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

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HIGHLIGHTS
• Al3+, Fe2+, Ca2+ can inhibit alkaline phosphatase (AP) activities.
• Excess S2− reduces AP activities by removing Zn2+ active sites.
• The inhibition of Al3+ and Fe2+ on AP activities can be alleviated by S2−.
• Organic P monoester excluding recalcitrant P chelates can be hydrolyzed by AP.

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 PO43− 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. Fe2+ inhibited the APA via a mechanism involving free radical oxidation. The main mechanism by which Ca2+ inhibited the APA was by competing with Mg2+ and Zn2+ for the active sites of the enzyme. Excessive S2− could reduce the APA by removing Zn2+ from the active sites of the enzyme. The inhibition of APA could be reversed if some metal ions (e.g., Fe2+) were precipitated by S2− under reducing conditions. Therefore, in an-aerobic 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 PO43−, 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 31P nuclear magnetic resonance spectroscopy (31P 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
monooester (e.g., α- or β-glycerophosphate, mononucleotides, choline phosphate, inositol hexaphosphates, and glucose-6-phosphate), and orthophosphate diester (e.g., DNA, phospholipids, and RNA), can be characterized by 31P 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 monooesters into PO4 3− and organic matter (Zhou et al., 2002). However, excess of PO4 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 phosphomonooesterase 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 (Zn2+) occupy the M1 and M2 sites, and a magnesium ion (Mg2+) occupies the M3 site (Tang et al., 2019). The M4 is a peripheral binding site occupied by Ca2+; it is conserved in human and mouse APs (Le Du et al., 2002). However, the M4 site is not present in bacterial enzymes (Le 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 Aasegirsen, 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 Zn2+, resulting in the inhibition of APA (Salar et al., 2017). Arsenate can inhibit AP through its strong binding to AP and/or its structural similarity with PO4 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 AP 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 S2−) can be eliminated by precipitation processes (Jong and Parry, 2003). During anaerobic sulfate reduction, the accumulated S2− would couple with Fe, promoting PO4 3− release from Fe oxides and/or hydroxides (Kraal et al., 2017). Generally, S2− 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 Zavaczkí et al. have reported the toxicity of S2− towards APA, the damage caused by S2− to the active sites of AP is still unclear (Zavaczkí et al., 2011). Therefore, understanding the effects of S2− 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 anoxic 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 °C until analysis. The sediments were lyophilized and homogenized prior to analysis. Details regarding the location of sample collection (JHS) 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 31P 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 0.4-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 28–30 °C in duplicate for 24 h. At specific time points (0, 2, 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 PO4 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 28–30 °C).

2.2.2. Sequential extraction of OP and 31P 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 sediment samples, which were passed through a 100-mesh sieve, and extracellular enzyme that is secreted by 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.

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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
were removed by free dissolved sulfides. Seven sets of Na2S concentration gradient experiments were established in parallel: 4.0 g of the sediments were extracted with 200 mL of Na2S solutions (0 mM, 5 mM, 10 mM, 30 mM, 60 mM, 100 mM, and 200 mM); the S2−-contents corresponded to 0, 1, 2, 6, 12, 20, and 40 mmol, respectively. The slurries were incubated at 28–30 °C for 24 h with slight shaking (180 rpm). The control sample without Na2S (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 Al3+, Fe3+, and Ca2+ 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 Na2S. 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 28–30 °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 Al3+, Fe3+, and Ca2+ with and/or without the addition of S2− at a pH of 8 and 28–30 °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: SI > 0, no precipitate; SI = 0, equilibrium; and SI < 0, generating precipitates (Migaszewski et al., 2018).

\[ SI = \frac{\text{IAP}}{K_{\text{sp}}} \]  

(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 PO43− 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 PO43− 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 PO43− concentration in the slurry following AP addition was significantly higher (p < 0.05). Additionally, the PO43−-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 PO43−-P following AP addition almost plateaued, while it still showed a slight increase in the control sample (after 2.1 mg L−1 to the final 2.2 mg L−1). At the end of the experiment, the average amounts of PO43− 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 PO43− 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
slurries containing 0, 0.2, 2, and 20 U mL\(^{-1}\) of AP was 0.27, 410, 6100, and 15,000 mg L\(^{-1}\) 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 mg L\(^{-1}\) h\(^{-1}\), respectively.

