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Regulation of the activity and kinetic properties of alkaline phosphatase in eutrophic water bodies by phosphorus and heavy metals

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ABSTRACT

Phosphatase-catalyzed hydrolysis of organic phosphorus is an important pathway for providing bioavailable phosphorus to aquatic organisms. In this paper, a eutrophic water body was used to investigate how phosphorus and heavy metal ions affect alkaline phosphatase activity (APA) and its kinetic properties. The results show that phosphate (PO_4^{3-}), hexametaphosphate ($(PO_3)_6^{6-}$), pyrophosphate ($P_2O_7^{4-}$), and β-glycerophosphate ($C_3H_4O_6P^{2-}$) inhibit APA and the inhibition effects increased with increasing concentration at the range of 0.01–1 mmol L^{-1}. Addition of heavy metal ions, that is, Co^{2+}, Pb^{2+}, and Cr^{6+} at concentrations of 1 mmol L^{-1}, increased the APA 3.1-fold, 2.3-fold, and 2.7-fold, respectively. The Cu^{2+}, Zn^{2+}, and Ni^{2+} exhibited an inhibitory effect on APA in water. For 1 mmol L^{-1} Cu^{2+}, the APA deceased by 37.7% relative to the control. As the concentrations of Cu^{2+}, PO_4^{3-}, $(PO_3)_6^{6-}$, and $P_2O_7^{4-}$ increased, the V_{max} of the alkaline phosphatase (AP) remained stable and K_m increased, indicating that these ions are competitive inhibitors of AP. In addition, V_{max}/K_m decreased with increasing concentrations of Cu^{2+}, PO_4^{3-}, $(PO_3)_6^{6-}$, $P_2O_7^{4-}$, and $C_3H_4O_6P^{2-}$, demonstrating the decreased catalytic efficiency of the AP. In summary, phosphorus and heavy metal significantly affect conversion of organic phosphorus into bioavailable inorganic phosphorous by changing the APA in eutrophic water bodies.

Keywords: Alkaline phosphatase; Kinetic characteristics; Heavy metal; Swan Lake

1. Introduction

Water bodies are facing a major environmental issue worldwide called eutrophication. Eutrophication has occurred in most of the inland lakes and some of the estuaries and bays in China [1]. Phosphorus is a key contributor to eutrophication. In water bodies, phosphorus exists primarily in the form of dissolved organic phosphorus and suspended particulate phosphorus, which account for 12%–30% and 62%–82% of the total phosphorus, respectively, while orthophosphate, which can be readily used by organisms, only accounts for 5%–8% of the total phosphorus [2]. The bioavailable dissolved inorganic phosphorus content of water is often below the growth and reproduction requirements of aquatic organisms, so alkaline phosphatase (AP) is excreted to hydrolyze the less bioavailable organic phosphorus, releasing bioavailable inorganic phosphorus for use by organisms [3].

AP is a non-specific phosphomonoesterase that exists in almost all organisms, except for higher plants. It can directly participate in the metabolic processing of phosphorus, and it plays an important role in phosphorus digestion,

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absorption, and secretion. AP activity (APA) can be used as an indicator of the abundance of phosphorus in aquatic ecosystems and significantly affects the amount of bioavailable phosphorus in the water and on any subsequent eutrophication [4]. A high level of APA is an important indicator of systemic phosphorus limitation. Using in situ nutrient addition experiments, Perkins and Underwood [5] demonstrated that the APA can effectively reflect the phosphorus limitation of a body of water. Qu et al. [6] also found that the addition of exogenous phosphatase can promote the conversion of organic phosphorus into bioavailable inorganic phosphorus. The APA of a given water body is closely related to the concentration and speciation of the phosphorus in the water body. In the study of Xiamen Bay, Huang and Hong [7] showed that the APA in a water body is significantly negatively correlated with the concentrations of phosphates and some low-molecular-weight organic phosphorus (glycerol phosphate), indicating that the generation of AP is inhibited by these ions.

In addition to phosphorous concentration and speciation, the heavy metals in the environment can also affect the APA. The accumulation of heavy metals in soils, sediments, and water significantly reduces the APA in these media, which in turn affects the phosphorus conversion and bioavailability [8]. Xie et al. [9] studied the effect of heavy metal ions in municipal sludge on APA. Their results indicate that Zn^{2+}, Cu^{2+}, and Ag^{+} have strong inhibitory effects on the APA in the sludge; while Mg^{2+}, Co^{2+}, Pb^{2+}, and Cr^{6+} had different degrees of activating effects on the APA. Due to increasing anthropogenic inputs, heavy metal ions have continued to accumulate in water bodies and sediments, threatening the local ecosystem and environment [10]. These heavy metals and phosphorus-containing groups may affect the phosphatase activity and kinetic properties of AP in aqueous environments, which in turn affect the decomposition and bioavailability of phosphorus in the water, representing a large potential impact on the management and control of eutrophication in these environments. Currently, a very limited number of reports have been published on the effects of phosphorus and heavy metal ions on APA in water. Thus, in this study, we investigate the effects of various anions and cations on APA and its kinetic properties in a eutrophic water body in order to identify an enzyme indicator for environmental monitoring and a theoretical basis for the management and control of water eutrophication.

