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Phytoplankton communities determine the spatio-temporal heterogeneity of alkaline phosphatase activity: evidence from a tributary of the Three Gorges Reservoir

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In order to reveal the role of phytoplankton in the spatio-temporal distribution of alkaline phosphatase activity (APA), monthly investigations were conducted in the Xiaojiang River, a tributary of the Three Gorges Reservoir in China. Different APA fractions, environmental parameters, and phytoplankton communities were followed. High spatio-temporal variations of APA were observed, with the highest value in summer and the lowest in winter. The annual average APA_T (total alkaline phosphatase activity) ranged from 7.78–14.03 nmol·L^{-1}·min^{-1} with the highest in the midstream and the lowest in the estuary. The dominant phytoplankton phyla in summer and winter were *Cyanophyta* and *Bacillariophyta*, respectively. The mean cell density in the midstream and in the estuary was 5.2×10^7 cell·L^{-1} and 1.4×10^7 cell·L^{-1}, respectively. That $APA_{>3.0\mu m}$ was significantly higher than $APA_{0.45\text{-}3\mu m}$ indicating phytoplankton was the main contributor to alkaline phosphatase. Correlation analysis indicated the dominant species and cell density could determine the distribution pattern of APA. Turbidity, total phosphorus, chemical oxygen demand, water temperature (WT), pH and chlorophyll *a* were proved to be positively correlated with APA; soluble reactive phosphorus, conductivity, transparency and water level(WL) were negatively correlated with APA. It was concluded that spatio-temporal heterogeneity of APA determined by phytoplankton communities was related to WT and WL.

Phosphorus is always treated as a limiting nutrient in many freshwater ecosystems because it frequently limits the primary production[1,2]. The availability of phosphorus (P) is regarded as a crucial factor regulating the dynamic of phytoplankton *in situ*. There is no doubt that there are many types of phosphorus in waters, including the organic and inorganic phosphorus. Among the various forms of phosphorus, orthophosphate (o-P) or available soluble reactive phosphorus (SRP), the bioavailable form of phosphorus, can be depleted rapidly in freshwaters due to the rapid uptake[3], which tends to limit algal growth in freshwater ecosystems. So the low concentrations of SRP can modify the structure of plankton communities and constrain phytoplankton distribution[4,5]. Some phytoplankton species, zooplankton and bacterioplankton can produce extracellular phosphatase liberating SRP from dissolved organic P compounds, which is one mechanism allowing the living organisms to overcome P limitation[6,7].

Relationship between APA and phytoplankton has been paid more attention since 1960s[8,9]. Kalinowska tried to figure out the major contributor of APase through membrane filtration method[10]. Even if size fractionation by filtration is never completely absolute (i.e., overlapping size), it still provides useful insights on the major microorganisms possibly contributing to APA. The increase in APA can be attributed more to phytoplankton biomass than to the bacterial biomass[11]. Therefore, phytoplankton contributed greatly to APA production and

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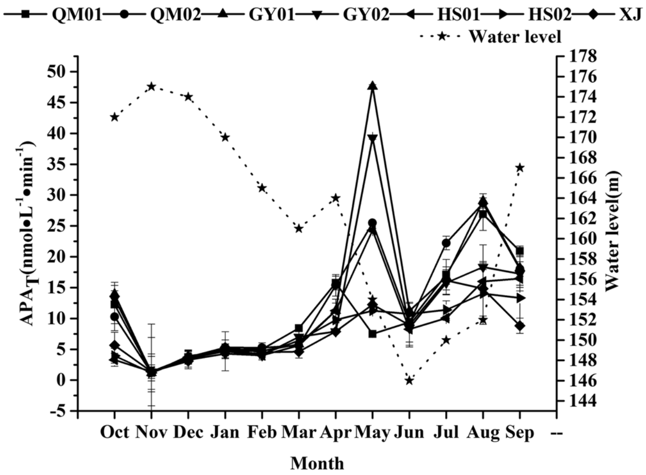

Figure 1. Monthly variations of the total APA concentrations in different sample sites and water level in the Xiaojiang River.

was significantly influenced by P bioavailability. Production of extracellular phosphatases has been detected in many phytoplankton species[12–14]. Various taxa are exhibiting differences in the presence, localization and labeling pattern of phosphatases. Both seasonal and short-term variations also have been detected in enzyme activity of phytoplankton[15]. Enzyme-labeled fluorescence (ELF) analysis revealed pronounced differences in the makeup of phytoplankton responsible for APA in San Francisco and Monterey bays[16].

