Effects of macro metals on alkaline phosphatase activity under conditions of sulfide accumulation

Guoqiang Zhao^{a,b}, Yanqing Sheng^{a,*}, Changyu Li^{a,b}, Qunqun Liu^{a,b}

^a Research Center for Coastal Environment Engineering Technology of Shandong Province, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai, China

^b University of Chinese Academy of Sciences, Beijing, China

HIGHLIGHTS

- Al^{3+} , Fe^{2+} , Ca^{2+} can inhibit alkaline phosphatase (AP) activities.
- Excess S^{2-} reduces AP activities by removing Zn^{2+} active sites.
- The inhibition of Al^{3+} and Fe^{2+} on AP activities can be alleviated by S^{2-} .
- Organic P monoester excluding recalcitrant P chelates can be hydrolyzed by AP.

GRAPHICAL ABSTRACT

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ABSTRACT

Alkaline phosphatase (AP) is commonly found in aquatic ecosystems as an extracellular enzyme closely related to the biogeochemical cycling of phosphorus. Although the AP activity (APA) is conventionally thought to be a main response to PO_4^{3-} starvation, significant effects of macro metal elements (Al, Fe, and Ca) and S on the APA were found in this study. The APA was reduced by Al primarily through the adsorption of the enzyme onto AlOOH colloids. Fe^{2+} inhibited the APA via a mechanism involving free radical oxidation. The main mechanism by which Ca^{2+} inhibited the APA was by competing with Mg^{2+} and Zn^{2+} for the active sites of the enzyme. Excessive S^{2-} could reduce the APA by removing Zn^{2+} from the active sites of the enzyme. The inhibition of APA could be reversed if some metal ions (e.g., Fe^{2+}) were precipitated by S^{2-} under reducing conditions. Therefore, in anaerobic ecosystems, the effects of macro metals on APA under conditions of sulfide accumulation may have innovative implications for phosphorus management.

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1. Introduction

Organic phosphorus (OP) can be hydrolyzed into PO_4^{3-} , which constitutes a large proportion of the total P in aquatic ecosystems

(Ahlgren et al., 2005). The hydrolysis of OP is closely related to its fraction and structure; OP hydrolysis has been widely studied by sequential extraction procedures and solution ^{31}P nuclear magnetic resonance spectroscopy (^{31}P NMR), as well as enzymatic hydrolysis experiments (Zhu et al., 2013). Generally, OP can be classified as readily labile OP (LOP), moderately labile OP (MLOP), and non-labile OP (NLOP) based on the fractionation method (Ivanoff et al., 1998). The species and classes of OP, which mainly comprise phosphonate, orthophosphate

* Corresponding author.

E-mail addresses: gqzhao@yic.ac.cn (G. Zhao), yqsheng@yic.ac.cn (Y. Sheng), cyli@yic.ac.cn (C. Li), qunqliu@yic.ac.cn (Q. Liu).

monoester (e.g., α - or β -glycerophosphate, mononucleotides, choline phosphate, inositol hexaphosphate, and glucose-6-phosphate), and orthophosphate diester (e.g., DNA, phospholipids, and RNA), can be characterized by ^{31}P NMR (W. Zhang et al., 2016). Alkaline phosphatase (AP, EC 3.1.3.1) is commonly found in aquatic ecosystems as an ectoenzyme and extracellular enzyme that is secreted by phytoplankton and bacteria (Dai et al., 2017). AP can hydrolyze labile orthophosphate monoesters into PO_4^{3-} and organic matter (Zhou et al., 2002). However, excess of PO_4^{3-} can rapidly inhibit the AP activity (APA) (Huang et al., 2018). Therefore, APA has been used as an indicator for the degree of eutrophication in aquatic ecosystems (X. Zhang et al., 2016). However, many factors, such as the amount of trace metals, suspended particulate matter, and pH, can significantly affect the APA (Labry et al., 2016; Tan et al., 2017; Tian et al., 2018; X. Zhang et al., 2016). Thus, the identification of factors that influence OP hydrolysis by AP is crucial for understanding the dynamics of phosphorus in aquatic ecosystems.

AP is a homodimeric, nonspecific phosphomonoesterase with different active sites (Takano et al., 2006). Each active site has three closely spaced metal-binding sites identified as M1, M2, and M3, and/or an additional non-catalytic binding site (M4) (Millan, 2006). Two zinc ions (Zn^{2+}) occupy the M1 and M2 sites, and a magnesium ion (Mg^{2+}) occupies the M3 site (Tang et al., 2019). The M4 is a peripheral binding site occupied by Ca^{2+} ; it is conserved in human and mouse APs (Le Du and Millan, 2002). However, the M4 site is not present in bacterial enzymes (Le Du et al., 2001; Mornet et al., 2001). Generally, AP derived from *Escherichia coli* has been selected as a research object rather than mammal-derived APs, because in natural ecosystems, APs are mainly secreted by microorganisms (Dai et al., 2017). AP-mediated catalysis requires M1 site occupancy and is greatly enhanced by M2 site occupancy (Hjorleifsson and Asgeirsson, 2016). The AP-catalyzed reaction shows a maximum rate at an alkaline pH (Holtz and Kantrowitz, 1999). Furthermore, sulfonamides can interact with the active sites in AP occupied by Zn^{2+} , resulting in the inhibition of APA (Salar et al., 2017). Arsenate can inhibit APA through its strong binding to AP and/or its structural similarity with PO_4^{3-} (Tian et al., 2018). AP can also be strongly adsorbed by goethite and montmorillonite, resulting in some active sites being occupied by organic matter and/or mineral ions (Zhu et al., 2016). Similarly, the inhibition of APA can also be achieved by Cd via the reduction of the enzyme's affinity to substrates, as well as the decrease of the maximum reaction velocity (Tan et al., 2018). Although the effects of some trace metal ions (Zn, Mn, Co, Ni, Cd, and As ions) on APA have been intensively studied (Brunel and Cathala, 1973), most studies have focused on APs secreted by large mammals. However, AP in aquatic ecosystems is mainly derived from phytoplankton and bacteria (Dai et al., 2017; Labry et al., 2016; Ragot et al., 2015). The molecular weight, structure, and activity of microbial APs are different from those of mammalian APs (Hoylaerts et al., 2015). In addition to trace metals, macro metals such as Al, Fe, and Ca may also affect the APA. However, there are only a few relevant studies on APs in aquatic ecosystems, especially with regards to the effects of these metals on the activity of microbial APs.

