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# Soil mineral alters the effect of Cd on the alkaline phosphatase activity

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ABSTRACT

The toxicity of heavy metals (HMs) to soil enzymes is directly influenced by the status of the enzyme (free vs. immobilized on minerals) and the duration of exposure. However, little information is available on the interaction effect of HMs, mineral, and exposure time on soil enzyme activities. We investigated the interaction mechanism of alkaline phosphatase (ALP) with minerals (montmorillonite and goethite) and the response of free and immobilized ALP to cadmium (Cd) toxicity under different exposure times. The adsorption isotherms of ALP on both minerals were L-type. The maximum adsorption capacity of goethite for ALP was 3.96 times than montmorillonite, although both had similar adsorption constant (*K*). Goethite showed a greater inhibitory effect on ALP activity than montmorillonite. The toxicity of Cd to free- and goethite-ALP was enhanced with increasing exposure time, indicating a time-dependent inhibition. However, Cd toxicity to montmorillonite-ALP was not affected by the exposure time. The inhibition of Cd to soil enzyme activity is influenced by the properties of mineral complexes and the duration of exposure. A further understanding of the time pattern of HMs toxicity is helpful for accurately assessing the hazards of HMs to soil enzyme activity.

## 1. Introduction

Heavy metals (HMs) have become one of the most serious pollutants in soil due to intensive human activities, such as rapid industrialization, urbanization, and agricultural intensification (Birke et al., 2017; Chen et al., 2014). This has led to severe farmland resource destruction and soil environment degradation (Tang et al., 2016). Elevated concentrations of soil HMs can potentially threaten human and animal health via the food chain (Zhao et al., 2015), as well as can have a detrimental effect on soil ecological functions and pose a potential risk to environmental health (Giller et al., 1998; Smolders et al., 2009). Soil microorganisms are the most active and sensitive component of the soil ecosystem, which play an important role in soil carbon and nutrient cycling (Burns et al., 2013). Therefore, a growing number of studies confirm that soil microbial activity can be used as a valuable indicator to assess the soil quality (Bouchez et al., 2016; Kenarova and Boteva, 2015).

Soil extracellular enzymes are a product of microbial cellular metabolism and can serve as proxies for microbial activity (Sinsabaugh et al., 2009). The activity of soil enzymes has been shown to be sensitive to natural and anthropogenic changes in the soil ecosystem, therefore has been widely used to assess the effect of HMs on soil microbial function (Bastida et al., 2008; Cepeda et al., 2016; Rao et al., 2014). Previous studies have shown that soil enzyme activities (SEAs) decrease exponentially or logistically with increasing HMs concentration (Smolders et al., 2009; Speir et al., 1995). However, the extent of SEAs in response to the same heavy metal toxicity may vary with soil properties (Oorts et al., 2006; Tan et al., 2017; Welp and Brümmer, 1997). For example, the $ED_{50}$ (ecological dose causing 50% inhibition in activity) of cadmium (Cd) has been shown to range from 4 to 5484 mg $kg^{-1}$ for soil phosphatase activity in different soils (Doelman and Haanstra, 1989; Renella et al., 2004a; Tan et al., 2014). Soil properties (e.g., organic matter, pH, cation exchange capacity, and soil texture) can significantly influence the bioavailability of HMs in soil and thereby SEAs (Vig et al., 2003). Further, the response of SEAs to HMs is highly dependent on the status of soil enzymes (free and

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immobilized enzyme) (Burns et al., 2013; Li et al., 2013; Zimmerman and Ahn, 2011) and its duration of exposure (Matyja et al., 2016; Smolders et al., 2009).