The Three Gorges Reservoir (TGR) is the biggest deep river-type reservoir in the world. More than 170 tributaries carry runoff and bring nutrients and pollutants into it, which affected the trophic status and resulted in water blooms in some tributaries of the TGR[17]. Though many studies have been conducted to screen APase in different water bodies, little knowledge was obtained in the Three Gorges Reservoir (TGR). Due to the complicated relationship between APA and ecological factors, it is necessary to screen the distribution pattern of APA and its influencing factors in the TGR. Xiaojiang River is one of the tributaries in the TGR, which is suffered from water blooms frequently. Eutrophication in Xiaojiang River is very serious after the Three Gorges Dam (TGD)'s impoundment since 2003[18]. In this study, Xiaojiang River was selected as the delegate of the tributary in the TGR, phytoplankton and APA in Xiaojiang River were screened. Based on the related researches focused on the complicated relationship between APA and phytoplankton mentioned above, it was assumed that the phytoplankton community successions might lead to the spatio-temporal heterogeneity of alkaline phosphatase activity. In order to verify this hypothesis, monthly investigation was conducted, different APA fractions (APA_T, $APA_{<0.45\mu m}$, $APA_{0.45\text{-}3\mu m}$ and $APA_{>3.0\mu m}$), environmental parameters and phytoplankton communities were screened synchronously. The role of phytoplankton communities in the spatio-temporal heterogeneity of APA and its influence factors in the Three Gorges Reservoir were demonstrated. The results of this study can help to know how APA production changes with phytoplankton communities' successions in the TGR.

Results

APAT distribution pattern. The APA_T ranged from 1.19–47.6 $nmol \cdot L^{-1} \cdot min^{-1}$ (Fig. 1). The lowest level of APA_T was observed in winter. Besides, the average APA_T in summer and autumn were higher than in other seasons. The mean water level was high in winter(169.7 ± 4.5 m) and low in summer(149.3 ± 3.1 m), the variations of water level presented different trends with that of APA_T at temporal scales.

The highest value of annual average APA_T in GY01 and lowest in XJ were also showed in Fig. 1. The average APA_T of GY01 in summer and autumn are higher than those of HS02 and XJ.

Size-fractionation of APA. The average size-fractionated APA indicated that $APA_{<0.45\mu m}$ accounted for the major portion of APA_T, whereas the average $APA_{>3.0\mu m}$ was higher than $APA_{0.45\text{-}3\mu m}$ (Fig. 2a). The average $APA_{>3.0\mu m}$ accounted for 28.1% of APA_T and $APA_{0.45\text{-}3\mu m}$ accounted for 16.7%. In addition, the size-fractionated APA ($APA_{<0.45\mu m}$, $APA_{0.45\text{-}3\mu m}$ and $APA_{>3.0\mu m}$) in summer and autumn are higher than those in winter.

At spatial scales, the average APA_T consisted of 30.2% $APA_{>3.0\mu m}$ and 20.4% $APA_{0.45\text{-}3\mu m}$ in all sites. The $APA_{<0.45\mu m}$ kept a relatively stable and high level. Both $APA_{0.45\text{-}3\mu m}$ and $APA_{>3.0\mu m}$ in midstream (GY01) are higher than those in estuary (XJ).

Phytoplankton communities. *Bacillariophyta* was the dominant group in winter and spring (72.7% of total cell density on average, Fig. 3a) except *cyanophyta* are dominant in April. In summer and autumn, phytoplankton mainly consisted of *Cyanophyta* (65.6% of total cell density on average) except the *Cryptophyta* accounted for 88.4% in August. The mean algal cells density was the highest in July 2014 ($1.27 \times 10^8 cell \cdot L^{-1}$), and the lowest in January 2014 ($1.3 \times 10^6 cell \cdot L^{-1}$). The cell density was higher in summer and autumn than in spring and winter. *Cyanophyta* dominated the phytoplankton in upstream (QM01, QM02, GY01, GY02, 69.9% of total cell density on average). In the downstream (HS01, HS02, XJ), phytoplankton mainly consisted of *Bacillariophyta* (35.3% of total cell density on average, Fig. 3b).