Sulfate reduction is an important biogeochemical process related to metal ion cycling and the toxicity of sulfides. Many trace metal ions and freely dissolved sulfides (referred to as S^{2-}) can be eliminated by precipitation processes (Jong and Parry, 2003). During anaerobic sulfate reduction, the accumulated S^{2-} would couple with Fe, promoting PO_4^{3-} release from Fe oxides and/or hydroxides (Kraal et al., 2017). Generally, S^{2-} is recognized as a biological toxin that poisons cells and nerves, and inhibits the activity of enzymes due to its acidity and by inducing oxidative stress (Chen et al., 2019; Zhou et al., 2018). However, most studies have merely focused on oxidases, peroxidases, and some enzymes related to gene expression in plants and animals, rather than microbial APs (Corpas et al., 2019; Nicholls et al., 2013; Thompson et al., 2003). Although Zavaczki et al. have reported the toxicity of S^{2-} towards APA, the damage caused by S^{2-} to the active sites of AP is still unclear (Zavaczki et al., 2011). Therefore, understanding the effects of S^{2-} and major metal ions on the activity of microbial APs may provide

significant insights into the mechanism underlying OP hydrolysis during hypoxic conditions.

The objectives of this study were to: (1) investigate the effects of APA gradients on the hydrolysis efficiency of OP; (2) explore the mechanism underlying the effects of the macro metal elements Al, Fe, and Ca on the APA; and (3) study the inhibition and/or recovery of APA under conditions of sulfate reduction. This research will provide new insights into the regulation of the APA in anaerobic ecosystems under conditions of sulfate reduction.

2. Materials and methods

2.1. Sediment sampling and chemicals

The surface sediments (0–10 cm) were collected from the Jiehe River (JH), a heavily polluted river with abundant OP and metal ion levels in the sediments (Zhao et al., 2019). The black and foul-smelling sediments were collected with a Van Veen grab and the samples were immediately (in ~1 min) stored in plastic zip lock bags (flushed with nitrogen prior to use), kept cold using ice packs, and transported to the laboratory within 12 h, where they were frozen in a refrigerator at $-20\text{ }^{\circ}\text{C}$ until analysis. The sediments were lyophilized and homogenized prior to analysis. Details regarding the location of sample collection (JH5) and the sediment properties are illustrated in Fig. S1 and Table S1. The AP from *Escherichia coli* (Sigma P5931) and standard substances used for obtaining the ^{31}P NMR spectra were purchased from Sigma-Aldrich.

2.2. Enzymatic hydrolysis experiment

2.2.1. Hydrolysis of OP and inactivation of AP

To compare the effects of AP on the hydrolysis of OP, four sets of slurries were maintained in parallel; four of the 4.0-g portions of the lyophilized sediment samples, which were passed through a 100-mesh sieve, were added into 200 mL of 0 unit (U) mL^{-1} , 0.2 U mL^{-1} , 2 U mL^{-1} , and 20 U mL^{-1} AP solutions. The AP solutions were prepared in Tris-HCl buffer (0.1 M, pH 8). All slurries were incubated at $28\text{--}30\text{ }^{\circ}\text{C}$ in duplicate for 24 h. At specific time points (0, 2, 4, 6, 4, 6, 10, 16, and 24 h), 2.0 mL of each mixture was sampled and immediately centrifuged (4000 rpm, 15 min). Approximately 1 mL of the supernatants was analyzed for PO_4^{3-} production using the molybdenum blue method. The residual supernatants were analyzed for APA by a spectrophotometric method (Sayler et al., 1979). Detailed information regarding these procedures is provided in the supporting information.

All enzymatic hydrolysis experiments focused on the enzymatic response to actual environmental conditions; thus, the incubation conditions were kept as close as possible to the natural conditions (pH 8, temperature $28\text{--}30\text{ }^{\circ}\text{C}$).

2.2.2. Sequential extraction of OP and ^{31}P NMR determination

At the end of the enzymatic hydrolysis experiment, the remainder of the slurry in each reaction system was lyophilized to obtain the dry sediments that had been hydrolyzed by AP. The OP in the lyophilized sediments was analyzed using the optimized Ivanoff method and ^{31}P NMR (Zhao et al., 2019). Detailed information regarding the procedures of the Ivanoff method and ^{31}P NMR analyses is provided in the supporting information.

