SCIENTIFIC REPORTS

OPEN

Forms and Lability of Phosphorus in Algae and Aquatic Macrophytes Characterized by Solution ^{31}P NMR Coupled with Enzymatic Hydrolysis

Weiying Feng[1,2], Yuanrong Zhu[1], Fengchang Wu[1], Zhongqi He[3], Chen Zhang[1] & John P. Giesy[1,4]

Solution Phosphorus-31 nuclear magnetic resonance (^{31}P NMR) spectroscopy coupled with enzymatic hydrolysis (EH) with commercially available phosphatases was used to characterize phosphorus (P) compounds in extracts of the dominant aquatic macrophytes and algae in a eutrophic lake. Total extractable organic P (P_o) concentrations ranged from 504 to 1643 mg kg^{-1} and 2318 to 8395 mg kg^{-1} for aquatic macrophytes and algae, respectively. Using ^{31}P NMR spectroscopy, 11 P_o species were detected in the mono- and diester region. Additionally, orthophosphate, pyrophosphate and phosphonates were also detected. Using EH, phytate-like P was identified as the prevalent class of enzyme-labile P_o, followed by labile monoester- and diester-P. Comparison of the NMR and EH data indicated that the distribution pattern of major P forms in the samples determined by the two methods was similar ($r = 0.712$, $p < 0.05$). Additional ^{31}P NMR spectroscopic analysis of extracts following EH showed significant decreases in the monoester and pyrophosphate regions, with a corresponding increase in the orthophosphate signal, as compared to unhydrolyzed extracts. Based on these quantity and hydrolysis data, we proposed that recycling of P_o in vegetative biomass residues is an important mechanism for long-term self-regulation of available P for algal blooming in eutrophic lakes.

Lake eutrophication is a serious environmental concern in world, including the lakes from middle and lower reaches of the Yangtze River watershed and southwest China[1–3]. Limiting phosphorus (P) supply to water can be an effective strategy for restoration of eutrophic lakes[1]. While the reduction of P inputs from industrial and agricultural sources is required for this purpose, internal recycling of death plants-associated P and sediment-associated P can slow eutrophic lake remediation[4–6]. Previous studies on the role of internal P cycling in lake eutrophication and its remediation have focused primarily on soluble and sediment-associated inorganic P (P_i)[7–10]. However, organic P (P_o) is also an important component of total phosphorus (TP) in the overlying water[11], sediments[12] and debris derived from decomposition of algae and aquatic plants[13]. The P_o concentration in biomass from algae and aquatic macrophytes was found to be 12.9-fold and 1.8-fold greater than in sediments, respectively[13], which represents a significant source of internal P for a eutrophic lake. In aquatic systems, P_o can be converted to bioavailable P (e.g., HPO_4^{2-}) for algal blooming through a series of redox-driven solubilization reactions and phosphatase-mediated hydrolytic processes[14,15]. In addition, some forms of P_o can be directly assimilated by algae[16]. Thus, P_o plays a key role in nutrient availability and algal blooming in lakes, irrespective of attempts to limit external P inputs. Despite the importance of lake internal P_o in the eutrophication process, the P composition of algae and aquatic macrophytes and their contribution to internal loading and cycling of P in lakes remains poorly understood.

In the last few decades, phosphorus-31 nuclear magnetic resonance spectroscopy (^{31}P NMR) has proven to be a useful tool to identify and quantify P_o in samples from environmental samples[3,17–21]. Three major classes of P_o have been identified in aquatic environments: orthophosphate monoesters, orthophosphate diesters and phosphonate[9,13,22,23]. Characterization of P_o with ^{31}P NMR can identify specific orthophosphate esters, such as sugar

[1]State Key Laboratory of Environmental Criteria and Risk Assessment, Chinese Research Academy of Environmental Sciences, Beijing 100012, China. [2]College of Water Sciences, Beijing Normal University, Beijing 100875, China. [3]USDA-ARS, Southern Regional Research Center, New Orleans, LA 70124, USA. [4]Department of Biomedical Veterinary Sciences and Toxicology Centre, University of Saskatchewan, Saskatoon, SK S7N 5B3, Canada. Correspondence and requests for materials should be addressed to Y.Z. (email: zhuyuanrong07@mails.ucas.ac.cn)

phosphates, inositol phosphates [IHP], phospholipids, and nucleic acid [deoxyribonucleic acid (DNA) and ribonucleic acid (RNA)][21,24]. Enzymatic hydrolysis (EH) with commercially available phosphatases has also been used to characterize P forms and evaluate their bioavailabilities in environmental samples, including animal manures, soils and sediments, and water[15,17,21,25–27]. For example, bioavailable P_o forms, such as monoester P, diester P and phytate-like P, were functionally classified using combinations of three commercially available phosphatases: alkaline phosphatase (AP), phosphodiesterase (PDE), and phytase (Phy)[2]. Importantly, the combination of solution ^{31}P NMR spectroscopy and EH has been applied to provide supplemental and integrated information on P forms and their labilities in environmental samples[9,16,17,28].

In the current study, solution ^{31}P NMR spectroscopy coupled with EH was applied to analyze P forms and their bioavailabilities in the debris derived from algae and aquatic macrophytes in lakes. The objectives of the study were to i) quantitatively compare differences in P forms (extracted by NaOH-EDTA) of common species of algae and aquatic macrophytes by solution ^{31}P NMR and EH, and ii) evaluate potential biogeochemical cycling of P_o derived from algae and aquatic macrophytes in Lake Tai, a eutrophic lake in the lower reaches of Yangtze River watershed, China. The eventual goal of this study is to better understand internal P loading and cycling relevant to lake eutrophication and remediation.

Results and Discussion

Forms of phosphorus in aquatic macrophytes and algae characterized by solution ^{31}P NMR.