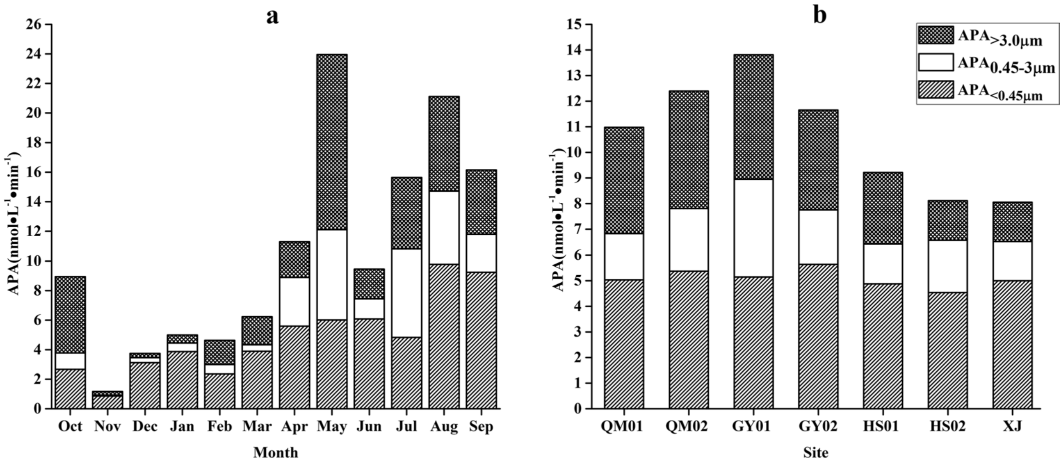

Figure 2. Seasonal (**a**) and spatial (**b**) variations of average size-fractionated APA in the Xiaojiang River. $APA_{>3.0\mu m}$: the alkaline phosphatase activity in algal fraction; $APA_{0.45\text{-}3.0\mu m}$: the alkaline phosphatase activity in bacterial fraction; $APA_{<0.45\mu m}$: picoplankton/dissolved alkaline phosphatase activity.

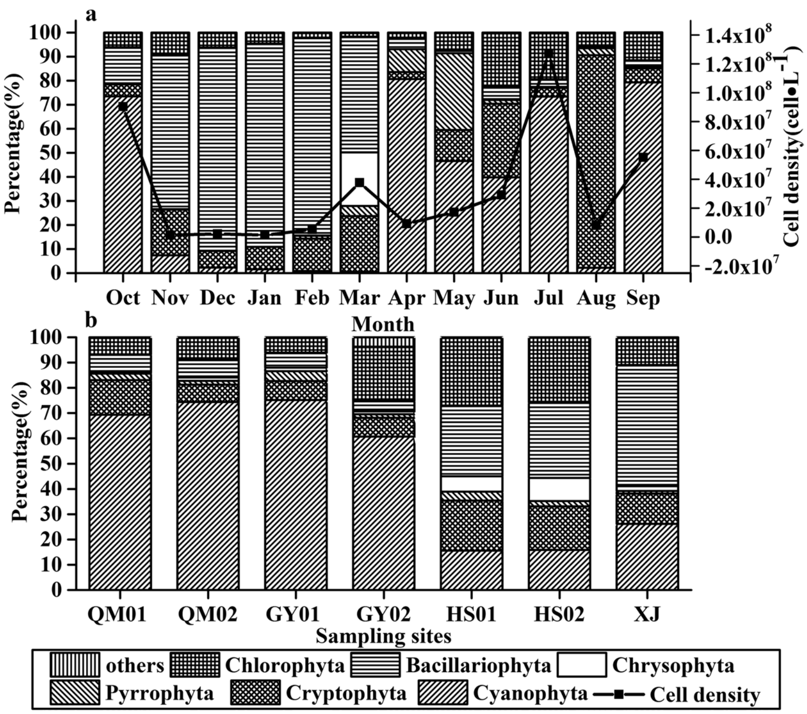

Figure 3. Seasonal (**a**) and spatial (**b**) variations of algal composition and algal cell density in the Xiaojiang River.

Spatio-temporal characteristics of chlorophyll *a* and environmental parameters. Spatio-temporal variations of chlorophyll *a* (Chl *a*) and environmental parameters could be observed in Fig. 4. The values of Chl *a*, total phosphorus (TP), chemical oxygen demand (COD) in spring were apparently higher than the values of other seasons, because the river suffered a *Microcystis* sp. bloom in May, which also resulted in the minimum values of SRP and transparency (SD) emerged. The levels of TP, COD, Chl *a*, water temperature (WT), turbidity (Turb), dissolved oxygen (DO) and pH stayed low in winter, contrary to the values of SRP and SD. The values of SRP fluctuated more frequently than other parameters in different seasons. At spatial scale, the concentrations of SRP and Chl *a* were higher in estuary than in upstream.

Relationships between APA and environmental parameters. SRP concentrations showed negative correlation to $APA_{<0.45\mu m}$ (Fig. 5a), $APA_{0.45\text{-}3\mu m}$ (Fig. 5b), $APA_{>3.0\mu m}$ (Fig. 5c) and APA_T (Fig. 5d). The Spearman correlations among environmental variables and $APA_{<0.45\mu m}$, $APA_{0.45\text{-}3\mu m}$, $APA_{>3.0\mu m}$ and APA_T were presented in Table 1. Turb, TP, COD, WT and pH were positively correlated with APA fractions. Cond., SD and WL were negatively correlated with APA.