The changes of OP speciation in the hydrolyzed sediments following treatment with AP are shown in Table S2. The concentration of NaHCO\(_3\)-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 H\(_2\)SO\(_4\)-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 \(\beta\)-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 \(-2400\) mg L\(^{-1}\) h\(^{-1}\) in the control to \(-200\) mg L\(^{-1}\) h\(^{-1}\) and \(-210\) mg L\(^{-1}\) 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 \(-200\) mg L\(^{-1}\) h\(^{-1}\) and 1500 mg L\(^{-1}\) h\(^{-1}\) to \(-560\) mg L\(^{-1}\) h\(^{-1}\) and 2000 mg L\(^{-1}\) 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 Al(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...
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 μmol L−1 and 1.5 μmol L−1, respectively, without the Na2S extraction.

![Fig. 3. 31P 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: α-α-glycerol phosphate; β-Gly: β-glycerophosphate; G6P: glucose 6-phosphate; AM: adenosine 5’ monophosphate; PC: phosphocholine; GTP: glucose 1-phosphate.](image-url)
After the extraction with S$^{2−}$, the Zn$^{2+}$ content dropped to 1.5 μmol L$^{−1}$ (67% removal). However, the Mg$^{2+}$ concentration remained constant before and after the extraction, showing that Na$_2$S 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$_2$S 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$_2$S than that in the absence of Na$_2$S when the concentration of S$^{2−}$ was <30 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 ± 0.38 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.
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$_2$S 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 MINITeq software. As shown in Table S5, 99% of the Al in the solution was precipitated as diaspore (AlOOH). Most of the Fe was present in the dissolved state (Fe$^{2+}$, 97%); only 3% was present as Fe(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. 4. Inhibition of the alkaline phosphatase activity (APA) by adding 1, 3, and 6 mM of Al$^{3+}$, Fe$^{2+}$, Ca$^{2+}$, SO$_4$$^{2−}$, and S$^2$−, respectively. Each value interval represents the average ± standard deviation (n = 3). Reactivation of the inhibitory APA by adding S$^2$− into the tubes contained 1 mM of Al$^{3+}$, Fe$^{2+}$, Ca$^{2+}$, and SO$_4$$^{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.

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 ± standard deviation (n = 3) (G).
throughout the experiment. Na₂S was added into the AlCl₃ solution to yield both inorganic and organic matter when it came in contact with the AP. The activity of AP due to its stable structure (Lü et al., 2016). Because NLOP was mainly resistant to hydrolysis (Turner et al., 2012; Zhu et al., 2015). The phosphonate could not be hydrolyzed by AP because it reacted with metal ions to form recalcitrant complexes (Turner et al., 2002). The results of the 31P NMR studies showed that most of the remaining (β-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 PO₄³⁻ 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 adsorbed 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 macromolecules found in the sediments. The contents of these macromolecules (Ca, Fe, and Ca) 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 Al³⁺ in the solution, accounting for 0.001% of the total Al. Thus, the enzymatic inactivation observed following Al³⁺ addition could not have been caused by Al³⁺. 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. 8. Concentrations of Fe, Al, and Ca released from sediments during the extraction by Na2S. Each value interval represents the average ± standard deviation (n = 3). The seven parallel 40 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 ± standard deviation (n = 3). The seven parallel 40 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.

According to the thermodynamic state analysis, Al³⁺ and S²⁻ hardly reacted in the solution during incubation; however, S²⁻ could induce the coagulation of the AOOH 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 AOOH 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 AOOH colloids and S²⁻, which occurred following the adsorption and coagulation of the AOOH 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⁻ could be removed as the high-potential hydroxides being reductively dissolved by the free sulfide (hydr)oxides being reductively dissolved by the free sulfide, thereby releasing Fe(II) combined with the free sulfide to generate FeS, thereby allowing 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.

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 AOOH colloids were removed from the supernatant by S²⁻. Therefore, S²⁻ accumulation could reduce the AI 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 Na2S (≥ 30 mM) were lower than those in the control samples; this was probably because the Fe2+ released from the sediments and most of the AIOOH 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 S2−. However, excessive Na2S (> 200 mM) could also reduce the APA, compared to the case when the resuspended sediments were extracted using 100 mM Na2S. This could be explained by the S2−-mediated inactivation of the AP in the presence of excessive amounts of S2−. In contrast to the macro metals (Al, Fe, and Ca), SO42− 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 the PO43− overload from causing eutrophication, but S2− generation following sulfate reduction can alleviate the Al and Fe ion-mediated inhibition of APA via the elimination of AIOOH colloids and Fe2+ in anoxic conditions. This can increase the APA, which improves the hydrolysis of OP. However, high APA can be reduced only when excess amounts of dissolved S2− are accumulated, because dissolved S2− can inhibit the APA via the removal of two Zn2+ from the active sites (M1 and M2) of AP. Therefore, the risk of PO43− release from OP by the action of AP should be taken seriously during the initial stage of the anaerobic production of S2− 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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