2. Materials and methods

2.1. Sample site description

Swan Lake (Fig. 1), also known as Yue Lake, is located in Rongcheng in Shandong at 122°33′0″–122°34′30″ E and 37°19′31″–37°22′30″ N. The accumulated sediment in the harbor forms an elliptical semi-closed lagoon, with an area of about 6 km^2 and an average water depth of 1–2 m. As a semi-closed lagoon, Swan Lake has only one shallow inlet that allows the exchange of lake water and coastal ocean water. As a result, lake water exchange is stifled, and the water quality has deteriorated quickly in recent years. In addition, pollutants flow into Swan Lake as a result of increased anthropogenic inputs, and algae such as *Cladophora oligoclona* and *Zostera marina* proliferate in the lake. The water in Swan Lake is in a eutrophic state with a total phosphorus content of 0.1 mg/L, a dissolved phosphorus content of 0.011 mg/L, and a chlorophyll content of 1.9 mg/L [11]. The averaged concentration of major anions and heavy metals in Swan Lake are described in Table 1.

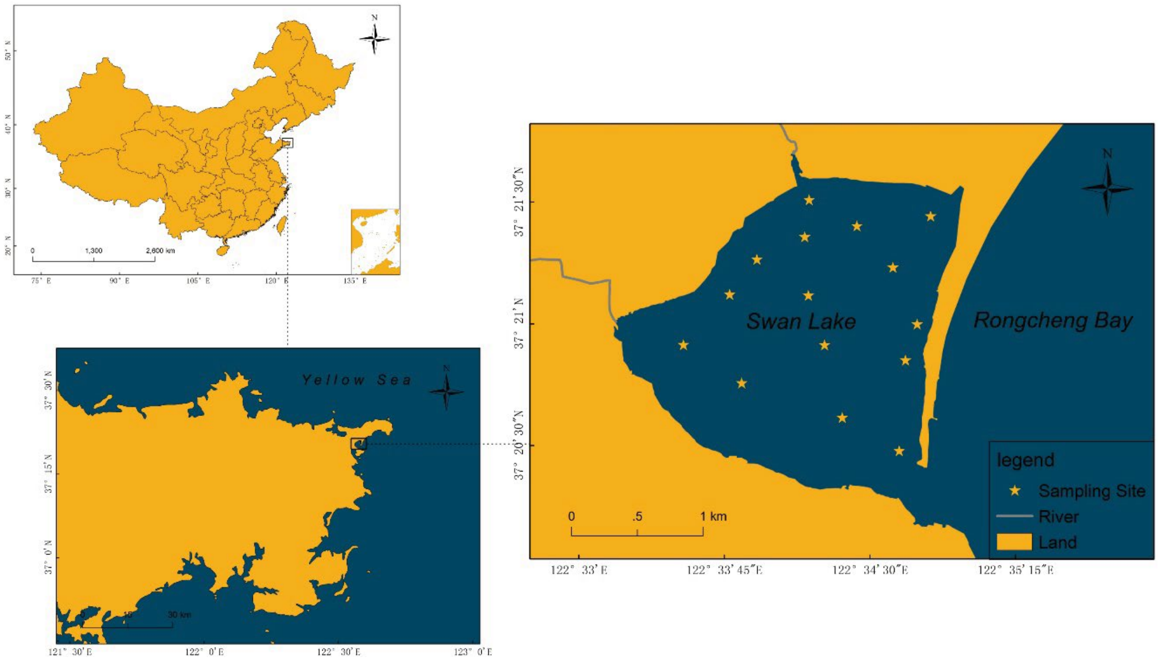

Fig. 1. Sample sites in the present study.