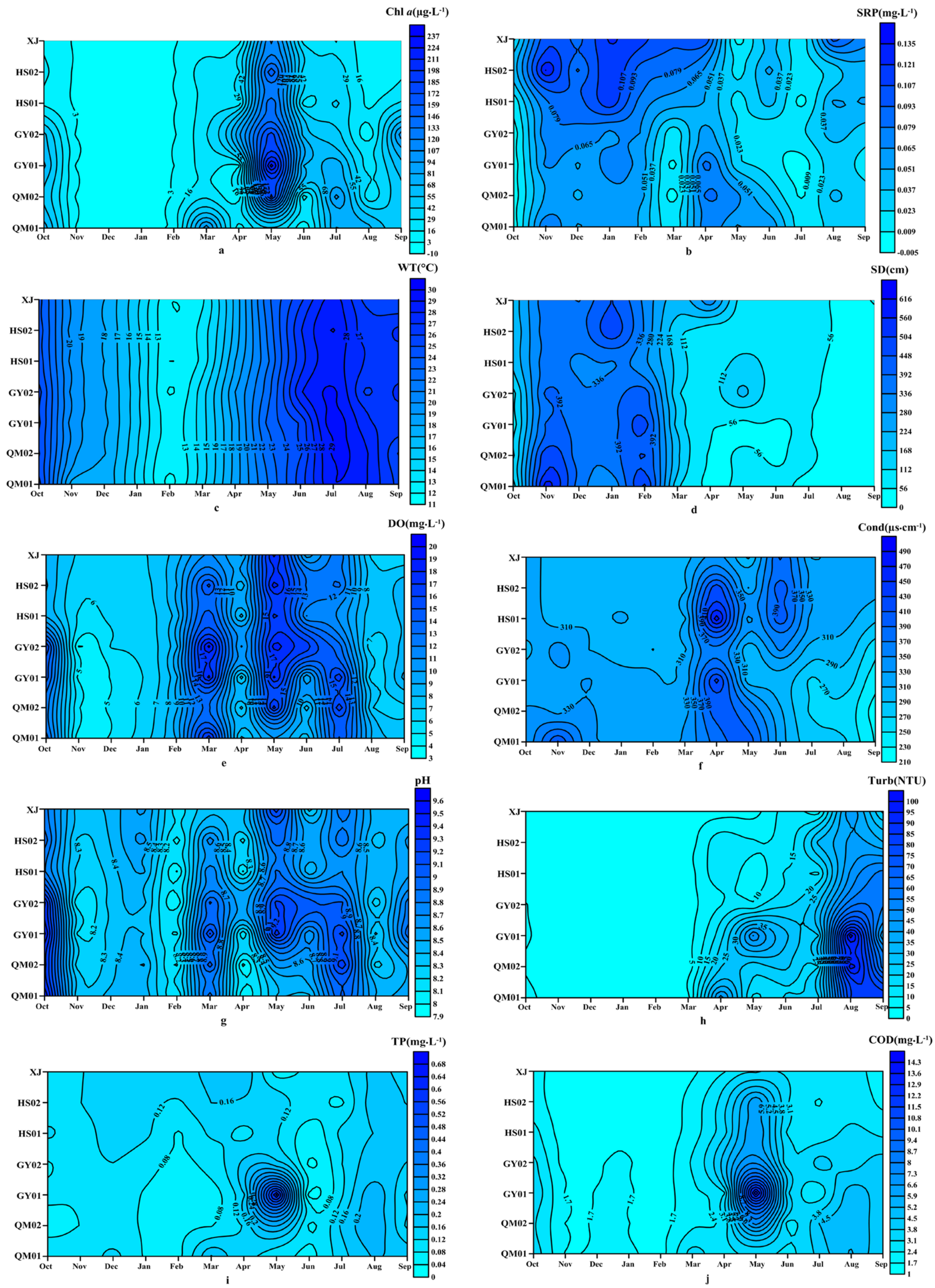

Figure 4. Temporal and spatial variations of a: chlorophyll (**a**) (Chl *a*) and other environmental parameters. (**b**) Soluble reactive phosphorus (SRP); (**c**) water temperature (WT); (**d**) transparency (SD); (**e**) dissolved oxygen (DO); (**f**) conductivity (Cond); (**g**) pH; (**h**) turbidity (Turb); (**i**) total phosphorus (TP) and (**j**) chemical oxygen demand (COD).

Redundancy analysis (RDA) was performed to analyze the relationship between environmental parameters and size-fractionated APA. The ordination diagrams of environmental variables and size-fractionated APA for axis 1 and axis 2 were shown in Fig. 6. The Monte Carlo test revealed that the first canonical axis and all canonical axes were significant ($F = 25.932$, $P = 0.002$; $F = 3.086$, $P = 0.002$; 499 random permutation). For environmental variables and size-fractionated APA, all canonical axes cumulatively explained 83.3% of the variance in APA–environment relationships, and the first two canonical axes accounted for 26.5% and 31.5% of the variance separately. The first axis was positively correlated with DO (0.57), COD (0.65) and negatively correlated with SRP