Extracellular enzymes in the soil often bind to soil particles through different pathways, forming mineral-enzyme, organic matter-enzyme, or clay-organic matter-enzyme complexes (Zimmerman and Ahn, 2011). Thus, the adsorbed enzyme shows significant differences in enzyme proprieties (such as stability and activity) compared to the free enzyme due to modification in enzyme molecular conformations and catalytic characteristics (Burns, 2013; Huang et al., 2009). In general, adsorption increases the enzyme tolerance to environmental stress (temperature, pH, inhibitor, etc.) (Allison, 2006; Zimmerman and Ahn, 2011). But this tolerance can vary with the nature of adsorbent, resulting in the differential response of apparent SEAs to the toxicity of HMs (Gianfreda et al., 1995b; Shindo et al., 2002; Wang et al., 2017). For instance, copper and zinc show higher inhibition on the activity of acid phosphatase (ACP) immobilized by kaolinite than goethite (Huang and Shindo, 2000a). However, the activity of goethite-alkaline phosphatase (G-ALP) had decreased slightly more than that of montmorillonite-ALP (M-ALP) in the presence of arsenate (Wang et al., 2017). These results suggest that it is still an open question with regards to how the interactions of soil enzymes and minerals affect the toxicity of HMs. Therefore, it is vital to clarify the response pattern of enzyme adsorbed on soil mineral and/ or organic matter to HMs toxicity to gain a better understanding of the effect of HMs on SEAs (Fan et al., 2018).

Mechanistically, enzymatic reactions display a slow onset of inhibition rather than responding instantly to the presence of an inhibitor, which is called slow-binding inhibition (Goliĉnik and Stojan, 2004; Marangoni, 2002). As a consequence, the toxicity of HMs to enzyme activity may vary depending on the duration of exposure. Unusually, most of the studies in ecotoxicology were conducted after a certain exposure time during the long-term incubation, which makes it difficult to predict how HMs directly influence SEAs during the exposure period (Baas et al., 2010; Epelde et al., 2016; Frossard et al., 2017). The microbial community can alter the amount and property of soil enzymes regarding their capacity to resist the effects of HMs contamination (Epelde et al., 2016; Frossard et al., 2017). A recent study found that the toxicity of HMs on intracellular enzymes (e.g., dehydrogenase) vary depending on the exposure time (Matyja et al., 2016). However, the time-dependent changes in toxic effects of HMs on extracellular enzymes, which have distinctly different properties from intracellular enzymes, have rarely been taken into account. A better understanding of the influence of exposure time on toxic effects of an inhibitor in the absence of microbial interference would be helpful to better assess the toxicity of HMs on SEAs (Baas et al., 2010; Matyja et al., 2016).

Alkaline phosphatase (ALP, EC 3.1.3) plays a vital role in the mineralization of organic phosphorous compounds in neutral and alkaline soils and is known to be sensitive to Cd toxicity (Renella et al., 2004b; Tan et al., 2014; Tian et al., 2017). Cadmium is one of the most toxic and commonly occurring metals in developing countries (Chen et al., 2014). Due to its high toxicity and mobility, Cd has enormous potential to impact nearly all biological processes (Chen et al., 2014). It is demonstrated that the effects of Cd on soil ALP are significantly different across particle-size fractions (Fan et al., 2018). We anticipated that the properties of ALP in various particle-size fractions (e.g., ALP adsorbed on different minerals) might contribute to this discrepancy. However, to our knowledge, there is no information available on how the interactions between soil minerals and ALP affect the Cd toxicity.

Thus, we investigated the influence of exposure duration to contamination on the Cd toxicity to free and immobilized ALP to address the following questions: (i) how the ALP is adsorbed on different minerals? (ii) What is the difference between free and immobilized ALP on different minerals to Cd toxicity; (iii) how does the exposure time affect the toxicity of Cd to free and immobilized ALP activity? This study will shed new light on variation in the patterns of ALP activity in different soils under Cd contamination and provide important basis to evaluate and monitor the toxicity of Cd on soil ecological functions.

## 2. Materials and methods

### 2.1. Minerals and alkaline phosphatase

Deionized water (DIW) was prepared using the water purification system (ULUP-III-60L, China), with the electrical resistivity being 18.25 MΩ $cm^{-1}$ at 22 °C.