With 0.5 M NaOH + 25 mM EDTA as the extracting agent, the recovery of TP and P_o in aquatic macrophytes was 92.4% and 88.1%, respectively. The recoveries of TP and P_o from algae were 96.4% and 90.8%, respectively. The application of the NaOH-EDTA extraction technique on algae and aquatic macrophytes for solution ^{31}P NMR analysis was reported in a previous work[13]. The recoveries of TP and P_o in algae and aquatic macrophytes in the current and previous study were similar, which indicated sample homogeneity and repeatability of the extraction procedure.
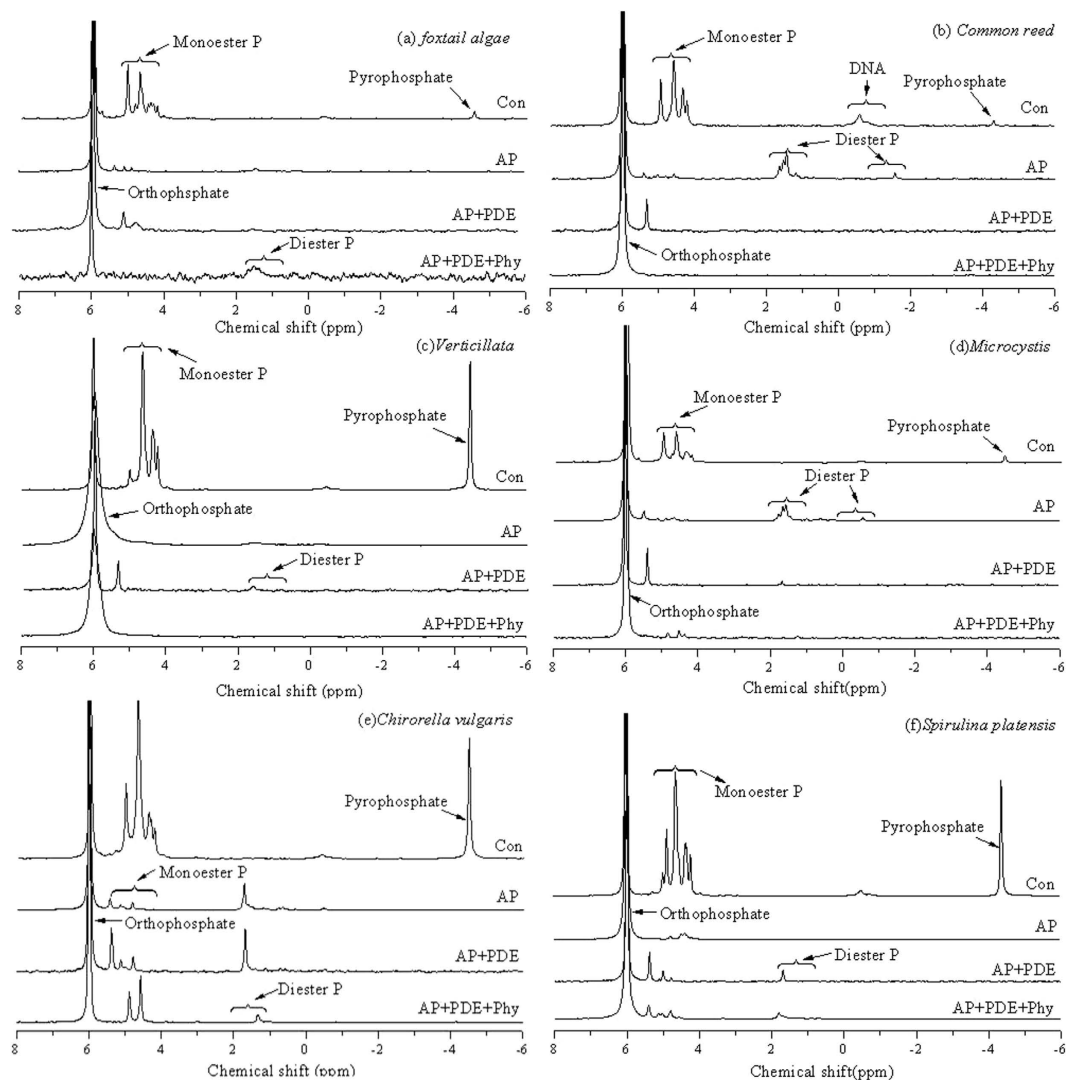
Solution ^{31}P NMR spectra of NaOH-EDTA extracts of aquatic macrophytes and algae are shown in Fig. 1 (i. e., Con: Control without EH). Integration of these peaks, and chemical shifts in the spectra, provided quantitative data of specific P species in the six aquatic organisms samples (Supplementary Information, S.I. Table 1). Up to 11 different P_o species were identified (Table 1, Fig. 1). Concentrations of extracted P_o ranged from 504 to 1643 mg kg^{−1} in aquatic macrophytes and from 2318 to 8395 mg kg^{−1} in algae. These data indicated that there was a large difference in P content between aquatic macrophytes and algae, but the relative distribution (i.e., percentage) of specific forms of NaOH-EDTA extractable P_o was similar among the two sample types. The detected P_o forms included monoester P, diester P and phosphonate. Monoester P (the sum of phytate and other monoester P) comprised the largest P_o fraction. The percentage of phytate in NaOH-EDTA extractable P in non-hydrolyzed samples ranged from 5.6 to 41.9% (average of 23.0%). The percentage of other monoester P in these samples ranged from 8.6 to 44.3% (average of 22.6%). Other monoester P was present as glycerides (i.e., α- and β-glycerophosphate), nucleotides (i.e., guanosine 2′ monophosphate, cytidine 5′ monophosphate, adenosine 5′ monophosphate) and sugars (i.e., fructose 6-phosphate, glucose 1-phosphate and glucose 6-phosphate) (Table 1, Fig. 1). Among them, glycerophosphate was assumed to derive from spontaneous hydrolysis of phospholipids in the alkaline extracts, as phospholipids constituted the main component of plant cell membranes[13,29]. The nucleotide category includes the degradation products of RNA and possibly DNA, both of which are involved in cellular metabolic and reproductive activities[19]. Sugar phosphates are important components in providing energy for cellular processes. This wide variety of monoester P forms found in macrophytes and algae has also been found in lake sediments and water[2], implying that debris from decomposed algae and macrophytes were released into the lake water body and deposited as sediments. The percentage of diester P in extracts was generally low compared to monoester P (Table 1, Fig. 1). A small amount of phosphonate was detected in *Spirulina*. Orthophosphate and pyrophosphate were the forms of P_i detected by NMR analysis and found in all samples investigated. The percentage of orthophosphate and pyrophosphate in non-hydrolzyed samples ranged from 27.1% to 67.5% and from 0.9 to 18.8%, respectively.

In order to compare NMR data with the P identities revealed by EH, we generalized the NMR-identified P species into six categories (i.e., orthophosphate, phytate, other monoester P, diester P, pyrophosphate and phosphonate). In aquatic macrophytes, the majority of extracted P was orthophosphate (27.3 to 56.6% of total extracted P), phytate (12.2 to 38.9%), other monoester P (10.8 to 33.5%), diester P (1.6 to 6.2%) and pyrophosphate (0.9 to 18.8%). Similarly, algae were dominated by orthophosphate (27.1 to 67.5%), phytate (5.6 to 41.9%), other monoester P (8.6 to 44.3%), diester P (1.3 to 14.4%), pyrophosphate (1.8 to 2.3%), with minor amounts of phosphonate (0 to 0.7%).

Forms of phosphorus in aquatic macrophytes and algae characterized by enzymatic hydrolysis.

Using EH, P in NaOH-EDTA extracts was classified into five groups: P_i, labile monoester P, diester P, phytate-like P, and enzyme-stable P (Table 2). Although the content of extractable P_i was greater in algae (3220 mg kg^{−1}) than in macrophytes (1126 mg kg^{−1}), the percentage of total extractable P present as P_i was lower in the algae sample (35.5%). For P_o, aquatic macrophytes contained an average of 180 mg kg^{−1} labile monoester P, which accounted for approximately 8.0% of extracted P. Labile monoester P was present at an average of 1566 mg kg^{−1} in algae samples, which accounted for approximately 16.5% of extracted P. *Spirulina* (an algal species) contained diester P at 695 mg kg^{−1} (~6.8% of extracted P). *Common reed*, a macrophyte, contained 45 mg kg^{−1} diester P (~3.8% of extracted P). The content of phytate-like P of macrophytes was 380 mg kg^{−1} (~14.0% of extracted P) in macrophytes, and 1235 mg kg^{−1} in algae (~14.5% of extracted P). Concentrations of enzyme-stable P in aquatic macrophyte and algal extracts accounted for 25.7% and 31.2% of extracted P, respectively.

The general distribution pattern of P was P_i > enzyme-stable P > phytate-like P > labile monoester P > diester P in NaOH-EDTA extracts of both aquatic macrophytes and algae. The distribution pattern was consistent with

Figure 1. Solution ^{31}P NMR analysis of the NaOH-EDTA extracts of aquatic macrophytes and algae before (Con) and after treatments with AP, AP + PDE and AP + PDE + Phy enzymes.

NaOH-EDTA extractable P from soil[30], dairy manure[17], and lake sediments[2], but different from that of poultry manure[17]. In general, phytate-like P represented the largest proportion of enzyme-hydrolysable P, followed by labile monoester P and diester P. Contents of total hydrolysable P_o ranged from 140 to 951 mg kg^{-1} in aquatic macrophytes and 1369 to 4387 mg kg^{-1} in algae. Additionally, 238 to 975 mg kg^{-1} and 2324 to 3726 mg kg^{-1} of enzyme-stable P, respectively, in macrophytes and algae could not be hydrolyzed by the enzymes used in this study. The percentage of enzyme-stable P in extracted P of aquatic macrophytes were similar to that of animal manure[26] and lake sediments[9].

The percentage of enzyme-stable P in extracted P of algae was slightly higher than macrophytes, perhaps due to differing abilities of the two organism types to mineralize P_o[8]. Enzyme-stable P in NaOH-EDTA extracts likely consisted of P_o in high molecular weight (e.g., humic acid) or combined with high molecular weight with metal bridge, which would be inaccessible by active sites of enzymes[3,30]. The percentage of P_i in extracted P of macrophytes was slightly lower than that reported for lake sediments (60.9%)[9] and were consistent with previous reports[31]. The percentages of P_i in extracted P of algae were lower than aquatic macrophytes likely due to the more complex cellular and organ systems of macrophytes. Thus, more P_i would exist in complex forms than in algae.

Comparison of P forms by ^{31}P NMR and Enzymatic Hydrolysis.