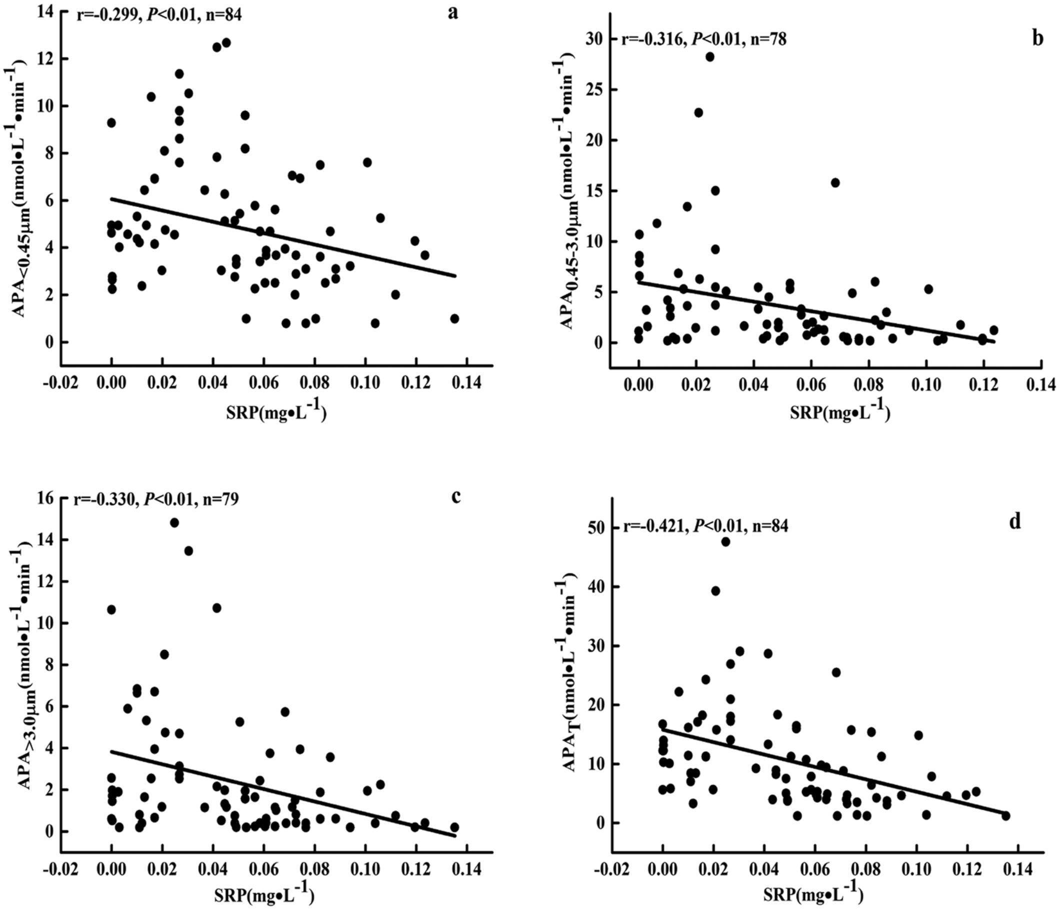

Figure 5. Relationship between soluble reactive phosphorus (SRP) concentrations and $APA_{<0.45\mu m}$ (**a**), $APA_{0.45\text{-}3\mu m}$ (**b**), $APA_{>3.0\mu m}$ (**c**) and APA_T (**d**) in the Xiaojiang River.

	APA_T(n = 84)	$APA_{<0.45\mu m}$(n = 84)	$APA_{>3.0\mu m}$(n = 78)	$APA_{0.45\text{-}3\mu m}$(n = 79)
WT	0.642**	0.562**	0.404**	0.609**
SD	−0.844**	−0.815**	−0.586**	−0.698**
DO	0.478**		0.382**	0.368**
Cond	−0.251*		−0.256*	
pH	0.405**		0.271*	0.271*
Turb	0.858**	0.834**	0.582**	0.753**
TP	0.388**	0.357**	0.346**	0.413**
COD	0.858**	0.684**	0.646**	0.751**
WL	−0.678*	−0.699*		−0.713**

Table 1. Spearman correlations between APA and 10 environmental variables: water temperature (WT); transparency (SD); dissolved oxygen (DO); conductivity (Cond); pH, turbidity (Turb); total phosphorus (TP); chemical oxygen demand (COD); water level (WL) in the Xiaojiang River. $^*P < 0.05$. $^{**}P < 0.01$.

(−0.41), SD (−0.38) and WL (−0.30). The second axis was mainly negatively correlated with Cond (−0.13) and WT (−0.16). $APA_{<0.45\mu m}$ and $APA_{>3.0\mu m}$ was the major portion of APA_T. $APA_{>3.0\mu m}$ and $APA_{0.45\text{-}3\mu m}$ were located on the right-hand side of the biplot. They were correlated negatively with WL, SD, SRP and Cond, and positively with other parameters.