Table 1
Concentration means and standard deviation of major anions and heavy metals in water samples in Swan Lake

Element	Unit	Mean ± SD
Cl^-	g/L	18.72 ± 2.34
NO_3^-	mg/L	0.58 ± 0.04
SO_4^{2-}	g/L	0.93 ± 0.17
PO_4^{3-}	mg/L	0.11 ± 0.02
K^+	g/L	0.37 ± 0.03
Na^+	g/L	10.56 ± 1.65
Cu^{2+}	μg/L	2.54 ± 0.77
Pb^{2+}	μg/L	3.18 ± 0.57
Zn^{2+}	μg/L	26.9 ± 14.2
Cr^{6+}	μg/L	0.40 ± 0.26
Mo^{2+}	μg/L	6.72 ± 0.09
Ni^{2+}	μg/L	1.27 ± 0.05
Co^{2+}	μg/L	0.05 ± 0.01

2.2. Sample collection and experimental setup

Fifteen sample sites were evenly distributed throughout Swan Lake (Fig. 1). In May 2016, surface (0–50 cm) water samples were collected in 200 mL bottles using a plexiglass sampler at all 15 sample sites. After all of the water samples were collected from the 15 sample sites, they were mixed to form one sample, which was transferred to the laboratory and stored at 4°C.

The water in Swan Lake is affected by both wastewater and seawater because Swan Lake is located at the intersection of the land and ocean. Heavy metal ions tend to be present in seawater at concentrations in the pmol/L range; however, their concentrations are generally orders of magnitude higher in wastewater, on the order of tens of mmol/L. The total phosphorus concentration of seawater ranges from 1 to 10 μmol/L; while that of sewage effluent has been measured as 1–10 mmol/L [11]. As a result, 0.01–1 mmol/L of phosphorus-containing anions (phosphate (PO_4^{3-}), hexametaphosphate ($(PO_3)_6{}^{6-}$), pyrophosphate ($(P_2O_7)^{4-}$), and β-glycerophosphate ($C_3H_4O_6P^{2-}$)), and heavy metal ions (Cu^{2+}, Zn^{2+}, Ni^{2+}, Mn^{2+}, Co^{2+}, and Pb^{2+}) were added to the lake water samples, and the APA was measured after equilibrating for 24 h. In order to remove the contribution from the counter ions, the effects of Cl^-, NO_2^-, NO_3^-, SO_4^{2-}, K^+, and Na^+ on the APA were also investigated. The equilibrated water was passed through a 0.45 μm membrane before the APA and kinetic determinations.

2.3. Activity and kinetic analysis of alkaline phosphatase

The APA was determined using the disodium *p*-nitrophenyl phosphate (p-NPP) hydrolysis method described by Zhou et al. [12]. Briefly, 1 mL of 0.05 mol/L Tris-HCl buffer (pH 8.40) was added to 2 mL of sample water, and then, 2 mL of p-NPP (5 mmol/L) was added. The mixture was maintained at 30°C for 6 h, and then, 1 drop of 1 mol/L NaOH was added to terminate the reaction. Then, the concentration of p-nitrophenol was measured using a spectrophotometer at 410 nm to characterize the APA. To determine the kinetic properties of the AP, 0.05, 0.065, 0.1, 0.2, 0.5, 1.0, and 2.0 mmol/L of p-NPP were used to determine the APA in the water sample. Then, the kinetic parameters were calculated using the Michaelis–Menten equation [12].

All of the samples were run in triplicate, and the experimental data were expressed as the average of the three measurements. The experimental accuracy was determined using a 1.08 UN/mL (1 UN can hydrolyze 1 mmol of p-NPP) solution prepared from the standard (Sigma, P7640). The calibration result was 1.02 ± 0.006 UN/m^{l}. In order to ensure the precision of the analysis, if the coefficient of variation of the three replicates was >5%, the data were re-collected until the coefficient of variation was <5%.

2.4. Calculations and analysis

The APA value of the original water sample was set to 100. The APA values measured after adding the different anions and cations were converted into the relative enzyme activity (*B*) to allow for the comparison of the effects of the different anions and cations on the APA.

$$\text{Relative activity } (B) = \frac{M}{M_0} \times 100\%. \tag{1}$$

where M is the enzyme activity (μ mol/h) corresponding to the different concentrations of the metal ions; and M_0 is the corresponding enzyme activity (μ mol/h) when no metal ions have been added.

In the kinetic experiments, the maximum reaction rate ($V_{\max}$) and the Michaelis constant (K_m) of the reaction that AP underwent at different substrate concentrations were determined using the Michaelis–Menten equation. After re-arrangement, the Michaelis–Menten equation is as follows:

$$\frac{1}{V} = \frac{K_m}{V_{\max}} \times \frac{1}{[S]} + \frac{1}{V_{\max}} \tag{2}$$

where $V_{\max}$ is the maximum reaction rate (μ mol/L h); and K_m is the Michaelis constant (μ mol/L). S is the substrate concentration (mmol/L); and V is the reaction rate of the enzyme at this substrate concentration (μmol/L·h).