Three aquatic macrophytes were *Foxtail algae* (A1), *Common reed* (A2), *Verticillata* (A3), respectively, and three algal species were *Microcystis* (B1), *Chirorella vulgaris* (B2), *Spirulina* (B3), respectively. Four forms of P in aquatic macrophytes and algae were similarly identified by ^{31}P NMR and EH: (1) orthophosphate or P_i, (2) phytic acid (phytate) or phytate-like P, (3) other monoester P or labile monoester P, and (4) diester P. There was no significant difference in P_i content among three samples (A2, B2, B3) measured by the two methods, but there was a significant difference in P_i content in other three samples (A1, A3, B1) measured by the two methods (Fig. 2). On the whole, the quantity of P_i measured

	Pi			Po																			
				Monoester P															Diester P				
				Phytate				Other Monoester P															
Class	Ortho-[a]	Pyro-	∑Pi	chiro-	neo-	other-phy	∑Phy	Fru-	α-gly	β-gly	Gua-	Cyc-	Glu-1	Glu-6	AMP	P-acid	P-ine	∑other	Lipids	DNA	∑Die	C-P	∑Po
A1 + 0e[b]	990(45.9)[c]	52(2.4)	1042(48.3)	424(19.6)	64(3.0)	353(16.3)	841(38.9)	—[d]	79(3.7)	—	71(3.3)	—	—	—	83(3.8)	—	—	233(10.8)	—	44(2.0)	44(2.0)	—	1117(51.7)
A1 + 1e	2004(92.8)	—	2004(92.8)	—	—	66(3.0)	66(3.0)	—	25(1.2)	—	—	—	—	—	—	6.5(0.3)	—	32(1.5)	58(2.7)	—	58(2.7)	—	155(7.2)
A1 + 2e	1851(85.7)	—	1851(85.7)	—	—	159(7.4)	159(7.4)	—	—	—	—	—	—	—	—	121(5.6)	—	121(5.6)	29(1.3)	—	29(1.3)	—	308(14.3)
A1 + 3e	2068(95.9)	—	2068(95.9)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	88(4.1)	—	88(4.1)	—	88(4.1)
A2 + 0e	670(56.6)	10(0.9)	680(57.4)	77(6.5)	68(5.7)	—	145(12.2)	—	96(8.1)	—	—	—	—	—	—	—	190(16.1)	286(24.2)	—	74(6.2)	74(6.2)	—	504(42.6)
A2 + 1e	932(78.7)	—	932(78.7)	—	—	33(2.8)	33(2.8)	—	5(0.4)	—	—	—	—	—	—	5.8(0.5)	2.6(0.2)	13(1.1)	187(15.8)	18(1.5)	206(17.4)	—	252(21.3)
A2 + 2e	1082(91.4)	—	1082(91.4)	—	—	102(8.6)	102(8.6)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	102(8.6)
A2 + 3e	1162(98.1)	—	1162(98.1)	—	—	—	—	—	—	—	—	—	—	—	22(1.9)	—	—	22(1.9)	—	—	—	—	22(1.9)
A3 + 0e	832(27.3)	573(18.8)	1405(46.1)	351(11.5)	226(7.4)	—	576(18.9)	881(28.9)	116(3.8)	—	24(0.8)	—	—	—	—	—	—	1021(33.5)	—	49(1.6)	49(1.6)	—	1643(53.9)
A3 + 1e	2902(95.2)	—	2902(95.2)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	88(2.9)	58(1.9)	146(4.8)	—	146(4.8)
A3 + 2e	2606(85.5)	—	2606(85.5)	—	—	338(11.1)	338(11.1)	—	—	—	—	—	—	—	—	—	—	—	104(3.4)	—	104(3.4)	—	442(14.5)
A3 + 3e	3048(100)	—	3048(100)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
B1 + 0e	5094(67.5)	137(1.8)	5232(69.3)	596(7.9)	128(1.7)	825(10.9)	1549(20.5)	652(8.6)	—	—	—	—	—	—	—	—	—	652(8.6)	—	116(1.5)	116(1.5)	—	2318(30.7)
B1 + 1e	5915(78.4)	—	5915(78.4)	14(0.2)	—	270(3.6)	285(3.8)	100(1.3)	47(0.6)	—	9(0.1)	—	—	35(0.5)	—	36(0.5)	—	227(3.0)	1049(13.9)	73(1.0)	1123(14.9)	—	1634(21.7)
B1 + 2e	6657(88.2)	—	6657(88.2)	—	—	719(9.5)	719(9.5)	—	—	—	—	—	—	—	—	—	—	—	174(2.3)	—	174(2.3)	—	892(11.8)
B1 + 3e	7183(95.2)	—	7183(95.2)	—	—	—	—	—	—	94(1.2)	86(1.1)	—	—	—	—	129(1.7)	—	309(4.1)	57(0.8)	—	57(0.8)	—	366(4.9)
B2 + 0e	2646(27.1)	1497(15.3)	4143(42.4)	3498(35.8)	391(4.0)	206(2.1)	4095(41.9)	—	979(10.0)	—	—	431(4.4)	—	—	—	—	—	1410(14.4)	—	130(1.3)	130(1.3)	—	5636(57.6)
B2 + 1e	7695(78.7)	—	7695(78.7)	—	—	680(7.0)	680(7.0)	—	—	—	—	—	—	—	—	216(2.2)	19(0.2)	235(2.4)	1119(11.4)	51(0.5)	1170(12.0)	—	2084(21.3)
B2 + 2e	7056(72.2)	—	7056(72.2)	494(5.1)	—	1228(12.6)	1722(17.6)	—	—	—	—	—	—	—	—	—	—	—	1001(10.2)	—	1001(10.2)	—	2723(27.9)
B2 + 3e	7540(77.1)	—	7540(77.1)	—	—	—	—	784(8.0)	—	—	—	—	—	—	—	—	1138(11.6)	1923(19.7)	317(3.2)	—	317(3.2)	—	2239(22.9)
B3 + 0e	4236(32.8)	297(2.3)	4533(35.1)	90(0.7)	594(4.6)	39(0.3)	723(5.6)	2583(20.0)	1227(9.5)	555(4.3)	1266(9.8)	90(0.7)	—	—	—	—	—	5721(44.3)	—	1860(14.4)	1860(14.4)	90(0.7)	8395(65.0)
B3 + 1e	10732(83.1)	220(1.7)	10952(84.8)	—	—	1072(8.3)	1072(8.3)	—	39(0.3)	—	426(3.3)	—	—	—	—	—	—	465(3.6)	220(1.7)	—	220(1.7)	220(1.7)	1963(15.2)
B3 + 2e	10952(84.8)	—	10952(84.8)	116(0.9)	—	1421(11.0)	1537(11.9)	—	—	—	—	—	—	—	—	—	—	—	—	439(3.4)	—	439(3.4)	—	1976(15.3)
B3 + 3e	12295(95.2)	—	12295(95.2)	129(1.0)	—	—	129(1.0)	—	—	—	—	—	245(1.9)	245(1.9)	—	—	—	491(3.8)	—	—	—	—	620(4.8)

Table 1. P components in the NaOH-EDTA extracts of aquatic macrophytes and algae affected by enzymatic hydrolysis treatments prior to NMR analysis. [a]Ortho-: Orthophosphate; Pyro-: Pyrophosphate; ∑P$_i$: the sum of orthophosphate and pyrophosphate; chiro-: chiro-IHP, neo-: neo-IHP; other-phy: other phytate; ∑Phy: the sum of phytate(IHP); Fru-: D-Fructose 6-phosphate; α-gly: α-Glycerophosphate; β-gly: β-Glycerophosphate; Glu-1: Glucose 1-phosphate; Glu-6: D-Glucose 6-phosphate; Gua-: Guanosine 2′ monophosphate; Cyc-: Cytidine 5′ monophosphate; AMP: Adenosine 5′ monophosphate; P-acid: 3-sn phosphatidic acid; P-ine: O-phosphorylethanolamine; ∑other: the sum of other monoesters P; Lipids: Lipids phosphate; DNA: deoxyribonucleic acid; ∑Die: the sum of diesters P; C-P: Phosphonates; ∑Po: the sum of P$_o$. [b]A1, A2, A3, B1, B2, B3: *Foxtail algae, Common reed, Verticillata, Microcystis, Chirorella vulgaris, Spirulina*; 0e, 1e, 2e, 3e: without enzyme treatments, +AP; +AP + PDE; +AP + PDE + Phy. [c]Values in parentheses are percentages of individual P compounds in NaOH-EDTA extractable P. [d]"—": not detected or negative value.