Relationships between $APA_{>3.0\mu m}$ and algal cell density. $APA_{>3.0\mu m}$ reached the highest in mid-stream (GY01) in May (28.24 $nmol \cdot L^{-1} \cdot min^{-1}$), and undetectable in estuary (XJ) in December. Values ranged from 0.19–22.71 $nmol \cdot L^{-1} \cdot min^{-1}$at the other sites. The mean cell density was the highest in midstream (GY02, $5.2 \times 10^7 cell \cdot L^{-1}$) and the lowest in estuary (XJ,$1.4 \times 10^7 cell \cdot L^{-1}$). A significant positive relationship was found between $APA_{>3.0\mu m}$ and cell density among all sites (Fig. 7).

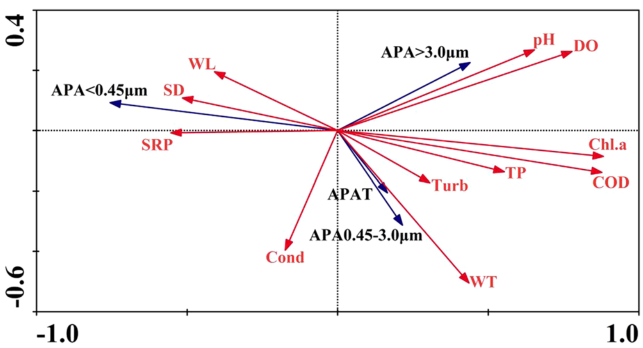

Figure 6. Biplot diagrams for RDA of the relationship between 11 environmental variables (red lines) and $APA_{<0.45\,\mu m}$, $APA_{>3.0\,\mu m}$, $APA_{0.45\text{-}3\,\mu m}$ (blue lines).

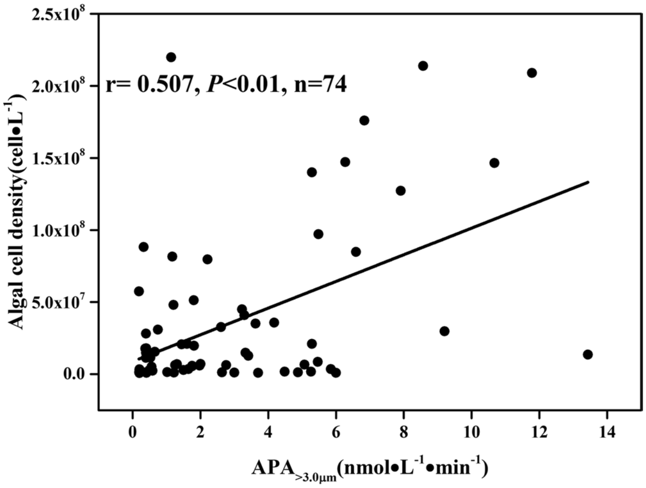

Figure 7. Relationships between $APA_{>3.0\,\mu m}$ and cell density in all sites.

Discussion

APase has different sources, different kinds of bacteria, phytoplankton and zooplankton can excrete extracellular phosphatase[19]. Specific APA was related to different phosphatase producing organisms. In our investigation, $APA_{>3.0\,\mu m}$ contributed in average 28.1% in the APA_T, while bacterial APA accounted for 16.7%, APA in algal fraction ($APA_{>3.0\,\mu m}$) was higher than that in the picoplankton/bacterial fraction ($APA_{0.45\text{-}3\,\mu m}$). Though many (not all) phytoplankton cells have a host heterotrophic bacteria inhabiting or in close association with cells, and the overlapping size on the filter also influenced the final data, which making it difficult to assign the different size fractionation by filtration to individual cells alone, it could be admitted that the coarser fraction ($APA_{>3.0\,\mu m}$), mainly from algae, was conventionally defined as "algal APA"[20] due to phytoplankton was the main contributor according to its size, biomass and physiological activity. It was confirmed $APA_{>3.0\,\mu m}$ accounted for the major portion of total APA (55–87.9%) than $APA_{0.45\text{-}3\,\mu m}$[21]. It could be deduced that the phytoplankton was the major contributor of bulk APA based on the larger proportion of $APA_{>3.0\,\mu m}$(52.73%) than $APA_{0.45\text{-}3\,\mu m}$(21.09%)[22]. Therefore, the phytoplankton contributed greatly to APA production. Meanwhile, the picoplankton/dissolved APA ($APA_{<0.45\,\mu m}$) kept a relative stable and high level (53.4% of the APA_T). Some studies showed that the picoplankton/dissolved APA represents a significant part of the total activity. For example, Labry *et al.* reported that picoplankton/dissolved APA represented 13% to 44% of APA_T in the Bay of Biscay (on the French Atlantic coast)[23]. Higher proportions were recorded in the northern Red Sea (42–74%)[24]. The dissolved APase can be liberated into the environment through the lysis of dead phytoplankton cells and from cells damaged by zooplankton grazing[25]. The high values may result from physical damage of cells by water current and zooplankton grazing on phytoplankton. Nevertheless, some study found that the dissolved APA might origin from bacteria[26]. In order to elucidate the origins of dissolved APA, the dissolved alkaline phosphatase was capsulated into reverse micellar media, and it was proved that the different behaviors of dissolved phosphatase of surface and overlying water might be due to the different origins, with the former being algae and the latter being bacterial[27]. The results of Song *et al.* (2005) couldn't identify the exact origins form, the fraction <0.45 μm contains some pico-bacteria and some pico-phytoplankton and can't be called as the dissolved fraction. Here we changed it as the picoplankton/dissolved fraction. Besides, the positive relationships between APA and the environmental parameters that have been treated as the indexes of the productivity and trophic status, such as Chl *a*, Turb and COD, and the negative relationship between APA and SD can also indicate that the phytoplankton is the main contributor of APA.