3. Results and discussion

In eutrophic water (e.g., Swan Lake), the bioavailable phosphorus content was generally lower in growth season than that in non-growth season. In order to satisfy the phosphorus requirements of the organism, the alkaline phosphatase was produced by phytoplankton, zooplankton and bacteria to convert organic phosphorus into bioavailable phosphorus [12]. The APA has been concluded to be related to the productivity of aquatic ecosystems [13]. APA is affected by the following environmental conditions: temperature, pH, UV-B radiation, and the presence or lack of activators and inhibitors. In this study, we present and discuss the regulation of the activity and kinetic properties of alkaline phosphatase in eutrophic water bodies by phosphorus and heavy metal ions.

3.1. Effects of different anions on the alkaline phosphatase activity

As can be seen from Fig. 2, the inorganic anions (Cl^-, NO_2^-, NO_3^-, and SO_4^{2-}) had almost no impact on the APA, even at high concentrations. This is consistent with the results of Xie et al. [9], who concluded that high concentrations of Cl^-, NO_2^-, NO_3^-, and SO_4^{2-} had no significant effect on APA in sludge. However, the phosphorus-containing ions (PO_4^{3-}, $(PO_3)_6^{6-}$, $P_2O_7^{4-}$, and $(C_3H_4O_6P)^{2-}$) exhibited strong inhibitory effects on the APA. For PO_4^{3-}, $(PO_3)_6^{6-}$, $P_2O_7^{4-}$, and $(C_3H_4O_6P)^{2-}$ concentrations of 1 mmol/L, the APA values reached only 49.2%, 44.2%, 47.6%, and 85.6% of the APA measured in the control, respectively. AP is an inducible enzyme, that is, an increase in phosphorus can inhibit the production and secretion of phosphatase, which is known as the product inhibition mechanism in the "inhibition-induction mechanism". Kuenzler and Perras [14] have reported that a variety of algae produce AP when they are phosphorus deficient, whereas the synthesis of these enzymes is inhibited when phosphorus levels are excessive. Gao et al. [2] designed a simulation of Taihu Lake, demonstrating that for a bioavailable phosphorus concentration of >6.25 μmol/L, the increase in the phosphate concentration substantially inhibited the APA, dramatically reducing it. Nausch [15] studied the relationship between phosphatase and the phosphate concentration and concluded that phosphatase activity is inhibited when the phosphorus concentration exceeds 0.2 μmol/L. Labry et al. [16] reported that phosphate concentrations less than 0.05 μmol/L induce the synthesis of phosphatase, and that APA is inhibited when the phosphate concentration exceeds 0.05 μmol/L. However, in other lakes, this inhibitory effect on the APA was not observed when the bioavailable phosphorus content was as high as 10 μmol/L [2]. This indicates that the APA threshold varies in different water bodies.

The degradation rates of numerous organic phosphorus compounds and polyphosphorus compounds by AP are lower than that of the substrate p-NPP in enzyme test reactions [17]. As a result, the addition of organic phosphorus and polyphosphate may lead to a decrease in the catalytic activity of the AP, thereby exhibiting an inhibitory effect on the AP. Bogé et al. [18] studied oligotrophic waters and

