro-P and Poly-P under alkali conditions. Additionally, there was no significant correlation between P compounds and metal elements in aquatic macrophytes and phytoplankton (Table 5). However, metal ions would be an important factor that influence cycling of P, such as forming of Fe-, Ca-, Al-bound inorganic P or Fe-phytate, then precipitated or even buried in the sediments (Zhu et al., 2013b).

### 3.5. Cyanobacterial blooming and recycling of P by alkaline phosphatase

P<sub>o</sub> was one of the main constituents of total P in aquatic environments (Baidwin, 2013). Aquatic macrophytes and phytoplankton were the sources of five categories of P in eutrophic lakes (Figs. 1, 2). The major form of P<sub>o</sub> in aquatic macrophytes and phytoplankton was Mono-P (Table 2). In this study, Mono-P in aquatic macrophytes was dominated by α-glycerophosphate and phytate, while in phytoplankton

**Table 5**  
Correlation coefficients(r) between metal elements and P species identified by solution <sup>31</sup>P NMR spectroscopy.

	P	Ca	Mg	K	Fe	Mn	Al	Ortho-P	Mono-P	Di-P	Pyro-P
Ca	-0.602										
Mg	0.565	0.136									
K	-0.317	0.348	0.331								
Fe	-0.414	<b>0.886*</b>	0.393	0.610							
Mn	-0.508	0.775	-0.274	-0.225	0.447						
Al	-0.483	<b>0.933**</b>	0.372	0.614	<b>0.981**</b>	0.506					
Ortho-P	<b>0.909*</b>	-0.512	0.673	-0.176	-0.389	-0.510	-0.384				
Mono-P	<b>0.958**</b>	-0.582	0.441	-0.290	-0.423	-0.407	-0.492	<b>0.840*</b>			
Di-P	<b>0.957**</b>	-0.649	0.387	-0.339	-0.514	-0.440	-0.573	<b>0.852*</b>	<b>0.994**</b>		
Pyro-P	0.495	-0.369	0.012	-0.285	-0.123	-0.229	-0.309	0.150	0.091	0.792	
Poly-P	0.464	-0.352	-0.008	-0.280	-0.108	-0.217	-0.295	0.056	0.490	0.768	<b>0.999**</b>

Ortho-P, orthophosphate; Mono-P, monoester P; Di-P, diester P; Pyro-P, pyrophosphate; Poly-P, polyphosphate.

\*\* Correlation is significant at the 0.01 level (2-tailed, n = 6).

\* Correlation is significant at the 0.05 level (2-tailed, n = 6).

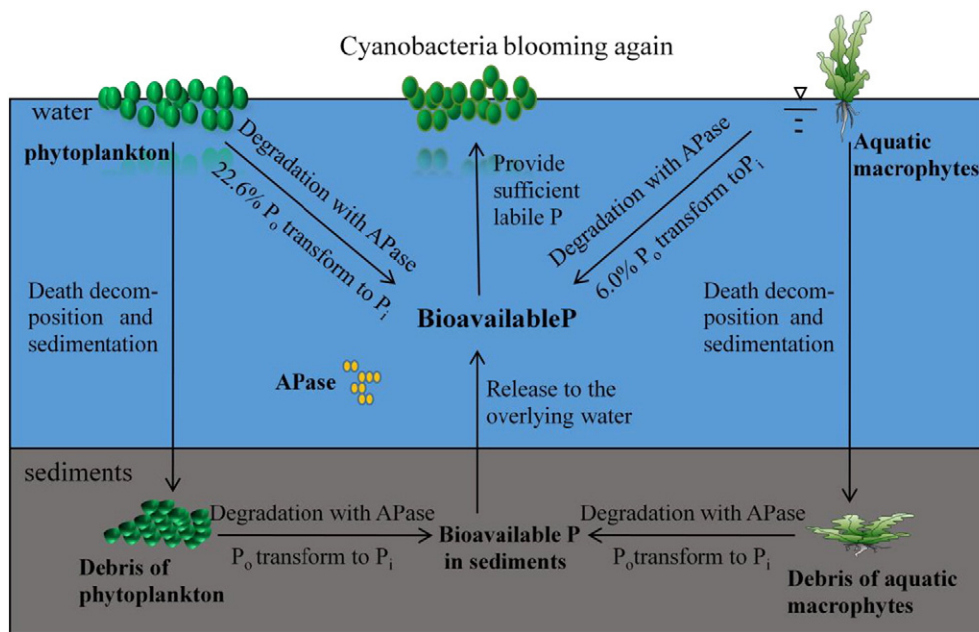


Fig. 3. Cycling of  $P_o$  from aquatic macrophytes and phytoplankton in eutrophic lakes.