in the aquatic macrophytes and algae were slightly higher with ^{31}P NMR (~49.8% of total extracted P) than EH (~43.3%). The content of phytate detected by the two methods was similar for three out of the six samples. Phytate content was lower in the NaOH-EDTA extracts of A1 and A2 and B2 with EH than measured with NMR. Using EH, significantly other monoester P was detected in the extracts of both the macrophytes and algae than with NMR. Similarly, the percentage of diester P determined by EH was less than by ^{31}P NMR, with the exception of B1. This may be attributed to the presence of unstable diester P that was easily biologically degraded to monoester P[32]. Other studies have shown the rapid hydrolysis (16 h) of RNA or bacterial compounds in alkaline extracts[19,32] and plant tissues[33]. In a word, not all P forms (i.e., enzyme-stable P) are easily hydrolyzed by commercially available phosphatases, they may be lack of specificity and non-ideal incubation conditions. Approximately 25.7% and 31.2% of total extractable P in macrophytes and algae were characterized as enzyme-stable P in aquatic macrophyte and algae, respectively (Table 2). As a result, these causes led to the differences in total detectable P$_o$ concentrations between the NMR and EH methods.

Overall, these data indicated that the distribution patterns of major P forms in aquatic macrophytes and algae determined by EH and solution ^{31}P NMR were similar ($r = 0.712$, $p < 0.05$, n = 5). While NMR provided information on the relative abundance of specific P compounds and classes, it is not an economically viable approach for many researchers and requires expert interpretation of the resulting spectra[9]. Enzymatic hydrolysis provided a valid estimate of hydrolyzable, and thus potentially bioavailable P$_o$[2]. However, the EH method also has some disadvantages. For example, the EH method may not effectively hydrolyze some forms of P$_o$ (i.e., enzyme-stable P). Besides, P was only classified into five groups in the EH method (Table 2). In contrast, NMR analysis could identify 18 specific P compounds (Table 1). In this study, the combination of EH and NMR for analyses of specific P

Code	Sample	P_i	Total hydrolyzable P_o	Phytate-like P	labile Monoester P	Diester P	Enzyme-stable P
		% of total P extracted					
A1	*Foxtail algae*	33.4 ± 5.1[a]	26.9 ± 3.4	23.5 ± 2.6	3.4 ± 0.5	—[b]	39.7 ± 5.4
A2	*Common reed*	58.7 ± 4.8	11.8 ± 2.1	—	8.0 ± 1.5	3.8 ± 0.5	29.5 ± 2.8
A3	*Verticillata*	61.0 ± 3.6	31.2 ± 2.9	18.5 ± 1.4	12.7 ± 0.4	—	7.8 ± 0.1
B1	*Microcystis*	30.0 ± 4.8	41.3 ± 5.8	26.4 ± 5.8	14.9 ± 2.8	—	28.7 ± 4.1
B2	*Chlorella vulgaris*	42.3 ± 5.6	15.5 ± 2.3	12.2 ± 0.9	3.3 ± 0.5	—	42.2 ± 0.8
B3	*Spirulina*	34.2 ± 2.4	42.9 ± 1.8	4.8 ± 0.2	31.3 ± 2.1	6.8 ± 0.5	22.8 ± 1.7
		$mg\,kg^{-1}$					
A1	*Foxtail algae*	820 ± 125	660 ± 83	577 ± 64	83 ± 12	—	975 ± 133
A2	*Common reed*	697 ± 57	140 ± 25	—	71 ± 6	45 ± 6	350 ± 33
A3	*Verticillata*	1860 ± 110	951 ± 88	564 ± 43	387 ± 12	—	238 ± 3
B1	*Microcystis*	2429 ± 389	3344 ± 470	2138 ± 470	1206 ± 227	—	2324 ± 332
B2	*Chlorella vulgaris*	3735 ± 494	1369 ± 203	1077 ± 79	291 ± 44	—	3726 ± 71
B3	*Spirulina*	3497 ± 245	4387 ± 184	491 ± 21	3200 ± 215	695 ± 51	2331 ± 174

Table 2. Phosphorus forms identified in NaOH-EDTA extracts of aquatic macrophytes and algae by enzymatic hydrolysis. [a]Mean with standard deviation, $n = 3$. [b]"—":not detected or negative value.

forms and bioavailability in aquatic macrophytes and algae were both complimentary and supplementary and provided detailed information related to endogenous P cycling in eutrophic lakes.

Lability of phosphorus in aquatic macrophytes and algae characterized by ^{31}P NMR coupled with enzymatic hydrolysis.

Incubation of NaOH-EDTA extracts of macrophytes and algae with commercially available phosphatases clearly changed peak distribution in the NMR spectra (Fig. 1). Addition of any phosphatase treatment (i.e., AP, AP + PDE, or AP + PDE + Phy) resulted in decreased NMR signal in the monoester (i.e., phytate and other monoester P) and pyrophosphate regions, with a corresponding increase in orthophosphate (Figs 1 and 3). As an example, the NMR spectral features of *foxtail algae* (A1) with three types of enzyme treatments were analyzed in detail (Table 1). Addition of AP significantly increased measured orthophosphate by $1014\,mg\,kg^{-1}$ (46.9%) compared to Control and significantly decreased concentrations of some P_o forms by 44.5% (phytate-35.9%; other monoester P-9.3%) and pyrophosphate by 2.4% in *foxtail algae*. There was a small increase in diester P (0.7%) after hydrolysis by AP (Table 1, Fig. 3). On the other hand, the peaks assigned to chiro-IHP, neo-IHP, Guanosine $2'$ monophosphate, AMP and DNA did not appear after the AP enzyme treatment. In addition, a new peak appeared at a chemical shift of 4.58 ppm in the monoester region after the treatment, which was assigned to O-phosphorylethanolamine (P-ine). Pyrophosphate, comprising 2.4% of total P in the untreated extract (the peak at -4 to -5 ppm), was completely hydrolyzed by the AP treatment (Table 1, Fig. 3).

Likewise, treatment with AP + PDE resulted in the complete loss of pyrophosphate along with some signals in the monoester region (3.5 to 6.0 ppm). Adding AP + PDE significantly decreased P_o forms by 39.8% (phytate-31.5%; other monoester P-8.9%; diester P-0.7%) and significantly increased orthophosphate by $861\,mg\,kg^{-1}$ (Table 1, Fig. 3). Treatment with AP + PDE additionally removed diester P (-1 to 2 ppm chemical shift), while treatment with AP alone did not affect this peak. Solution ^{31}P NMR analysis of the enzyme-treated extracts of *foxtail algae* (Fig. 1) showed that the anticipated substrate specificity of AP + PDE was also achieved in foxtail algae extracts. Specifically, in the extracts of *foxtail algae*, hydrolysis caused a significant increase in orthophosphate by $1078\,mg\,kg^{-1}$ and a small increase in diester P by $44\,mg\,kg^{-1}$ (2.1%) with a significant decrease in other three P forms by 52.1% (phytate-38.9%, other monoester P-10.8%, and pyrophosphate-2.4%).