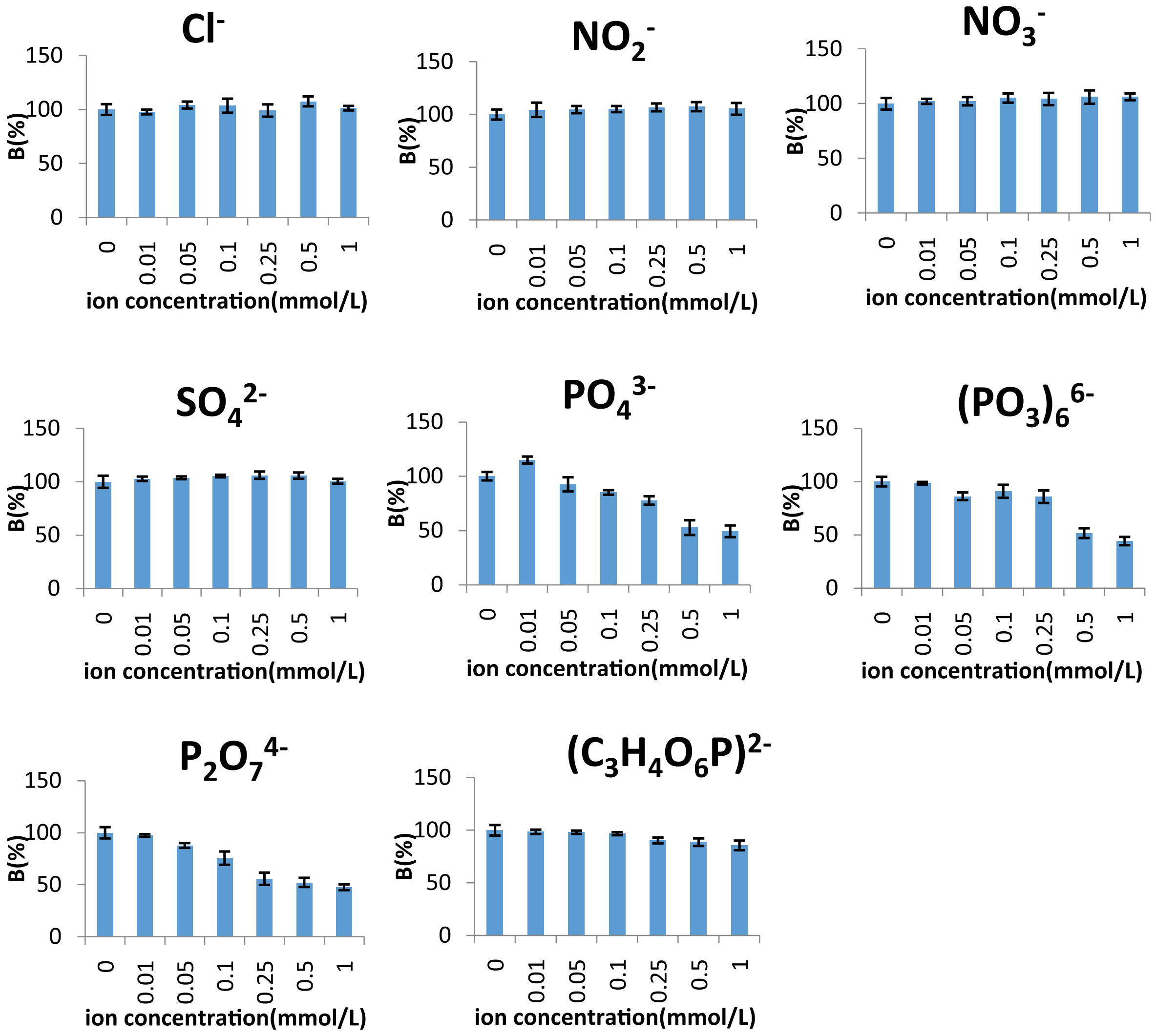

Fig. 2. Effects of different anions on the activity of alkaline phosphatase.

demonstrated that both dissolvable inorganic phosphorus and organic phosphorus control the APA. High concentrations of organic phosphorus have also been reported to increase the intercellular phosphate content and significantly inhibit APA [19]. $P_2O_7^{4-}$ exerts a strong inhibitory effect at high concentrations, most likely because it produces two phosphates during hydrolysis.

3.2. Effects of different heavy metals on alkaline phosphatase activity

As shown in Fig. 3, cations such as Na^+ and K^+ have no significant effect on the APA. In contrast, the effects of different heavy metals on APA can be divided into two categories: inhibition and activation effects.

The heavy metal ions Cu^{2+}, Zn^{2+}, and Ni^{2+} had inhibitory effects on the APA in water. Among these ions, Cu^{2+} exhibited a greater inhibitory effect than Zn^{2+} and Ni^{2+}. When the Cu^{2+} concentration reached 1 mmol/L, the APA was reduced to 62.3% of the control. The inhibitory effects of Cu^{2+} and Zn^{2+} on the APA in soil have been widely reported [20]. The inhibition mechanism involves the reaction of heavy metals with the enzyme–substrate mixtures or with active groups of enzyme proteins (e.g., reaction with the sulfhydryl group at catalytically active sites) to inactivate the enzyme activity [20]. The mode of inhibition depends on the type of substrate and the amount of heavy metal ions present [21,22]. In addition, heavy metals can reduce phosphatase synthesis by reducing the overall system's microbial activity [22]. Mn^{2+} and Co^{2+} can bind to the active sites in the AP to increase the APA [23]. In the present study, Pb^{2+} and Cr^{6+} exhibited strong stimulation effects on the APA. Xie et al. [9] reported that the addition of Pb^{2+} and Cr^{6+} increased the APA in activated sludge system. They concluded that when high concentrations of these toxic metal ions are added, the system tends to become more unstable, leading to an increase in the APA [9]. However, in soil, sediment or water system, Pb^{2+} and Cr^{6+} are assumed to have toxic effects on a large number of organisms. The toxic effects are founded to limit the growth of organism, then depress the production of AP from bacteria or algae [8]. The eutropic Swan Lake water is a mixture of different microorganisms, such as bacteria, zooplankton, fungi and algae. The interactions among metal ions, microorganisms and enzymes may complicate the effects of heavy metal ions on APA.