2.3. Enzymatic activity experiment

2.3.1. Determination of the interference of APA by metal ions and sulfide

To understand the interaction of AP with macro metal ions (Al, Fe, and Ca ions) in aquatic ecosystems under conditions of sulfate reduction, batches of enzymatic activity experiments were conducted in parallel. Five groups comprising samples in 10-mL reaction tubes were set; AP solutions (0.1 M Tris-HCl, pH 8.0) were added into each tube in

parallel (to obtain a final concentration of 1 U mL^{-1}). Then, Al^{3+} , Fe^{2+} , Ca^{2+} , SO_4^{2-} , and S^{2-} -containing solutions were mixed with the samples from the five groups. In each group, the concentration gradient of each ion (Al^{3+} , Fe^{2+} , Ca^{2+} , SO_4^{2-} , and S^{2-}) was set to 1, 3, and 6 mM. Four sets of experiments were performed in 10-mL reaction tubes to compare the APA changes after the reaction of the free dissolved sulfides with the metal ions (Al^{3+} , Fe^{2+} , and Ca^{2+}). First, 1 mL of 10 mM S^{2-} solution, followed by 1 mL of 10 mM Al^{3+} , Fe^{2+} , and Ca^{2+} solutions, were added into each tube in parallel. Then, 7 mL of 0.1 M Tris-HCl buffer solution (pH 8) was added into each tube to attain final Al^{3+} , Fe^{2+} , Ca^{2+} , and S^{2-} concentrations of 1 mM, and the reaction was initiated by adding 1 mL of 10 U mL^{-1} AP solution into each tube, to attain a final enzyme concentration of 1 U mL^{-1} . A control experiment was conducted in parallel with the addition of 1 U mL^{-1} AP.

The AP, AlCl_3 , FeCl_2 , CaCl_2 , Na_2SO_4 , and Na_2S solutions were prepared in 0.1 M Tris-HCl buffer with a final pH of 8. Milli-Q water was purged with nitrogen prior to use. To maintain a reducing environment in the reaction tubes, the Tris-HCl buffer was mixed with 10% (wt%) ascorbic acid solution at a ratio of 50:1 (v/v). All samples were incubated at $28\text{--}30^\circ\text{C}$ for 24 h in a sealed bag filled with nitrogen. After incubation, 1 mL of the supernatants from each reaction mixture was sampled for the determination of the APA.

2.3.2. Identification of metal sulfide products

In the presence of dissolved sulfides, the metal ions (Al^{3+} , Fe^{2+} , Ca^{2+}) may precipitate as Al-S , Fe-S , and Ca-S , respectively. To study the mechanism underlying the removal of metal ions from the reaction system by dissolved sulfides, the precipitates were collected by filtering the samples through a $0.45\text{-}\mu\text{m}$ membrane (Pall®, GFF) at the end of the experiment, rinsed with ultrapure water, and lyophilized for further identification using scanning electron microscopy (SEM), energy dispersive X-ray spectrometry (EDS), and X-ray diffraction (XRD). Although there was no precipitation in the tube containing Ca^{2+} following the addition of S^{2-} , the clear solution was filtered through the $0.45\text{-}\mu\text{m}$ membrane and the Ca^{2+} levels were determined. Control experiments without S^{2-} addition were also performed in parallel.

To study the effect of S^{2-} on aluminum colloids, the particle sizes of the precipitates formed in the AlCl_3 solution-containing sample with and/or without the addition of Na_2S were investigated. The Na_2S solution was added into the AlCl_3 solution to yield final AlCl_3 and Na_2S concentrations of 10 mM. A control was prepared without Na_2S addition, where only the particle size of the precipitates was determined and the other conditions were kept constant throughout the experiment. The Na_2S and AlCl_3 solutions were prepared with Milli-Q water. The final pH of the Na_2S and AlCl_3 solutions was adjusted to 8.0 using NaOH. Subsequently, the particle size of the precipitate in the supernatant was analyzed by using a laser particle-size analyzer (Malvern 2000F, Malvern, UK) after the centrifugation of the supernatant at 4000 rpm for 15 min.

2.3.3. Enzyme deactivation by Na_2S

To investigate the mechanism underlying the deactivation of AP by dissolved sulfides, AP solution was added into 10-mL tubes to attain a final concentration of 5 U mL^{-1} . Then, Na_2S solution was added into these tubes to attain a final S^{2-} concentration of 10 mM. Control experiments without Na_2S addition were also performed in parallel. After incubation at $28\text{--}30^\circ\text{C}$ for 24 h, the solution in each tube was centrifuged at 13,000 rpm for 15 min and then filtered through a $0.45\text{-}\mu\text{m}$ membrane. Concentrations of Zn^{2+} and Mg^{2+} in the filtered solutions were measured using inductively coupled plasma-mass spectrometry (ICP-MS, PerkinElmer ELAN DRC II, US).

2.4. Extraction of sediments using Na_2S

The sediments were extracted with different concentrations of Na_2S to investigate whether the APA can be reactivated after the metal ions

were removed by free dissolved sulfides. Seven sets of Na_2S concentration gradient experiments were established in parallel: 4.0 g of the sediments were extracted with 200 mL of Na_2S solutions (0 mM, 5 mM, 10 mM, 30 mM, 60 mM, 100 mM, and 200 mM); the S^{2-} contents corresponded to 0, 1, 2, 6, 12, 20, and 40 mmol, respectively. The slurries were incubated at $28\text{--}30^\circ\text{C}$ for 24 h with slight shaking (180 rpm). The control sample without Na_2S (0 mM) was extracted using the Tris-HCl buffer. At the end of the experiment, 2 mL of slurry from each conical flask was centrifuged at 4000 rpm for 15 min. The amounts of Al^{3+} , Fe^{2+} , and Ca^{2+} in the supernatants were analyzed inductively coupled plasma optical emission spectroscopy (ICP-OES) to characterize the removal efficiency of the metal ions by the dissolved sulfides.

The remaining portions of the slurries were lyophilized to obtain the dry sediments that had been immobilized by Na_2S . Then, 4.0 g of the lyophilized sediments were added into seven 200-mL aliquots of AP solution (1 U mL^{-1} , pH 8), which was prepared using the Tris-HCl buffer. Then, the seven slurries were incubated at $28\text{--}30^\circ\text{C}$ for 24 h. Data regarding the APA from the seven reaction systems were collected at 0, 2, 4, 6, 10, 16, and 24 h after the reaction to study the effects of sulfate reduction on the APA under reducing conditions.