Adding AP alone and AP + PDE + Phy slightly increased detected diester P (Fig. 3). This observation might be due to the fact that glycerophosphate in extracts was derived from phospholipid hydrolysis during alkaline extraction[29]. In the current case, this process was likely to be reversed by phosphorylation activity[2,21]. Interestingly, guanosine $2'$ monophosphate and α-glycerophosphate were completely hydrolysed by the AP and AP + PDE + Phy (Table 1). A portion of these compounds may have been transformed to diester P, which is supported by the measured increases in diester P with AP alone and AP + PDE + Phy treatments. The three P_o classes were quantified by the increases in *molybdate-reactive P (i. e. MRP)* in enzyme treated samples compared to the control (non-enzyme amended) (all values in $mg\,kg^{-1}$ of *foxtail algae* dry biomass) as phytate: 841, other monoester P: 233, diester P: 44. In addition, the NMR spectra showed some new peaks with enzyme treatments, as shown in Table 1, such as glucose 6-phosphate (B1 + 1e), cytidine $5'$ monophosphate (B1 + 1e, + 3e, B3 + 3e), AMP (A2 + 3e), 3-sn phosphatidic acid (P-acid) (A1 + 1e, etc.) and P-ine (B1 + 1e, B2 + 1e, + 3e). Phytate has many stereo-isomers, of which chiro-, neo-, myo-, scyllo-IHP have been found to be most common in soils[21] and sediments[5]. In this study, chiro-IHP and neo-IHP were identified in macrophytes and algae (Fig. 1, Table 1). While only a portion of the exacted phytate with EH during the 16 h incubation in the current study, in this work, most of the phytate disappearance was reported after a 40 d incubation under anaerobic conditions[34].

Overall, the vast majority of monoester P, including phytate, and total pyrophosphate were transformed into orthophosphate with EH. As shown in Fig. 4, Phytate (average of 18.9%, of extractable P), other monoester P (20.7%) and pyrophosphate (6.6%) were transformed into orthophosphate with AP. Phytate (12.3%), other monoester P (20.8%) and pyrophosphate (7.0%) were transformed into orthophosphate with AP + PDE. Phytate (22.8%), other monoester P (17.7%) and pyrophosphate (7.0%) were transformed into orthophosphate
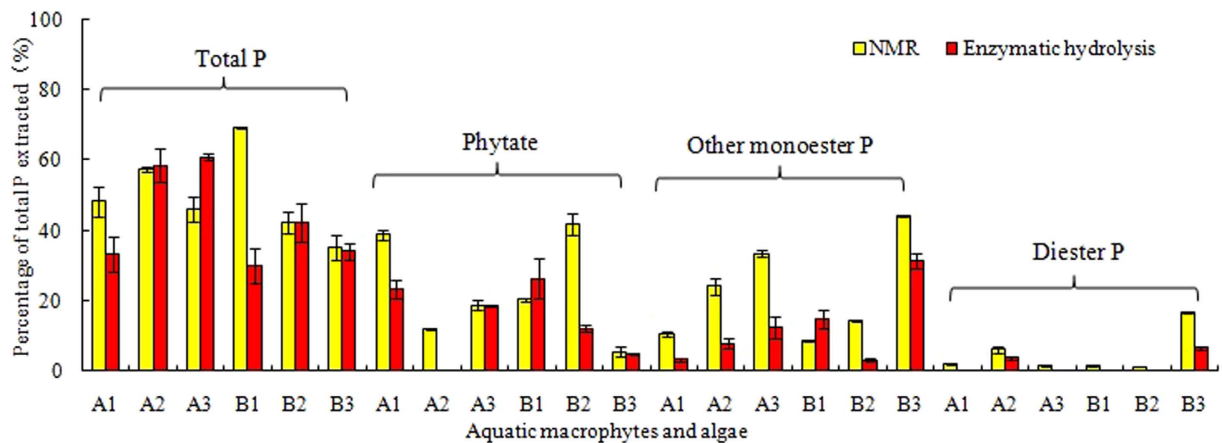
Figure 2. Comparison of P forms in the NaOH-EDTA extracts of aquatic macrophytes and algae biomass samples identified by solution ^{31}P NMR spectroscopy and enzymatic hydrolysis. A1, A2, A3, B1, B2, and B3 stand for *Foxtail algae, Common reed, Verticillata, Microcystis, Chiorella vulgaris and Spirulina*, respectively.
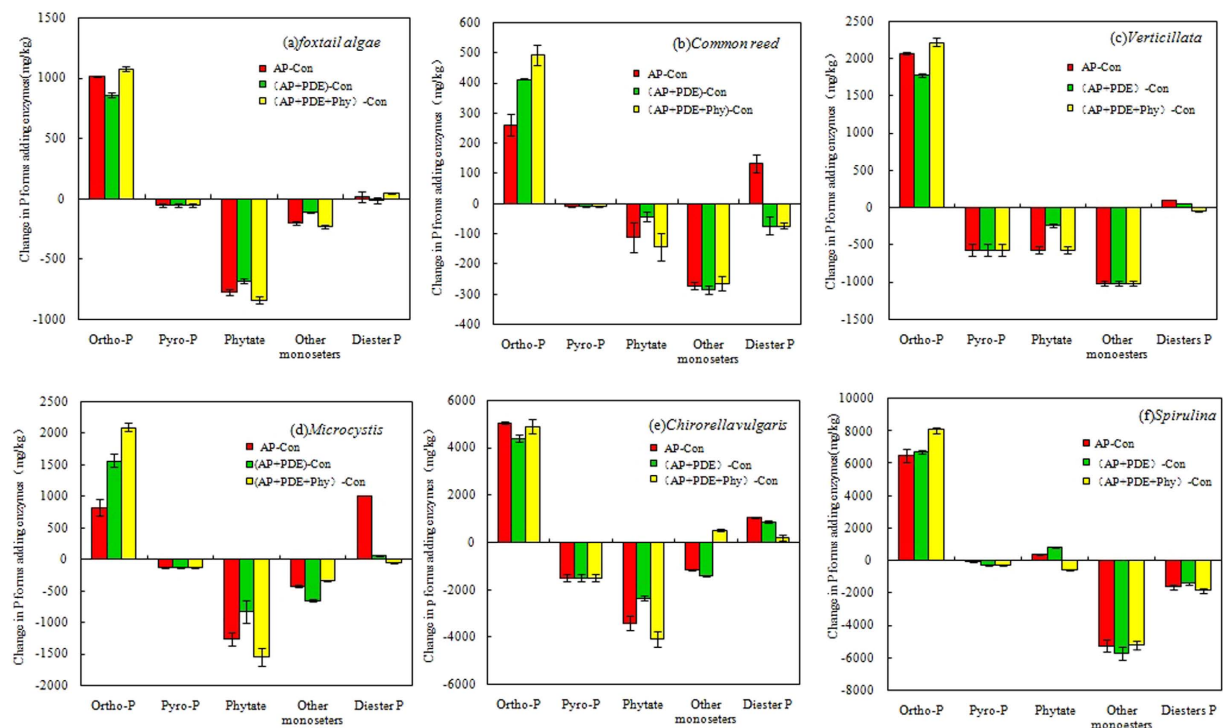
**Figure 3. Degradation characteristics of P in aquatic macrophytes and algae revealed per ^{31}P NMR spectroscopic changes of the NaOH-EDTA extracts due to the EH treatments.** These treatments were added AP, AP + PDE and AP + PDE + Phy enzymes.

with AP + PDE + Phy. These data suggests high potential for release of monoester P and pyrophosphate during decomposition of macrophytes and algae and that these P forms could be further enzymatically converted into orthophosphate in aquatic systems. In most instances, P is a limiting nutrient in freshwater systems and orthophosphate concentrations are linked to cyanobacterial proliferation in eutrophic lakes[35]. In contrast, some forms of P_o are bioavailable to algae. Therefore, this cyclic process would be cause algae outbreak again. These results suggest that bioavailable P_o derived from decomposing aquatic macrophytes and algae could play an important role in maintaining the eutrophic status of lakes and could present complications to mitigation efforts.