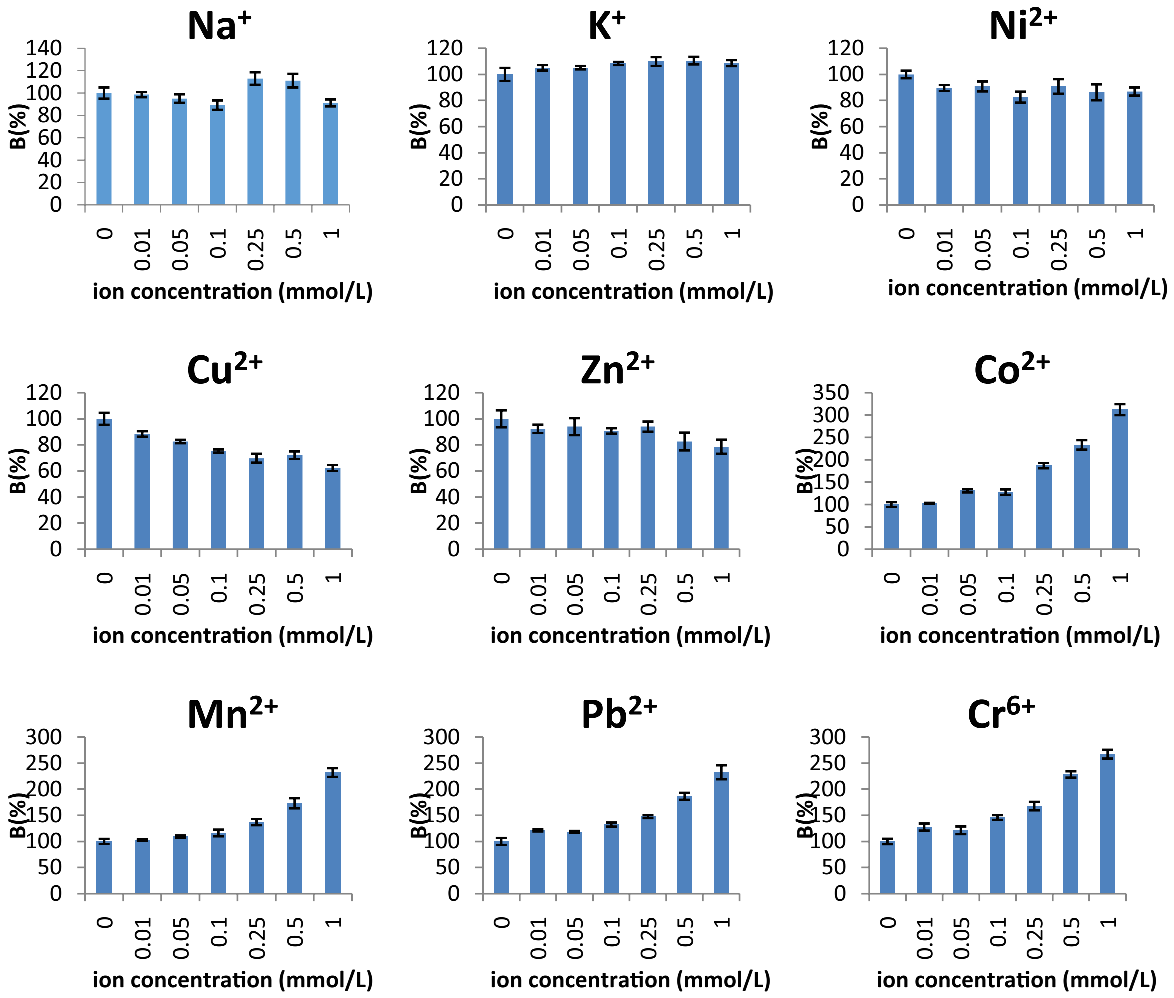

Fig. 3. Effects of different heavy metals on the activity of alkaline phosphatase.

3.3. Kinetics of alkaline phosphatase in the presence of different ions

The measurements of the Michaelis constants at different ion concentrations are shown in Fig. 4. The values of the Michaelis constants, K_m and V_{max}, are listed in Table 2. The kinetics of the AP was determined using the different reaction rates measured at different substrate concentrations. K_m and V_{max} are important parameters for studying enzymatic reactions. V_{max} represents the intrinsic catalytic rate of the enzyme and characterizes the maximum reactivity of the AP. The larger the value of V_{max}, the stronger the catalytic ability of the enzyme [24]. When no ions were added, the V_{max} of the AP in the Swan Lake water was 29.4 µmol/h. V_{max} decreased dramatically with increasing Cu^{2+} and $(C_3H_4O_6P)^{2-}$ concentration (0–0.5 mmol/L). Therefore, incorporation of Cu^{2+} and $(C_3H_4O_6P)^{2-}$ into water decreased efficiency of the AP catalytic process which was consistence with increasing K_m and decreasing V_{max}/K_m values. However, with increasing