2.5. Simulation of reaction equilibria by visual MINTEQ

The thermodynamic states of Al^{3+} , Fe^{2+} , and Ca^{2+} with and/or without the addition of S^{2-} at a pH of 8 and $28\text{--}30^\circ\text{C}$ were simulated by using the software Visual MINTEQ 3.0. As per the simulation conditions, the pH and temperature were fixed. The thermodynamic state of each ion in the solution was calculated as the solubility product (Ksp), ion activity product (IAP), and saturation index (SI), according to the Visual MINTEQ software (Liu et al., 2018). The following criteria were used: $\text{SI} < 0$, no precipitate; $\text{SI} = 0$, equilibrium; and $\text{SI} > 0$, generating precipitates (Migaszewski et al., 2018).

$$\text{SI} = \log \frac{\text{IAP}}{K_{\text{sp}}} \quad (1)$$

2.6. Statistical analysis

Significance of data were analyzed using one-way analysis of variance (ANOVA) followed by means testing between treated groups and the control and/or between different treated groups by using SPSS 20.0 (IBM SPSS Inc.). All significance levels mentioned in the text are $p < 0.05$.

3. Results

3.1. Release of PO_4^{3-} and OP hydrolysis

Effects of APA gradients on the hydrolysis of OP in the sediments are shown in Fig. 1. Approximately 2 min after the hydrolysis experiment, the initial concentrations of the PO_4^{3-} released in the slurry following the addition of 0, 0.2, 2, and 20 U mL^{-1} of AP were 0.59, 0.62, 0.70, and 0.70 mg L^{-1} , respectively. Compared to the control sample, the initial PO_4^{3-} concentration in the slurry following AP addition was significantly higher ($p < 0.05$). Additionally, the PO_4^{3-} -release rates in all samples abruptly increased within the first 2 h, and then showed a slower increase until the 16-h time point. After 16 h, the concentration of PO_4^{3-} -P following AP addition almost plateaued, while it still showed a slight increase in the control sample (from 2.1 mg L^{-1} to the final 2.2 mg L^{-1}). At the end of the experiment, the average amounts of PO_4^{3-} in the slurries containing 0.2, 2, and 20 U mL^{-1} of AP were approximately 2.5, 2.6, and 2.7 mg L^{-1} , respectively; they were much higher than the PO_4^{3-} concentration in the control sample (2.2 mg L^{-1}).

The trend of APA variation with time during the OP hydrolysis experiment is presented in Fig. 2. The initial APA in the four centrifuged

Fig. 1. Concentration of $\text{PO}_4^{3-}\text{-P}$ released from sediments during the hydrolysis by alkaline phosphatase (AP). Each value interval represents the average $\pm$ standard deviation ($n = 3$). Four parallel 4.0-g lyophilized sediments were extracted by 200 mL of 0, 0.2, 2 U and 20 U mL^{-1} AP solutions for 24 h at 28–30 °C.

slurries containing 0, 0.2, 2, and 20 U mL^{-1} of AP was 0.27, 410, 6100, and 15,000 $\text{mg L}^{-1} \text{h}^{-1}$, respectively. The APA in the control sample (without AP addition) remained constant throughout the experiment. In contrast, the APA in the samples containing AP dropped significantly from 0 to 4 h. The APA fluctuated within a narrow range after 16 h in the tubes containing 0.2 and 2 U mL^{-1} of AP. However, in the tube containing 20 U mL^{-1} of AP, the APA reached an equilibrium after 4 h. At the end of the experiment, the final APA in the slurries containing 0, 0.2, 2, and 20 U mL^{-1} of AP was 0.27, 20, 4300, and 14,850 $\text{mg L}^{-1} \text{h}^{-1}$, respectively.

The changes of OP speciation in the hydrolyzed sediments following treatment with AP are shown in Table S2. The concentration of $\text{NaHCO}_3\text{-OP}$ (classified as labile OP) in the sediment not subjected to hydrolysis by AP was 42 mg kg^{-1} , while it dropped down to 21, 4.2, and 4.2 mg kg^{-1} in the sediments hydrolyzed by the addition of 0.2, 2, and 20 U mL^{-1} of AP, respectively. Additionally, the concentrations of both HCl-OP and Flv-OP, which were classified as moderately labile OP, in the hydrolyzed sediments decreased significantly ($p < 0.01$). For HCl-OP and Flv-OP, the hydrolysis rates were 16% and 11%, respectively, for 0.2 U mL^{-1} of AP, 33% and 17%, respectively, for 2 U mL^{-1} of AP,

and 67% and 44%, respectively, for 20 U mL^{-1} of AP. However, there was no significant drop in the concentrations of Hum-OP and $\text{H}_2\text{SO}_4\text{-OP}$, which were classified as non-labile OP, in the hydrolyzed sediments ($p > 0.1$).

The recovery of P in the NaOH-EDTA extracts is shown in Table S3. The extraction efficiencies of both total P and OP with NaOH-EDTA were higher than 80% in the sediment, which indicated that most of the P compounds had been successfully extracted from the sediments into the supernatants for NMR analysis. The ^{31}P NMR spectra and integral results of P compounds are illustrated in Fig. 3 and Table S4, respectively. The NMR results illustrated that the contents of phosphonate, myo-IHP, and DNA did not significantly decrease after incubation with 2 U mL^{-1} of AP, compared to the control sample. However, the $\beta\text{-Gly}$ and PC contents dropped from 1.62% and 0.42% to 1.02% and 0.35%, respectively, after the hydrolysis with 2 U mL^{-1} of AP.