they were dominated by nucleotides, which indicated that the main classes of Mono-P varied with compositions of aquatic plants. Mono-P was prone to hydrolysis during blooms of phytoplankton, especially in eutrophic lakes (Bai et al., 2017). Based on the composition of Mono-P, Mono-P in phytoplankton was more labile than that in aquatic macrophytes.

Sediments and debris from phytoplankton and aquatic macrophytes were all sources of endogenous P in eutrophic lakes. Based on  $^{31}\text{P}$  NMR analysis and APase treatment, the percentage of  $P_o$  transformed to Ortho-P were 22.6% and 6.0% (based on TP) in phytoplankton and aquatic macrophytes, respectively (Table 4). However, it was only 0.9% of  $P_o$  transformed to Ortho-P in sediment suspensions with APase treatment of Tai Lake (Zhu et al., 2016). Evaluated by APase hydrolysis, the contribution of bioavailability P in the overlying water could be in the order that phytoplankton (76.6%) > macrophytes (20.3%) > sediments (3.1%). Previous studies also suggested that most  $P_o$  was contributed by aquatic macrophytes and phytoplankton, so that the two types of plants were main sources for bioavailable P with less contributed by sediments (Feng et al., 2016b; Liu et al., 2016). Thus, debris from phytoplankton such as blooming algae would be an important and endogenous source of P for supporting blooms of phytoplankton again in eutrophic lakes.

Whereas part of the  $P_o$  in decomposing debris of aquatic macrophytes and phytoplankton was released to water and transformed to dissolved Ortho-P, part of the  $P_o$  was precipitated to sediments of lakes (Fig. 3), which also possibly mineralized to bioavailable P by phosphatase enzymes in sediments. These bioavailable forms of P were both sources for repeating blooming of cyanobacteria in eutrophic lakes.

#### 4. Conclusions

- 1) The order of abundance of forms of P in aquatic macrophytes was Ortho-P (82.5% of total dissolved P) > Mono-P (13.0%) > Di-P (4.5%). The order in phytoplankton was Ortho-P (58.8%) > Mono-P (28.6%) > Di-P (7.4%) > Pyro-P (3.5%) > Poly-P (1.8%). The major forms of Mono-P in extracts of aquatic macrophytes were  $\alpha$ -glycerophosphate and phytate. The major forms of Mono-P in phytoplankton were  $\alpha$ -glycerophosphate and nucleotides. Forms of Di-P were phospholipids, RNA and DNA in both aquatic macrophytes and phytoplankton.

- 2) Trends of  $P_o$  including condensed P hydrolyzed by APase were similar in aquatic macrophytes and phytoplankton. Almost all Mono-P (15.3% of total dissolved P), Pyro-P (1.8%), Poly-P (0.4%) and DNA (3.2%) were transformed into Ortho-P. The hydrolyzed Mono-P was mainly consisted of glycerophosphate (8.8%), nucleotide (2.5%), and other Mono-P (3.6%).
- 3) It was estimated that 22.6% and 6.0% of  $P_o$  derived from suspensions of phytoplankton and aquatic macrophytes hydrolyzed to Ortho-P by APase. When concentrations of dissolved Ortho-P were insufficient in the water column of lakes, these dissolved  $P_o$  in phytoplankton and aquatic macrophytes, especially phytoplankton would be mineralized rapidly to bioavailable P components.

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#### Author contributions

W.F., F.W. and Y.Z. designed the research and wrote the paper. F.S., Y.W., N. Q., C.Z., H. C. and F. S. analyzed the data. Z.H and J.P.G. analyzed the data, wrote and edited the manuscript. All authors discussed the results and reviewed the manuscript.



## Additional information

The authors declare no competing financial interests.

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