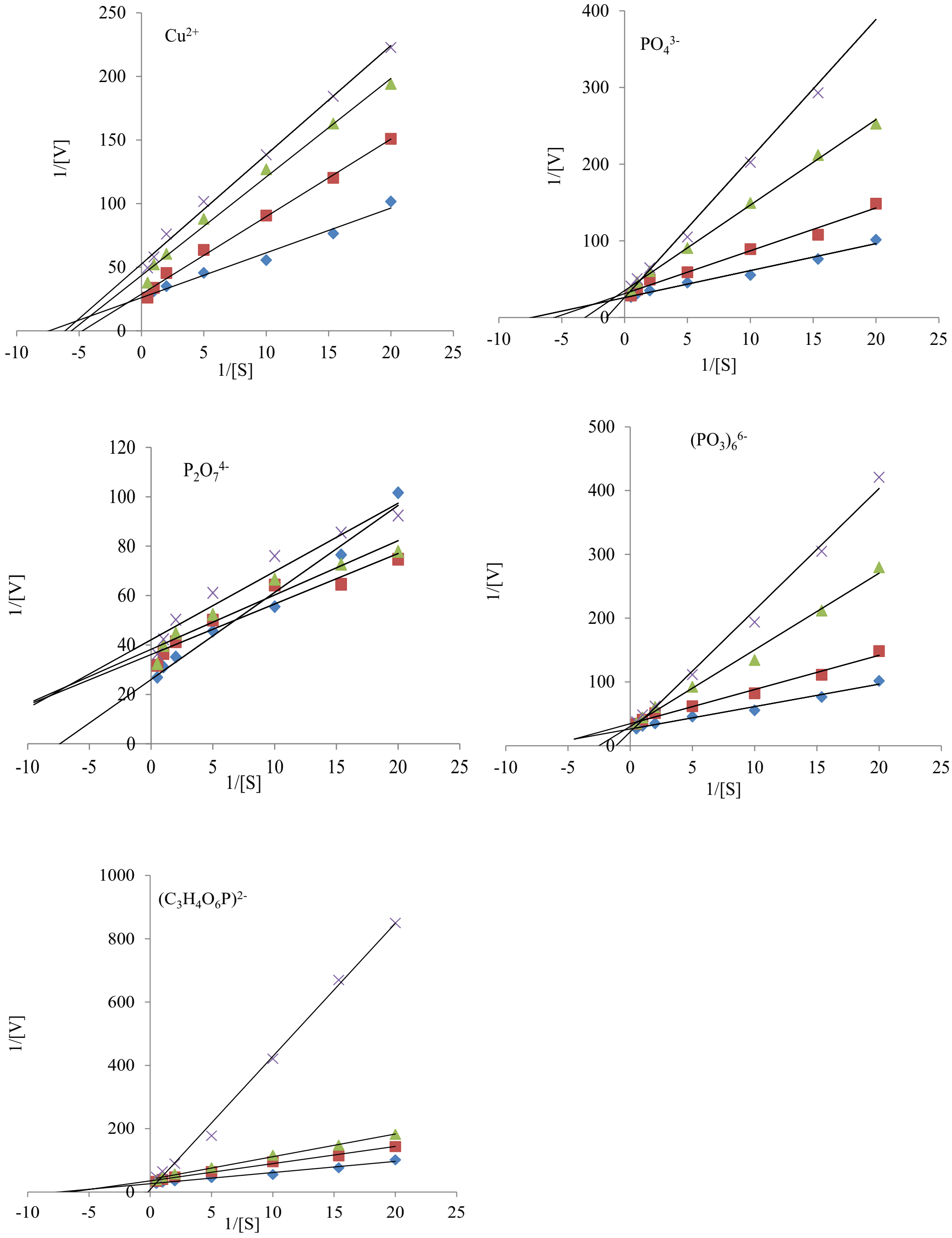

Fig. 4. Lineweaver–Burk plots of alkaline phosphatase activity at different ion concentrations. (◆0 mmol/L; ■0.1 mmol/L; ▲0.25 mmol/L; ×0.5 mmol/L).