3.2. Inhibition and reactivation of the APA

The results showed that Al^{3+} , Fe^{2+} , Ca^{2+} , SO_4^{2-} , and S^{2-} could inhibit the APA, compared to the case for the control sample (Fig. 4). Among these ions, Fe^{2+} and S^{2-} had strong inhibitory effects on the APA, while SO_4^{2-} had a slight effect on the APA. The APA was reduced from $\sim 2400 \text{ mg L}^{-1} \text{h}^{-1}$ in the control to $\sim 200 \text{ mg L}^{-1} \text{h}^{-1}$ and $\sim 210 \text{ mg L}^{-1} \text{h}^{-1}$ in the samples containing 1 mM of Fe^{2+} and S^{2-} , respectively. The addition of S^{2-} ameliorated the inhibition of APA by Fe^{2+} and Al^{3+} . The inhibited APA in the sample containing 1 mM of Fe^{2+} and Al^{3+} rose from $\sim 200 \text{ mg L}^{-1} \text{h}^{-1}$ and 1500 $\text{mg L}^{-1} \text{h}^{-1}$ to $\sim 560 \text{ mg L}^{-1} \text{h}^{-1}$ and 2000 $\text{mg L}^{-1} \text{h}^{-1}$, respectively, after the addition of 1 mM S^{2-} . The inhibition of APA by Ca^{2+} could not be reversed, but was instead, more pronounced after the addition of S^{2-} .

The characterization of the products of the reaction of Al^{3+} , Fe^{2+} , and Ca^{2+} with S^{2-} is presented in Fig. 5. The results depicted in Fig. 5A show that the precipitate from the sample in which Al^{3+} and S^{2-} were added was faint yellow. It was primarily composed of C, O, and Al, with molar ratios of 13%, 70%, and 16%, respectively. The percentage of S was low (only 0.22%). The precipitate in the sample containing Al was in a weak crystallinity phase, with a uniform and compact surface (Fig. 5B and E). The XRD spectrum confirmed that the precipitate was $\text{Al}(\text{OH})_3$ with AlOOH phases. In comparison, the product of the reaction between Fe^{2+} and S^{2-} in the enzymatic activity experiment was black, with a compact and striated surface. The main elements in the black precipitate were C, O, Fe, and S, with molar percentages of approximately 23%, 36%, 17%, and 20%, respectively (Fig. 5D). This

Fig. 2. The variation trend of alkaline phosphatase activity (APA) with time in the mixtures of sediments and alkaline phosphatase. Each value interval represents the average $\pm$ standard deviation ($n = 3$). Four parallel 4.0 g of lyophilized sediment was extracted by 200 mL of 0, 0.2, 2 and 20 U mL^{-1} alkaline phosphatase solutions, respectively, for 24 h at 28–30 °C.

Fig. 3. ^{31}P NMR spectra of enzymatic hydrolyzed sediment. (A) Full spectra were initially processed with 10 Hz line broadening (LB). (B) (C) (D) The region of orthophosphate monoester was subsequently processed with 1 Hz LB. *myo*-IHP: *myo*-inositol hexaphosphate; α -Gly: DL- α -glycerol phosphate; β -Gly: β -glycerophosphate; G6P: glucose 6-phosphate; AM: adenosine 5' monophosphate; PC: phosphocholine; G1P: glucose-1-phosphate.

precipitate was in an amorphous phase (Fig. 5F) after the addition of organic matter (Tris and AP). Thus, even without the addition of organic matter, precipitates could still be generated in the samples containing Fe and Al. The product of the reaction between Al and S was mainly composed of AlOOH (Fig. S2), while the precipitate generated from the reaction between Fe and S was likely FeS₂ and FeO(OH) (Fig. S3). A detailed description of the identification of the precipitates generated without the addition of organic matter is provided in the *supporting information*. The content of Ca²⁺ in the filtrate remained constant after Na₂S addition, compared to the control sample (Fig. 5G).

As shown in Fig. 5, AlOOH colloids were probably generated in the sample containing Al³⁺. Additionally, the addition of S²⁻ may affect the formation of AlOOH colloids. Therefore, the particle size of precipitates formed in the sample containing Al³⁺ in the presence and/or absence of S²⁻ was analyzed using a laser particle analyzer. The results

of the particle size determination are shown in Fig. 6. The particle size of the precipitates in the supernatant of the AlCl₃-containing sample formed following Na₂S addition was smaller, and the particle size range of these precipitates was higher than that of the precipitates formed in the absence of Na₂S (Fig. 6A). The particle sizes of all the precipitates were >200 nm in the supernatant containing Na₂S, while those of the precipitates formed in the samples not containing Na₂S after centrifugation were <100 nm (the scope of a colloid) (Fig. 6B).

The contents of Zn²⁺ and Mg²⁺ in AP were determined after the extraction using Na₂S to study whether the addition of S²⁻ could precipitate the Zn²⁺ and Mg²⁺ present in the active sites of AP. The concentrations of Zn²⁺ and Mg²⁺ in the active sites of AP in the presence and/or absence of Na₂S are shown in Fig. 7. The results showed that the contents of intrinsic Zn²⁺ and Mg²⁺ in the AP were 4.5 $\mu\text{mol L}^{-1}$ and 1.5 $\mu\text{mol L}^{-1}$, respectively, without the Na₂S extraction

Fig. 3 (continued).

process. After the extraction with S^{2-} , the Zn^{2+} content dropped to $1.5 \mu\text{mol L}^{-1}$ (67% removal). However, the Mg^{2+} concentration remained constant before and after the extraction, showing that Na_2S addition had almost no influence on the Mg^{2+} content.