Algal blooming and biogeochemical cycling of phosphorus in lakes. Based on results in this study, biogeochemical cycling of P driven by algal blooming in eutrophic Lake Tai was further discussed (Fig. 5). Approximately 32.7% to 41.3% and 39.5% to 46.0% of extractable P_o from algae and macrophytes, respectively, has potential for phosphatase hydrolysis to soluble orthophosphate and released into the water bodies.
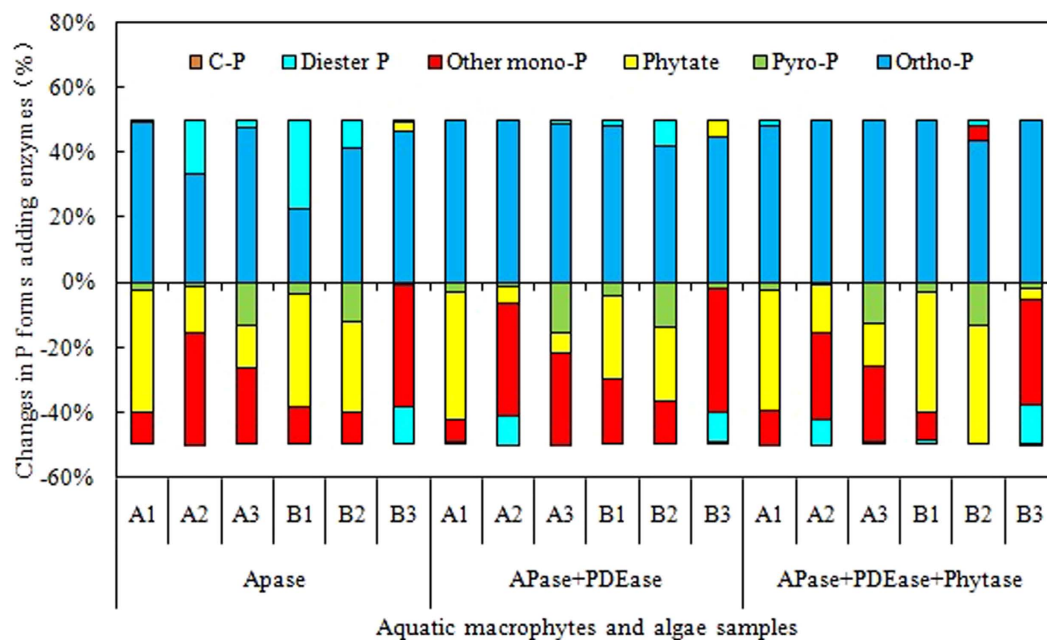
Figure 4. Percentage changes of P forms, as proportion of total extractable P, in aquatic macrophytes and algae after the treatments with AP, AP + PDE and AP + PDE + Phy enzymes.
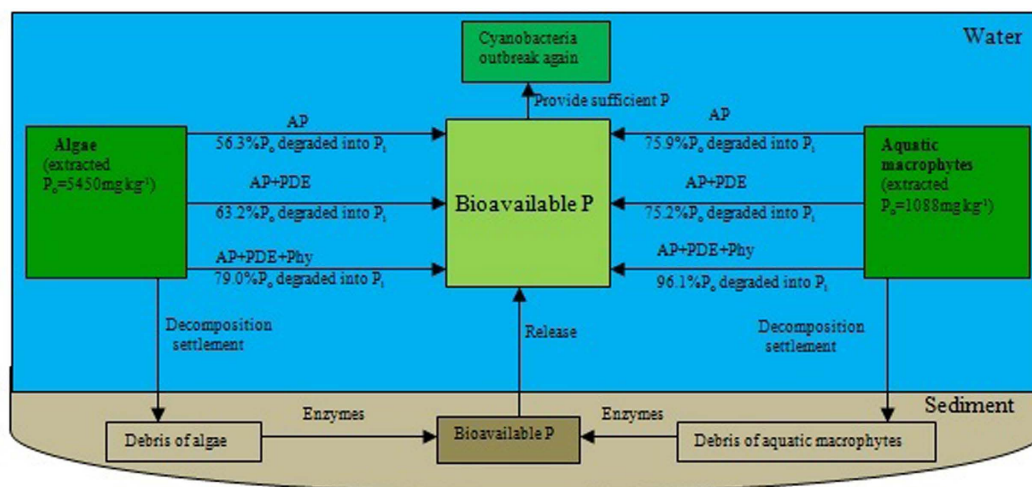
Figure 5. A schematic diagram of the cycling process of organic phosphorus (P$_o$) of aquatic macrophytes and algae in freshwater lakes. The proportions were percentage of hydrolyzed P$_o$ in NaOH-EDTA extractable P$_o$.

Orthophosphate can be directly assimilated and utilized by cyanobacteria and other organisms in eutrophic lakes. The majority of P$_o$ from decomposition would be deposited as sediments, which can then be or transformed into orthophosphate under the action of various enzymes and microorganisms within sediments and subsequently released to overlying water. Both water and sediment sources of bioavailable P could provide sufficient P for proliferation of algae and cyanobacteria. In turn, P incorporated into biomass during algal blooms could be readily replenished by the bioavailable P from the debris of aquatic macrophytes and algae. Thus, P cycling among sediment, water, and aquatic organisms is the primary mechanism of continuous (or repeated) algal and maintenance of eutrophic conditions.

As the biomass of algae and aquatic macrophytes were 13.57 mg kg^{-1} and 2606 g m^{-2} in Lake Tai in 2012, which has a water volume of 44.3×10^8 m^3 and area of 2338 km[26]. From this information, we estimated the total biomass of algae and aquatic macrophytes in Lake Tai were 6.01×10^4 Mg and 6.09×10^6 Mg, respectively. With the P$_o$ levels of algae and aquatic macrophytes we measured in this study (Fig. 5), we calculated the P$_o$ biomass of algae and aquatic macrophytes to be approximately 328 Mg and 7510 Mg, respectively. Of this amount of P$_o$, algae and macrophytes would contain 107 to 135 Mg and 2966 to 3455 Mg, respectively, of bioavailable P. This bioavailable

P would be released into the water body and promote repeated algal blooms. Therefore, the contribution of P in the debris from aquatic organisms must be considered in relation to a endogenous P cycling in eutrophic lakes.

It is reported that cyanobacteria salvage (a technology for removal of cyanobacteria from the eutrophic lakes by a salvage ship or machine) could effectively eliminate the phenomenon of algal blooming so that cyanobacteria salvage could reduce the concentration of nitrogen (N) and P in lakes[36]. In addition to the physical methods, cyanobacteria (e.g., *Microcystis aeruginosa*) could be removed efficiently by using quaternary ammonium salt of Gemini surfactant[37]. In order to prevent the release of P from the debris of aquatic macrophytes, which continue to proliferate and cause secondary pollution in lakes, the debris of aquatic macrophytes must be harvested in a timely fashion. Harvested aquatic organisms could be mechanically broken down and recycled as a source of organic fertilizer for crop production[38]. No matter what measures to take, the importance on preventing or reducing the P release from the debris of algae and aquatic macrophytes would make great contribution on controlling the phenomenon of eutrophication of lakes and repeated algal blooming from internal P cycling.

Conclusions

About 90% of TP and P_o in debris of algae and aquatic plants could be extracted by 0.5 M NaOH-25 mM EDTA. Solution ^{31}P NMR analysis found 11 P_o species in the mono- and diester region plus orthophosphate, pyrophosphate and phosphonate in these extracts. The bioavailability of these NaOH-EDTA extractable P components was further characterized by EH, which revealed that 2173 mg kg^{-1}, accounting for 28.3% of extracted P, was hydrolyzable with commercially available phosphatases. The hydrolyzable portions included phytate-like P (1809 mg kg^{-1}, 14.2% of extracted P), labile monoester P (808 mg kg^{-1}, 12.3% of extracted P) and diester P (123 mg kg^{-1}, 4.9% of extracted P). However, 1657 mg kg^{-1}, (~28.5%) of extracted P, was not enzyme hydrolyzable (enzymatic stable P).

Based on the results of ^{31}P NMR spectroscopic analysis of EH extracts, most proportions of P_o and pyrophosphate were hydrolyzable by phosphatases into orthophosphate. Enzymatically stable P was assumed to be macromolecular P_o that would accumulate and be preserved in lake sediments for long periods. According to lability of these P_o forms, recycling of P_o in the debris derived from aquatic macrophytes and algae, especially algae, would be an important internal source of bioavailable P in lakes. Recycling of concentrated bioavailable P in aquatic macrophytes and algae would continuously support algal blooming in a eutrophic lake, such as Lake Tai.