Table 2
Effect of copper, phosphate and organic phosphates on the kinetic properties of alkaline phosphatase

	0 mmol/L			0.10 mmol/L			0.25 mmol/L			0.50 mmol/L		
	K_m	$V_{\max}$	$V_{\max}/K_m$	K_m	$V_{\max}$	$V_{\max}/K_m$	K_m	$V_{\max}$	$V_{\max}/K_m$	K_m	$V_{\max}$	$V_{\max}/K_m$
Cu^{2+}	45.3	29.4	0.65	210.9	34.6	0.16	177.8	23.0	0.13	172.3	18.7	0.11
PO_4^{3-}	45.3	29.4	0.65	166.1	31.0	0.19	301.5	26.9	0.09	692.7	38.2	0.06
$(PO_3)_6^{6-}$	45.3	29.4	0.65	89.9	33.6	0.37	102.1	35.9	0.35	73.9	39.4	0.53
$P_2O_7^4$	45.3	29.4	0.65	156.0	29.1	0.19	398.8	33.2	0.08	885.2	46.4	0.05
$(C_3H_4O_6P)^{2-}$	45.3	29.4	0.65	121.2	19.1	0.16	157.6	18.4	0.12	543.9	10.5	0.02

PO_4^{3-}, $(PO_3)_6^{6-}$, and $P_2O_7^{4-}$ concentration (0–0.5 mmol/L), the $V_{\max}$ values tend to increase which reflected the increasing of catalytic rate of AP. This phenomenon was opposite with the result of increasing K_m and decreasing $V_{\max}/K_m$ values which reflected the decreasing affinity and efficiency of the catalytic process. Up to now, we still have not reasonable explanation for this inconsistency results.

The Michaelis constant (K_m) can further indicate the abundance of phosphorus in the water. It represents the affinity of the enzyme for the substrate. A smaller K_m value indicates a greater affinity of the enzyme for the substrate; while a higher K_m value indicates a lower affinity of the enzyme for the substrate, that is, a low possibility of the formation of an enzyme–substrate complex [24]. The K_m value increased with the additions of Cu^{2+}, PO_4^{3-}, $(PO_3)_6^{6-}$, and $P_2O_7^{4-}$. As the concentration increased, the K_m value increased, indicating that the additions of Cu^{2+}, PO_4^{3-}, $(PO_3)_6^{6-}$, and $P_2O_7^{4-}$ reduced the affinity of the enzyme for the organic phosphorus, which is not conducive to the catalytic hydrolysis of organic phosphorus.

The $V_{\max}/K_m$ value usually indicates the catalytic efficiency of the AP [25]. As can be seen from Table 2, in the concentration range of 0–0.5 mmol/L, the $V_{\max}/K_m$ value decreased and the catalytic efficiency decreased with increasing concentrations of Cu^{2+}, PO_4^{3-}, $P_2O_7^{4-}$, $(PO_3)_6^{6-}$, and $C_3H_4O_6P^{2-}$. However, the causes of this decline may be different. Cu^{2+} ions can form inhibitor–enzymes and inhibitor–enzyme–substrate complexes in the active sites of the AP and the enzyme–substrate complexes [9], respectively, thus causing a decrease in the catalytic ability of the enzyme. As one of the products of phosphatase hydrolysis, PO_4^{3-} can exhibit competitive inhibition with phosphatase and cause a decrease in the catalytic ability of the enzyme. The rate of degradation of most of the polyphosphates and organic phosphorus by phosphatases is lower than that of *p*-NPP, resulting in the reduced catalytic efficiency of phosphatase [9].

4. Conclusions

In this paper, the effects of phosphorus and heavy metal ions on the APA and kinetic properties of AP were studied in the eutrophic waters of Swan Lake, China. Phosphate species (PO_4^{3-}, $(PO_3)_6^{6-}$, $P_2O_7^{4-}$, and $C_3H_4O_6P^{2-}$) were observed to inhibit APA, while heavy metals exhibited both inhibitory and catalyzing effects. Cu^{2+}, Zn^{2+} and Ni^{2+} significantly inhibited the APA, with Cu^{2+} showing the strongest effect. However, Mn^{2+}, Co^{2+}, Pb^{2+}, and Cr^{6+} significantly increased the APA. Among all parameters of $V_{\max}$, K_m and $V_{\max}/K_m$, the parameter of $V_{\max}/K_m$ showed a most effective indicator of catalytic performance of phosphatase. Increasing concentrations of Cu^{2+}, PO_4^{3-}, $(PO_3)_6^{6-}$, $P_2O_7^{4-}$, and $(C_3H_4O_6P)^{2-}$ were found to cause a decrease in $V_{\max}/K_m$, and therefore a decrease in catalytic efficiency.

The results of this paper are of great significance for understanding the mechanism of the eutrophication of water bodies and for the management and control of eutrophication. Future studies are needed to further elucidate the complex effects of phosphorus–heavy metal–organic pollutants on phosphatase activity and the kinetic properties of AP in water.

Acknowledgements

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