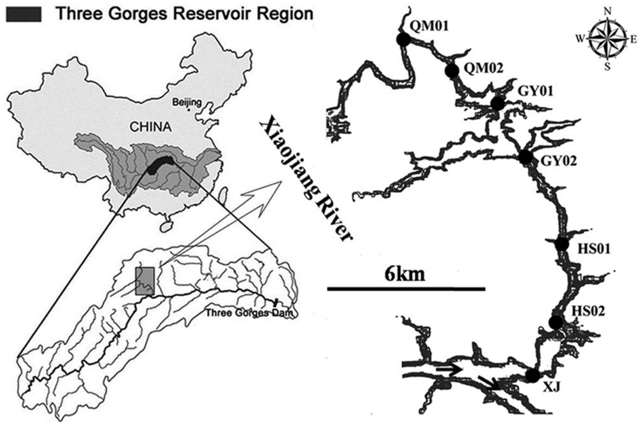

Figure 8. Maps of the location of the Three Gorges Reservoir Region, and the sampling sites in the Xiaojiang River (Created by Arcmap 10.0).

Different algal species showed significant different secreting ability of APase. In this study, phytoplankton communities were dominated by *Bacillariophyta* in winter. *Pyrrophyta*, *Bacillariophyta* and *Chlorophyta* can easily produce extracellular phosphatase as evidenced by ELFA labeling[21]. The low $APA_{>3.0\mu m}$ during this period may result from the low algal cell density of phytoplankton and the increased concentrations of SRP. When the *Pyrrophyta* subdominated the phytoplankton community in May, the $APA_{>3.0\mu m}$ peaked. Results in some shallow eutrophic lakes revealed that the species belonging to *Pyrrophyta* were regularly phosphatase-positive, while *Bacillariophyceae* were phosphatase negative except *Aulacoseira* sp[28]. *Dinoflagellates* were poor competitors for phosphate accumulation compared to diatoms; they have to excrete much more APase than diatom to hydrolyze DOP to satisfy their P demand, even when phosphate is adequate[29]. In nutrient addition experiments, a higher percentage of *dinoflagellates* were identified with cell-specific APA than diatoms[30]. It can explain why $APA_{>3.0\mu m}$ peaked when *Pyrrophyta* subdominated the phytoplankton.It was consistent with the results in Monterey Bay that *dinoflagellates* comprised only 14% of all cells counted and accounted for 78% of APase-producing cells examined[16]. *Microcystis aeruginosa* was confirmed can also synthesize APase[31]. It can explain that as the cell density of *Cyanophyta* increased in summer and autumn, the $APA_{>3.0\mu m}$ was also prompted. The dominating of *Cyanophyta* during the summer and autumn resulted in the high amount of APA. The synchronous pattern of alkaline phosphatase activity and algal cells amount can also be found in Jialing River[32]. The higher algal cell density in midstream than in estuary can also explain why the APA_T was higher in midstream. It could be concluded that phytoplankton communities determined the level of $APA_{>3.0\mu m}$, which determined the significant seasonal and regional variations of APA_T.

APA showed significant seasonal and regional variations, with lower value in inlet waters and higher value in the estuarine, and relatively low in winter and high in summer[7]. However, the distribution characteristics of APA in this study were not consistent strictly with the above mentioned. The APA_T fluctuated frequently from spring to autumn. Relative stable level of APA_T in winter can be seen in Fig. 1. This phenomenon may result from the fluctuant water level of the TGR. For the sake of flood control and hydropower, the water level in the TGR is subjected to the specific management of the TGD and is meant to seasonally fluctuate between 145 and 175 m a.s.l. It has been demonstrated that the turbulence promoted the phytoplanktonic APA and accelerated the biogeochemical cycle of P in Lake Taihu[33]. This was consistent with our results that the high APA was present during the significant water level fluctuated period from spring to autumn. Meanwhile, it has been proved that the APA increased with water temperature[34,35]. The positive relationship between WT and APA in this study (Table 1) supports the conclusion that WT determined the APA through its effects on the phytoplankton seasonally and the direct influences on APase.

Methods

Samples area and sites. Xiaojiang River, a tributary of the TGR, originates from Kaixian, Chongqing Municipality with a length of 180 km and watershed area of 5172.5 km^2. It flows from north to south; entering into the TGR in Yunyang County. The distance from the estuary to the TGD is 248 km.