Materials and Methods

Study Site and Sample Preparation.
Lake Tai (33°55′−31°32′N, 119°52′-120°36′E), a large shallow, eutrophic lake, is located in Jiangsu Province, China. Lake Tai was chosen as a study site because it is one of the eutrophic lakes where the algal blooms have been closely monitored for decades[39]. Research indicated that the water column in the algal blooming areas contains a high level of aquatic macrophytes and phytoplankton[31,38]. In an earlier study[13], we characterized P_o in the NaOH-EDTA extracts of three aquatic macrophytes and three algae samples by solution ^{31}P NMR spectroscopy. Our group also characterized the P forms and C functional groups of the water extracts of the macrophytes collected from the macrophyte-dominated zone in Lake Tai[31]. These studies found the presence of multiple monoester and diester P forms in the aquatic macrophyte and algal biomass, suggesting that P_o from the dying and dead biomass of these samples might be transformed into P_i that eventually contributes to algal blooming associated with eutrophication.

Three aquatic macrophytes [*Foxtail algae* (A1), *Common reed* (A2), *Verticillata* (A3)] were collected from Lake Tai, and three algal species [*Microcystis* (B1), *Chirorella vulgaris* (B2), *Spirulina* (B3)] were collected from Lake Tai and provided by Institute of Hydrobiology, Chinese Academy of Sciences. Three representative aquatic macrophytes (two submerged macrophytes and one emergent macrophyte) were investigated. Two submerged macrophytes (i. e. A1, A3) and one emergent macrophyte (i. e. A2) were collected in the region of Lake Tai during late October 2010. Whole aquatic macrophytes (i. e. roots, stems, leaves) were collected by use of a plant collector, then washed and took back to laboratory. These field samples were dried to a constant weight at 60 °C, ground, and sieved through a 2-mm screen[21,38]. The resulting powder samples were stored at −20 °C until use.

NaOH-EDTA extractions for solution enzymatic hydrolysis and ^{31}P-NMR spectroscopy.
Phosphorus extraction was followed the procedure of Feng[13]. Briefly, prepared samples of aquatic macrophytes and algae (0.5 g DM) were extracted with 30 mL of 0.5 M NaOH-25 mM EDTA at 22 °C for 16 h on an end-over-end shaker. After extraction, the slurry solutions were centrifuged at $10000 \times g$ for 30 min. Supernatants were filtered through 0.45 μm membrane filters to obtain the NaOH-EDTA extracts. Molybdate-reactive P (i. e. MRP) was measured by the molybdenum blue/ascorbic acid method[40,41]. The TP concentration of extracts was determined after digestion with potassium persulfate ($K_2S_2O_8$) in an autoclave at 121 °C for 30 min[42]. Concentrations of P_o in extracts were then calculated as the difference between concentrations of TP and MRP. The remaining extracts were freeze-dried and kept in at −20 °C until ^{31}P NMR analysis.

Enzymatic hydrolysis procedure.
Alkaline phosphatase (AP) (EC. 3.1.3.1), phosphodiesterase (PDE) (EC. 3.1.4.1), and crude phytase (Phy) (EC 3.1.3.26) were purchased from Sigma (St Louis, MO). Working solutions of AP and PDE were prepared in Tris-HCl buffer (0.1 M, pH 9.0) at concentrations of 1.0 and 0.02 U mL^{-1}, respectively. Crude phytase was purified by dialysis to remove phosphates[2,26]. The working phytase solution was prepared in Tris-HCl buffer (0.1 M, pH 7.0) at concentration of 0.06 U mL^{-1}. AP was used alone, but PDE was used in combination with AP to achieve complete hydrolysis of diester phosphates. Phytase was used in combination with AP and PDE (0.1 mol·L^{-1}, pH 9.0) to ensure that hydrolysis of dissolved P_o was as complete as possible[2].

The enzymatic incubation followed the procedure of Zhu[2]. The incubation solution consisted of 5 mL of pH-adjusted and diluted extracts[2] and 0.44 mL of the working enzyme solution (AP at pH 9.0, AP + PDE at pH 9.0, or AP + PDE + Phy at pH 7.0) in the appropriate buffer. These mixtures were incubated for 16 h at 37 °C with shaking at 220 rpm. The P_o hydrolyzed by each enzyme preparation was calculated as the difference in MRP

concentrations determined before and after incubation. Aliquots of the solution was used to determine P_i by the molybdenum blue/ascorbic acid method[40,41]. Sodium dodecyl sulfate (SDS) was added at 2% (v/v) prior to analysis to prevent enzyme precipitation[41]. A portion of the EH incubation solutions were freeze-dried and subsequently used for ^{31}P NMR analysis.

Four classes of P_o were quantified based on EH studies of He[21] and Zhu[2]. They are (1) labile monoester P, which was MRP released by AP; (2) diester P, which was the difference in MRP determined after incubation with AP + PDE, and AP alone; (3) phytate-like P[43], which was defined as net increase in MRP determined after incubation with AP + PDE + Phy, compared to MRP determined in step (2); and (4) enzyme-stable P that was the part of P_o unhydrolyzable by any of the enzyme treatments, it was the difference value between P_o with hydrolyzable P_o (i. e. labile monoester P, diester P, phytate-like P).

Solution ^{31}P NMR Spectroscopy.
Freeze-dried NaOH-EDTA extracts and incubation solutions were dissolved, respectively, in 1 mL 1 M NaOH-0.1 M EDTA/0.1 mL 99% deuterium oxide (D_2O), and allowed to stand for 30 min at room temperature. Samples were then centrifuged for 30 min at approximately $10000 \times g$, transferred to NMR tubes, and stored at 4 °C before analysis (<24 h). Solution ^{31}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, 0.2102 s acquisition, 5 s pulse delay, and 5-Hz spinning. The number of points was 8192. For each sample, the total NMR experiment lasted 15 to 20 h, collecting 24000 scans. Phosphorus compounds were identified by their chemical shifts, with the orthophosphate peak in all spectra standardized to 6.00 ppm.

Data Analysis.
Data were checked for deviation from normality of variance before analyses. To check whether there was a significant linear relationship between bioavailable P_o, Pearson correlation coefficients (r values, two-tailed) at $p < 0.01$ and $p < 0.05$ were determined using SPSS 11.5. Solution ^{31}P NMR Spectra were analyzed by MestReNova software 9.0. Figures were processed using CorelDraw Graphics Suite X4 (Corel Corp.) and OriginPro 8.0 (OriginLab Corp.).