3.3. Metal ion immobilization and APA changes

Concentrations of Fe, Al, and Ca released from the sediments during the extraction process using Na_2S are shown in Fig. 8A. The concentration of Al in the supernatant significantly decreased as the content of S^{2-} increased. The immobilization efficiency of Al was 88% when the S^{2-} concentration was 100 mM; however, it only increased by 8%

when the S^{2-} concentration was 200 mM, compared to the case when 100 mM S^{2-} was present. The amount of Fe in the supernatant presented a peak when the concentration of S^{2-} was 10 mM. The amount of Fe was greater in the presence of Na_2S than that in the absence of Na_2S when the concentration of S^{2-} was $<30 \text{ mM}$. During the whole extraction process, the Al concentration decreased as the S^{2-} concentration increased, while the Ca concentration remained within a narrow range, with the average concentration being $11.3 \pm 0.38 \text{ mg L}^{-1}$.

After the extraction, the slurries were lyophilized to obtain dry materials immobilized by S^{2-} . Pictures of the lyophilized materials are presented in Fig. S4. The immobilized sediments were resuspended in a solution of 1 U mL^{-1} of AP to investigate the changes in the APA. The

Fig. 4. Inhibition of the alkaline phosphatase activity (APA) by adding 1, 3, and 6 mM of Al^{3+} , Fe^{2+} , Ca^{2+} , SO_4^{2-} , and S^{2-} , respectively. Each value interval represents the average $\pm$ standard deviation ($n = 3$). Reactivation of the inhibitory APA by adding S^{2-} into the tubes contained 1 mM of Al^{3+} , Fe^{2+} , Ca^{2+} , and SO_4^{2-} , respectively. Control was the activity of 1 U mL⁻¹ of AP without any related ions addition. The activity of AP added in all experimental groups was also 1 U mL⁻¹ to be consistent with the control. The four parallel 1 mM of S^{2-} were added into the tubes with the presence of 1 mM of Al^{3+} , Fe^{2+} , Ca^{2+} and SO_4^{2-} , respectively.

trend of APA variation with time in the resuspended mixtures is illustrated in Fig. 8B. The results showed that the initial APA in each supernatant from the resuspended slurries was different. The initial APA gradually increased as the S^{2-} concentration increased (5 to 100 mM), while it dropped when S^{2-} reached a concentration of 200 mM. Moreover, the initial APA in the sample without Na_2S was higher than that of the immobilized slurries as the S^{2-} concentration dropped below 30 mM. Additionally, the APA in the resuspended sediments significantly decreased in the first 6 h; the APA decreased at differing rates. Generally, the largest decrease was seen in case of S^{2-} concentrations below 60 mM. The decrease was clearly slowed when the S^{2-} concentration increased to 100 mM, but accelerated again as the S^{2-} concentration reached 200 mM. After 6 h, the APA in nearly all the samples was stable within a narrow range. The final APA in the seven slurries showed the following order: 100 mM S^{2-} > 200 mM S^{2-} > 60 mM S^{2-} > 0 mM S^{2-} > 30 mM S^{2-} > 10 mM S^{2-} > 5 mM S^{2-} . The contents of Fe, Al, and Ca in the system were

approximately 2.1, 12.2, and 0.8 mmol, as calculated from the metal contents described in Table S1.

3.4. Thermodynamic analysis

The concentration and state of metal ions in the presence and/or absence of S^{2-} addition were calculated using the Visual MINTEQ software. As shown in Table S5, 99% of the Al in the solution was precipitated as diasporite ($\text{Al}(\text{OH})_3$). Most of the Fe was present in the dissolved state (Fe^{2+} , 97%); only 3% was present as $\text{Fe}(\text{OH})_2$. The Ca content was stable, because 100% of the Ca was still in the form of Ca^{2+} , showing no precipitation. The proportion and form of Al and Ca were stable after S^{2-} addition. All the added S^{2-} was present in the form of S^{2-} in the two samples containing Al and Ca. However, 99% of the Fe^{2+} was removed from the solution as mackinawite (FeS) after S^{2-} addition. The concentrations of both Fe^{2+} and S^{2-} reduced significantly, down to ~0.3%.

Fig. 5. Picture of precipitate of Al mixed with S and its SEM image (A), atomic composition by EDS (B), and XRD pattern (E). Figures of C, D, and F were the corresponding results on the products of Fe reacted with S. Changes in Ca contents of the filtered solution after the Ca and S reaction. Each value interval represents the average $\pm$ standard deviation ($n = 3$) (G).

Fig. 6. Particle size of the precipitates in AlCl_3 solution with and/or without Na_2S addition after the centrifugation. (A) The overall distribution of particle size, (B) The distribution of particle sizes within the colloid range. The solutions of Na_2S and AlCl_3 were prepared with Milli-Q water. The final pH of Na_2S and AlCl_3 solutions were adjusted to 8.0 with NaOH. The solution of Na_2S was added into the AlCl_3 solution to yield both in final concentrations of 10 mM. A control was prepared with 10 mM AlCl_3 without Na_2S , and other conditions remained constant throughout the experiment.

4. Discussion

4.1. Enzymatic hydrolysis and activity

AP is a nonspecific phosphomonoesterase that can hydrolyze phosphate monoesters into PO_4^{3-} and organic matter. Therefore, both the concentration and release efficiency of PO_4^{3-} in slurries containing AP were higher than those in the control sample, indicating that AP enhanced the PO_4^{3-} release. Additionally, the content of the PO_4^{3-} in the slurries treated with AP was greater than that in the control at the first time point (about 2 min), which indicated that the hydrolysis of OP by AP was rapid. A small amount of the OP that was adsorbed by the sediments was released into the AP solution during the mixing process. Then, the released OP could be rapidly decomposed into PO_4^{3-} and organic matter when it came in contact with the AP. The activity of AP gradually decreased with AP addition during the experiment; this explains why the rates of PO_4^{3-} release dropped significantly after 2 h. Comparison of the hydrolysis efficiency of various fractions of OP (Table S2) revealed that $\text{NaHCO}_3\text{-OP}$ was the most easily degraded by AP, followed by HCl-OP and Flv-OP . Compared to the control sample, the extra PO_4^{3-} in the slurries containing AP was mainly produced by the hydrolysis of $\text{NaHCO}_3\text{-OP}$, HCl-OP , and Flv-OP . Most of the NLOP (Hum-OP and the $\text{H}_2\text{SO}_4\text{-OP}$) could not be hydrolyzed into PO_4^{3-} by AP due to its stable structure (Lü et al., 2016). Because NLOP was mainly converted from the free labile OP associated with humic acid and/or metal ions (Al^{3+} , Fe^{3+} , Fe^{2+} , and Ca^{2+}), it was recalcitrant to hydrolysis