Montmorillonite ($(Al, Mg)_2[Si_4O_{10}](OH)_2 \cdot nH_2O$) (analytical reagent grade with a surface area of 240 $m^2$ $g^{-1}$) was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Goethite (α-FeOH) was synthesized according to Montes-Hernandez et al. (2011). Briefly, 10 g of NaOH and 13.5 g of $FeCl_3 \cdot 6H_2O$ were dissolved in 250 mL of DIW. The solution was heated at 70 °C for 7 h under vigorous stirring (500 r $min^{-1}$) in the presence of $N_2$. The precipitate was washed with DIW and 95% alcohol until free of $Na^+$ ions and finally dried under vacuum-pressure in a desiccator for 24 h. The dry solid product was ground to pass through a 200-mesh sieve for subsequent use. The surface area of goethite is 31.2 $m^2$ $g^{-1}$.

Alkaline phosphatase from bovine intestine mucosa was supplied by Sigma-Aldrich, Inc. (St. Louis, USA, Country). The free ALP activity is 10 U, and one unit of the enzyme hydrolyzes 1 μmol of 4-nitrophenyl phosphate per minute at pH 9.8 and 37 °C.

### 2.2. Preparation of mineral immobilized ALP

Mineral colloids (6 mg $mL^{-1}$) solution was prepared by adding 300 mg of montmorillonite or goethite to 50 mL of borax buffer (pH 7.4, 10 mL 0.05 M sodium borate mixed with 90 mL 0.2 M boric acid). The suspension was stirred for 5 min and then fully dispersed by ultrasonication in a ultrasonic homogenizer (Scientz, China) for 30 min (Rao et al., 2000).

A stock solution of free ALP (3 mg $mL^{-1}$) was prepared by adding 0.3 g of free ALP to 100 mL of Tris buffer (pH 8.8). The stock solution was stored at 4 °C prior to use.

To prepare immobilized ALP, 2 milliliters of montmorillonite colloids (6 mg $mL^{-1}$) or diluted goethite colloids (1 mg $mL^{-1}$) was added into 2 mL of diluted ALP stock solutions (0.06 mg $mL^{-1}$). The mixed suspensions were shaken (250 r $min^{-1}$) in the orbital shaker (YC-2102C, Shanghai) at 25 °C for 2 h to reach equilibrium and centrifuged (Eppendorf AG, Germany) at 9882 g for 15 min. The residue was washed 6 times with 3 mL of borax buffer to remove the weakly adsorbed ALP molecules. Finally, the residue was suspended in 3 mL of borax buffer before use (Wang et al., 2017).

### 2.3. Experimental design

#### 2.3.1. Adsorption of ALP on the minerals

The isotherm for ALP adsorption onto the minerals was determined as follows: 1 mL of different concentrations of ALP solutions (diluted ALP stock solution in Tris buffer) were placed into centrifuge tubes, and then 1 mL of montmorillonite or goethite colloids were added, followed by the addition of 1 mL of borax buffer (pH 7.4). The tubes were shaken at 250 r $min^{-1}$ and 25 °C for 2 h. The final ALP concentrations in the mixtures (A) were 0, 20, 40, 60, 80, and 100 μg $mL^{-1}$, respectively. Then, the tubes were centrifuged at 9982 g for 15 min. The residue was washed 6 times with 3 mL of borax buffer and then resuspended in 3 mL of borax buffer (pH 7.4) (C). The supernatant fraction and washings were collected (B). Subsequently, an aliquot (100 μL) of A, B, and C were mixed with 100 μL borax buffer (pH 7.4) and 2 mL of substrate solution to determine ALP activity.

The concentration of ALP in A, B, and C was determined indirectly as enzyme activity. Mechanically, the enzyme activity is proportional to the enzyme concentration at a constant concentration of substrate,