Water temperature (WT), pH, dissolved oxygen (DO) and conductivity (Cond.) were measured using a YSI model Professional Plus multiparameter probe (USA); Transparency (SD) was measured with a Secchi disk; and turbidity (Turb.) was measured with a WGZ-B turbidmeter (XinRui, Shanghai). Water level (WL) was recorded by GPS *in situ*. Surface water samples (0.5 m) were collected with a Van Dorn sampler at seven sampling sites (XJ, HS02, HS01, GY02, GY01, QM02, QM01) (Fig. 8) monthly from October 2013 to September 2014. All samples were run in triplicate. In order to avoid the physiological and biological parameters changed dramatically, the water samples for APA test were filtered immediately after collection and strong oscillation *in situ*, the filters were put into a portable refrigerator at 0 °C and analyzed within 24 h. All water samples for the other parameters measurement were also stored in a portable refrigerator at 0 °C after collected and tested within 24 h. Concentrations of chlorophyll *a* (Chl *a*), total phosphorus (TP), soluble reactive phosphorus (SRP), chemical oxygen demand (COD) were analyzed after samples collected within 24 h. Samples for quantitative phytoplankton analyses were fixed with neutral Lugol's solution, and concentrated after 48 h sedimentation[36].

Measurement of APA. APA was measured using a modified procedure[37,38]. A total of 2 ml water samples were incubated at 37 °C for 4 h in the presence of 1 ml 0.05 M Tris-HCl buffer (pH = 8.5) and 2 ml 0.3 mM p-nitronphenylphosphate (p-NPP) as substrate, subsequently, 0.1 ml 0.1 M NaOH was added into the mixture after 4 h. The release of p-nitrophenol from p-nitronphenylphosphate was determined by absorbance at 410 nm using a spectrophotometer (TU-1810), and APA was calculated in $nM \cdot L^{-1} \cdot min^{-1}$. APA was determined in unfiltered water (APA_T) and water samples filtered through 0.45 (the picoplankton/dissolved alkaline phosphatase activity, $APA_{<0.45\,\mu m}$) and $3.0_{\mu m}$ membrane filters ($APA_{<3.0\,\mu m}$). The activity in algal fraction ($APA_{>3.0\,\mu m}$) and in bacterial fraction ($APA_{0.45\text{-}3.0\,\mu m}$) were calculated as follows: $APA_{>3.0\,\mu m} = APA_T - APA_{<3.0\,\mu m}$, $APA_{0.45\text{-}3.0\,\mu m} = APA_{<3.0\,\mu m} - APA_{<0.45\,\mu m}$[39].

Measurement of SRP, Chl *a*, TP, COD and phytoplankton quantification. Water samples used for the Chl *a* measurement were filtered with Whatman GF/C filter, then the residuals on the filter were extracted using 90% acetone solution in the darkroom for 24 h at 4 °C, and Chl *a* was analyzed spectrophotometrically. The concentrations of SRP were measured after all water samples were filtered through pre-washed filters (Whatman GF/C, glass microfiber filters).The concentrations of SRP, total phosphorus (TP) and chemical oxygen demand (COD) were analyzed according to the standard methods[40]. Phytoplankton was quantified at 400 × magnification with a light microscope (OLYMPUS BX41). The identification of phytoplankton species is according to Hu and Wei[41].

Statistical analysis. Statistical analysis was carried out using the SPSS 13.0 package. Variance analysis (one-way ANOVA) was used to compare the means of APA in different seasons and sampling sites. Normal distribution of the data was ensured by visual inspection of Q–Q plots, and Levene's test was used to check for homogeneity of variances before ANOVA. Non-parametric correlation (Spearman) analyses were employed for determining relationships among $APA_{<0.45\,\mu m}$, $APA_{0.45\text{-}3\,\mu m}$, $APA_{>3.0\,\mu m}$, APA_T and the environmental factors. Detrended correspondence analysis (DCA) of the size-fractionated APA and environmental data was performed using CANOCO version 4.5 to determine whether linear or unimodal ordination methods should be applied[42]. Before the analysis, the abiotic and biological data were transformed by $\log(x + 1)$. We calculated an unconstrained ordination through the DCA. Using detrending by segments and Hill's scaling, the length of the longest axis estimated the beta diversity in the data set. Moreover, the value of the data set implies that it is appropriate to use the Redundancy analysis (RDA) method. RDA was performed to get an approximate ordering of the size-fractionated APA's optima for environmental variables. The significance of canonical axes and environmental variables to explain the variance of the size-fractionated APA was tested using Monte Carlo simulations with 499 permutations[43,44].

Data Availability. All data analyzed during this study are included in this published article.

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Author Contributions

Y.J.Y. finished the filed work and the laboratory analysis of samples. Y.J.Y. and Y.H.B. finished the manuscript. Y.H.B. and Z.Y.H. reviewed and discussed the manuscript.

Additional Information

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