by AP (Zhu et al., 2015). Al, Fe, and Ca ions were present in high concentrations in the sediment (Table S1). Therefore, most of the NLOP may be strongly bound to the metal ions; this may explain why it is difficult to hydrolyze. As shown in Table S4, the DNA and *myo*-IHP could not be decomposed by AP because their hydrolysis requires phosphodiesterase and phytase, respectively (Shinohara et al., 2012; Turner et al., 2012). Although *myo*-IHP is an orthophosphate monoester, it was not decomposed by AP because it reacted with metal ions to form recalcitrant complexes (Turner et al., 2002). The results of the ^{31}P NMR studies showed that most of the remaining $\beta\text{-Gly}$ and PC could not be hydrolyzed by AP, which probably indicates that these P compounds had interacted with metal ions and/or humic acid, which made them resistant to enzymatic hydrolysis (Turner et al., 2012; Zhu et al., 2015). The phosphonate could not be hydrolyzed by AP due to the C—P bond being more stable than the O—P bond (Luo et al., 2011). Specific phosphatases, rather than AP, would be required to break it down. Therefore, the OP species associated strongly with metal ions and/or humic acid, and DNA, *myo*-IHP, and phosphonate probably belong to the NLOP category.

As shown in Fig. 2, the activity of AP in each slurry gradually decreased during the experiments. This is partly because the gradually increasing PO_4^{3-} concentration in the AP solution can inhibit the APA (Huang et al., 2018). Another important reason for this is the immobilization of AP by the sediments. A previous report has suggested that when absorbed by sediments, the activity of AP could be reduced to 1.3%–5.3% of the activity of free AP (Zhu et al., 2016). APA can also be significantly reduced by trace metal ions in sediments, mainly through competition with the substrate for the active sites in AP, denaturation of the AP protein, and/or the generation of covalent bonds with the AP-substrate complexes (Vig et al., 2003). Various trace metal ions were abundant in the JH5 sediments (Zhang et al., 2014; Zhao et al., 2019). There were plenty of other light metals that are categorized as macroelements found in the sediments. The contents of these macroelements (Ca, Fe, and Cu) are higher than those of the trace metals by several orders of magnitude. Thus, whether and how they might affect the APA should be investigated.

4.2. Mechanism of enzymatic inactivation and reactivation

According to the thermodynamic analysis, nearly all the Al was converted into AlOOH during the incubation of the samples. There was a little amount of free Al^{3+} in the solution, accounting for 0.001% of the total Al. Thus, the enzymatic inactivation observed following Al^{3+} addition could not have been caused by Al^{3+} . Aluminum (hydr) oxides are often used to purify wastewater because they readily form colloids with a high surface area (Zamparas and Zacharias, 2014). Pollutant removal by AlOOH is mainly attributed to the bridging behavior of the

Fig. 7. Concentration of Zn and Mg was in alkaline phosphatase (AP) with and/or without the extraction by Na_2S . Each value interval represents the average $\pm$ standard deviation ($n = 3$). The control was 5 U mL^{-1} of AP solution without the addition of Na_2S , while the other group was the parallel AP solution with the addition of the same Na_2S .

Fig. 8. Concentrations of Fe, Al, and Ca released from sediments during the extraction by Na₂S. Each value interval represents the average $\pm$ standard deviation ($n = 3$). The seven parallel 4.0 g of sediments were extracted with 200 mL of Na₂S of 0 mM, 5 mM, 10 mM, 30 mM, 60 mM, 100 mM, and 200 mM, respectively, for 24 h at 28–30 °C under reducing condition. (B) The variation trend of alkaline phosphatase activity (APA) with time in the mixtures of alkaline phosphatase (AP) solutions and the lyophilized sediments extracted by Na₂S. Each value interval represents the average $\pm$ standard deviation ($n = 3$). The seven parallel 4.0 g of lyophilized sediments extracted with Na₂S were extracted by seven parallel 200-mL AP solutions (1 U mL⁻¹, pH 8) for 24 h at 28–30 °C under reducing condition.