References

1. Zhang, R. *et al.* Characteristics of organic phosphorus fractions in different trophic sediments of lakes from the middle and lower reaches of Yangtze River region and Southwestern Plateau, China. *Environ. Pollut.* **152,** 366–372 (2008).
2. Zhu, Y. *et al.* Characterization of organic phosphorus in lake sediments by sequential fractionation and enzymatic hydrolysis. *Environ. Sci. Technol.* **47,** 7679–7687 (2013).
3. Zhu, Y. *et al.* Influence of natural organic matter on the bioavailability and preservation of organic phosphorus in lake sediments. *Chem. Geol.* **397,** 51–60 (2015).
4. Mao, R., Zeng, D.-H., Zhang, X.-H. & Song, C.-C. Responses of plant nutrient resorption to phosphorus addition in freshwater marsh of Northeast China. *Sci. Rep.* **5,** 8097, doi: 10.1038/srep08097 (2015).
5. Zhu, Y., Wu, F. & He, Z. Bioavailability and preservation of organic phosphorus in freshwater sediments and its role in lake eutrophication. In *Labile Organic Matter—Chemical Compositions, Function, and Significance in Soil and the Environment.* SSSA Special publication 62 (Eds: He, Z. & Wu, F.), 275–294 (Soil Science Society of America 2015).
6. Yu, T. *et al.* Six-decade change in water chemistry of large freshwater lake Taihu, China. *Environ. Sci. Technol.* **47,** 9093–9101 (2013).
7. Carman, R., Edlund, G. & Damberg, C. Distribution of organic and inorganic phosphorus compounds in marine and lacustrine sediments: a ^{31}P NMR study. *Chem. Geol.* **163,** 101–114 (2000).
8. Li, Y. *et al.* An analysis of the relationship between phytoplankton internal stoichiometry and water column N: P ratios in a dynamic lake environment. *Ecol. Model.* **252,** 196–213 (2013).
9. Giles, C. D. *et al.* Characterization of organic phosphorus form and bioavailability in lake sediments using P nuclear magnetic resonance and enzymatic hydrolysis. *J. Environ. Qual.* **44,** 882–894 (2015).
10. Li, H. *et al.* Occurrence and distribution of phosphorus fractions in sediments of Liangzi Lake under typical hydrodynamic conditions. *Environ. Sci.: Processes Impacts.* **17,** 1433–1442 (2015).
11. Chen, M. *et al.* Global landscape of total organic carbon, nitrogen and phosphorus in lake water. *Sci. Rep.* **5,** 15043 (2015).
12. Mackay, E. B. *et al.* Contribution of sediment focussing to heterogeneity of organic carbon and phosphorus burial in small lakes. *Freshwater Biol.* **57,** 290–304 (2012).
13. Feng, W. *et al.* Characterization of phosphorus forms in lake macrophytes and algae by solution 31P nuclear magnetic resonance spectroscopy. *Environ. Sci. Pollut. Res.* **23,** 7288–7297 (2016).
14. Monbet, P., McKelvie, I. D. & Worsfold, P. J. Dissolved organic phosphorus speciation in the waters of the Tamar estuary (SW England). *Geochim. Cosmochim. Acta.* **73,** 1027–1038 (2009).
15. Wang, J. & Pant, H. K. Enzymatic hydrolysis of organic phosphorus in river bed sediments. *Ecol. Engineer.* **36,** 963–968 (2010).
16. Zhu, Y. *et al.* Interaction of alkaline phosphatase with minerals and sediments: activities, kinetics and hydrolysis of organic phosphorus. *Colloids Surfaces A: Physicochem. Engineer. Aspects.* **495,** 46–53 (2016).
17. He, Z. *et al.* Comparison of phosphorus forms in wet and dried animal manures by solution phosphorus-31 nuclear magnetic resonance spectroscopy and enzymatic hydrolysis. *J. Environ. Qual.* **36,** 1086–1095 (2007).
18. Tazisong, I. A., Senwo, Z. N., Cade-Menun, B. J. & He, Z. Phosphorus forms and mineralization potentials of Alabama upland cotton production soils amended with poultry litter. In *Applied Manure and Nutrient Chemistry for Sustainable Agriculture and Environment* (Eds: He, Z. & Zhang, H.), 191–209 (Springer 2014).
19. Turner, B. L., Mahieu, N. & Condron, L. M. Phosphorus-31 nuclear magnetic resonance spectral assignments of phosphorus compounds in soil NaOH–EDTA extracts. *Soil Sci. Soc. Am. J.* **67,** 497–510 (2003).
20. Godinot, C., Gaysinski, M., Thomas, O. P., Ferrier-Pages, C. & Grover, R. On the use of ^{31}P NMR for the quantification of hydrosoluble phosphorus-containing compounds in coral host tissues and cultured zooxanthellae. *Sci. Rep.* **6,** 21760. doi: 10.1038/srep21760 (2016).
21. He, Z., Olk, D. C. & Cade-Menun, B. J. Forms and lability of phosphorus in humic acid fractions of Hord silt loam soil. *Soil Sci. Soc. Am. J.* **75,** 1712–1722 (2011).
22. Zhang, R. *et al.* Phosphorus composition in sediments from seven different trophic lakes, China: a phosphorus-31 NMR study. *J. Environ. Qual.* **38,** 353–359 (2009).
23. Baldwin, D. S. Organic phosphorus in the aquatic environment. *Environ. Chem.* **10,** 439–454 (2013).
24. Cade-Menun, B. J. Improved peak identification in ^{31}P-NMR spectra of environmental samples with a standardized method and peak library. *Geoderma.* **257–258,** 102–114 (2015).
25. Feuillade, J., Bielicki, G. & Renou, J.-P. ^{31}P-NMR study of natural phytoplankton samples. *Hydrobiologia.* **300,** 391–398 (1995).

26. He, Z. & Honeycutt, C. W. Enzymatic characterization of organic phosphorus in animal manure. *J. Environ. Qual*. **30,** 1685–1692 (2001).
27. Zhu, Y. *et al.* Phosphorus fractions and bioavailability in relation to particle size characteristics in sediments from Lake Hongfeng, Southwest China. *Environ. Earth Sci.* **68,** 1041–1052 (2013).
28. Young, E. O. *et al.* Phosphorus speciation in riparian soils: A phosphorus-31 nuclear magnetic resonance spectroscopy and enzyme hydrolysis study. *Soil Sci. Soc. Am. J.* **77,** 1636–1647 (2013).
29. Doolette, A., Smernik, R. & Dougherty, W. Spiking improved solution phosphorus-31 nuclear magnetic resonance identification of soil phosphorus compounds. *Soil Sci. Soc. Am. J.* **73,** 919–927 (2009).
30. Jarosch, K. A. *et al.* Characterisation of soil organic phosphorus in NaOH-EDTA extracts: A comparison of ^{31}P NMR spectroscopy and enzyme addition assays. *Soil Biol. Biochem.* **91,** 298–309 (2015).
31. Liu, S. *et al.* Characteristics and degradation of carbon and phosphorus from aquatic macrophytes in lakes: Insights from solid-state ^{13}C NMR and solution ^{31}P NMR spectroscopy. *Sci. Total Environ.* **543,** 746–756 (2016).
32. Makarov, M. Haumaier, L. & Zech, W. Nature of soil organic phosphorus: an assessment of peak assignments in the diester region of ^{31}P NMR spectra. *Soil Biol. Biochem.* **34,** 1467–1477 (2002).
33. Saha, S. *et al.* Lyophilization of cotton tissue on quality of extractable DNA, RNA, and protein. *J. Cotton Sci.* **1,** 10–14 (1997).
34. Suzumura, M. & Kamatani, A. Origin and distribution of inositol hexaphosphate in estuarine and coastal sediments. *Limnol. Oceanogra.* **40,** 1254–1261 (1995).
35. Sharpley, A., Mcdowell, R. & Kleinman, P. Phosphorus loss from land to water: integrating agricultural and environmental management. *Plant & Soil* **237,** 287–307 (2001).
36. Zhou, B. *et al.* Effect of Scooping out blue algae on growth of Microcystis aeruginosa. *J. Ecol. Rural Environt.* **3,** 260–265 (2012).
37. Chai, S. *et al.* Removal of *Microcystis aeruginosa* by using quaternary ammonium salt of Gemini surfactant. *J. Environ. Engineer. Technol.* **6,** 8–15 (2016).
38. Qu, X. *et al.* Quantitative and qualitative characteristics of dissolved organic matter from eight dominant aquatic macrophytes in Lake Dianchi, China. *Environ. Sci. Pollut. Res.* **20,** 7413–7423 (2013).
39. Duan, H. *et al.* Two-decade reconstruction of algal blooms in China's Lake Taihu. *Environ. Sci. Technol.* **43,** 3522–3528 (2009).
40. Murphy, J. & Riley, J. P. A modified single solution method for the determination of phosphate in natural waters. *Analytica chimica acta.* **27,** 31–36 (1962).
41. He, Z. & Honeycutt, C. W. A modified molybdenum blue method for orthophosphate determination suitable for investigating enzymatic hydrolysis of organic phosphates. *Commun. Soil Sci. Plant Anal.* **36,** 1373–1383 (2005).
42. Do Nascimento, C. A. C., Pagliari, P. H., Schmitt, D., He, Z. & Waldrip, H. Phosphorus concentrations in sequentially fractionated soil samples as affected by digestion methods. *Sci. Rep.* **5,** 17967, doi: 10.1038/srep17967 (2015).
43. Giles, C. D. & Cade-Menun, B. J. Phytate in animal manure and soils: Abundance, cycling and bioavailability. In *Applied Manure and Nutrient Chemistry for Sustainable Agriculture and Environment* (Eds: He, Z. & Zhang, H.), 163–190 (Springer 2014).

Acknowledgements

Author Contributions

W.F., Y.Z. and F.W. designed the research and wrote the paper. C.Z. analyzed the data. Z.H. and J.P.G. analyzed the data, wrote the paper, and improved the language of the manuscript. All authors discussed the results and reviewed the manuscript.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Feng, W. *et al.* Forms and Lability of Phosphorus in Algae and Aquatic Macrophytes Characterized by Solution ^{31}P NMR Coupled with Enzymatic Hydrolysis. *Sci. Rep.* **6,** 37164; doi: 10.1038/srep37164 (2016).

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.