precipitates formed by the interaction between Al and the surface of the organic matter particles in water (Johnson et al., 2005). Because AP is a protein, its inactivation by Al could be attributed to the ability of the generated AlOOH polynuclear hydroxy complexes to bridge and adsorb the AP (Strathmann and Myneni, 2005). According to the particle size analysis, there were indeed colloids formed in the AlCl₃-containing sample in the absence of Na₂S because the particle size of some precipitates was <100 nm after centrifugation (Fig. 6B). In addition, 97% of the Fe was still in the ionic state. Fe²⁺ and NO₃⁻ can easily produce hydroxyl radicals ($\cdot$ OH), which degrade organic matters under natural solar irradiation (Hadjira et al., 2012; Shah et al., 2015). A previous study has reported that the Fe²⁺ + ascorbic acid-induced inhibition of the APA is related to the generation of the hydroxyl radical ($\cdot$ OH), the most potent free radical known (Pintus et al., 2018). More specifically, Ohyashiki et al. (Ohyashiki et al., 1994) have reported that the oxidative damage-induced reduction of APA will be induced by the Fe²⁺ + ascorbic acid system; this indicates that the APA is irreversibly inhibited via the modification of the Mg²⁺-binding sites of AP during the early steps of the oxidation process. Therefore, the inactivation of APA by Fe in anaerobic ecosystems is mainly attributed to the destruction of AP by $\cdot$ OH. The thermodynamic state of Ca²⁺ was maintained; Ca was present as dissolved ions without the production of precipitates. Ca²⁺ could substitute the Mg²⁺ in the active sites of the AP; as a result, the maximal APA was approximately 40% of the activity of unsubstituted AP. If Ca²⁺ is present in sufficient amounts, it competes with Zn²⁺ for binding to the active sites of AP and ultimately displaces Zn²⁺ from the active sites, virtually eliminating the APA (Hoylaerts et al., 2015). Additionally, a novel and interesting finding was reported in the present study: S²⁻ can significantly deactivate AP. Therefore, we further analyzed the samples after the reaction of AP with S²⁻ using ICP-MS. The results indicated that the molar ratio of the intrinsic Zn²⁺ and Mg²⁺ bound to the enzyme was 2.8:1 (Fig. 7), which is consistent with the finding of a previous study (Anderson et al., 1975). The content of Zn²⁺ significantly decreased after the extraction process with Na₂S because the Zn²⁺ embedded in the AP could be eliminated by S²⁻. S²⁻ strongly combines with Zn²⁺, forming ZnS precipitates during the incubation of the samples (Kaksonen et al., 2003). However, the Mg²⁺ content did not decrease following the extraction process with S²⁻ because the saturation index of the components in the samples containing Mg²⁺ and S²⁻ was negative, and 100% of the Mg²⁺ could be dissolved in these samples (Table S6 and Table S7).

According to the thermodynamic state analysis, Al³⁺ and S²⁻ hardly reacted in the solution during incubation; however, S²⁻ could induce the coagulation of the AlOOH colloids to form precipitates because Al hydroxide colloids have positive charges, whereas S²⁻ is negatively charged (Zhu et al., 2011). Therefore, the particle size of precipitates in the sample containing AlCl₃ following the addition of Na₂S was smaller than that of the precipitates formed without Na₂S addition because most AlOOH colloids had coagulated into larger particles due to the activity of S²⁻ and had separated from the solution during centrifugation (Fig. 6B). Therefore, the APA in the Al³⁺-containing sample (after S²⁻ addition) was recovered mainly due to the elimination of the AlOOH colloids and S²⁻, which occurred following the adsorption and coagulation of the AlOOH colloids. The APA was not restored in the Ca²⁺-containing samples after S²⁻ addition because the Ca²⁺ could not be combined with S²⁻. Both the intrinsic Ca²⁺ plus the newly added free S²⁻ could synergistically inhibit the APA, resulting in a lower APA value than that without S²⁻ addition. Based on the SEM-EDS and XRD results, the precipitate formed by Fe²⁺ and S²⁻ is probably FeS because the Fe/S ratio was close to 1:1, and this was confirmed by the results of the thermodynamic simulation analysis. Thus, the APA increased because both Fe²⁺ and S²⁻ could be simultaneously removed from the system.

4.3. Effect of sulfate reduction on enzyme activity

As shown in Fig. 8A, the content of Al decreased as the S²⁻ content increased because the AlOOH colloids were removed from the supernatant by S²⁻. Therefore, S²⁻ accumulation could reduce the Al content from the overlying water or pore water in the sediments. Additionally, the content of soluble Fe increased with the S²⁻ concentration (<10 mM) in the slurry, which was probably caused by the Fe(III) (hydr)oxides being reductively dissolved by the free sulfide. Therefore, Fe could be released from the sediment under reducing conditions (Kopáček et al., 2005). Subsequently, the Fe content began to decrease as the S²⁻ content increased (>10 mM), which indicated that the released Fe(II) combined with the free sulfide to generate FeS, thereby outpacing the reductive dissolution of Fe(III) (hydr)oxides. Therefore, sulfate reduction could promote the release of Fe at the beginning of the reaction; the Fe was then fixed by the accumulated sulfides as the sulfate reduction gradually became intense.

In Fig. 8B, the APAs in slurries extracted using Na_2S (<30 mM) were lower than those in the control samples; this was probably because the Fe^{2+} released from the sediments and most of the AlOOH colloids had not been removed by the free sulfides and other trace metal ions that had not been coupled due to the insufficient amount of free S^{2-} . However, excessive Na_2S (>200 mM) could also reduce the APA, compared to the case when the resuspended sediments were extracted using 100 mM Na_2S . This could be explained by the S^{2-} -mediated inactivation of the AP in the presence of excessive amounts of S^{2-} . In contrast to the macro metals (Al, Fe, and Ca), SO_4^{2-} inhibited the APA relatively weakly. The Fe and Al contents directly determined the final APA because Ca could not chemically combine with the free sulfides. Therefore, the APA increased when the molar ratio of the free sulfides to the sediments (which contained 1 Fe and 6 Al) was between 6 and 10, while the APA was inhibited when this ratio was <6 or higher than 10.

5. Conclusions

Macro metal ions (Al, Fe, and Ca ions) can markedly inhibit the APA and further reduce the efficiency of OP hydrolysis. This prevents PO_4^{3-} overload from causing eutrophication, but S^{2-} generation following sulfate reduction can alleviate the Al and Fe ion-mediated inhibition of APA via the elimination of AlOOH colloids and Fe^{2+} in anaerobic conditions. This can increase the APA, which improves the hydrolysis of OP. However, high APA can be reduced only when excess amounts of dissolved S^{2-} are accumulated, because dissolved S^{2-} can inhibit the APA via the removal of two Zn^{2+} from the active sites (M1 and M2) of AP. Therefore, the risk of PO_4^{3-} release from OP by the action of AP should be taken seriously during the initial stage of the anaerobic production of S^{2-} in anoxic ecosystems.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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