**A proposal of “core enzyme” bioindicator in long-term Pb-Zn ore
pollution areas based on topsoil property analysis**

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Abstract

To study the effects of long-term mining activities on the agricultural soil quality of Mengnuo town in Yunnan province, China, the heavy metal and soil enzyme activities of soil samples from 47 sites were examined. The results showed that long-term mining processes led to point source heavy metal pollution and Pb, Cd, Zn and As were the primary metal pollutants. Polyphenoloxidase was found the most sensitive soil enzyme activity and significantly correlated with almost all the metals ($P<0.05$). Amylase (for C cycling), acid phosphatase (for P cycling) and catalase (for redox reaction) activities showed significantly positive correlations ($P<0.05$) with Pb, Cd, Zn and As contents. The correlations between soil enzymes activities and Cd, Pb and Zn contents were verified in microcosm experiments, it was found that catalase activity had significant correlations ($P<0.05$) with these three metals in short-term experiments using different soils under different conditions. Based on both field investigation and microcosm simulation analysis, oxidoreductases activities (rather than a specific enzyme activity) were suggested to be used as “core enzyme”, which could simply and universally indicate the heavy metal pollution degrees of different environments. And hydrolases (for C, N, P and S recycling) could be used as a supplement to improve correlation accuracy for heavy metal indication in various polluted environments.

Keywords: Pb-Zn ore; heavy metal pollution; oxidoreductase; soil enzymes activities; bioindicator

Capsule

Oxidoreductases could be used as a better and more sensitive bioindicator for soil heavy metal pollution than hydrolases.

1. Introduction

Mining, processing, smelting, power generation, waste spills and fossil fuel combustion activities are the major reasons for heavy metal contamination in water resources and nearby agricultural soils (Mirzaei et al., 2014; Wang et al., 2007). Heavy metals, unlike organic contaminants, are generally immutable, nondegradable and persistent in soils and waters (Garau et al., 2007), threatening the surrounding ecosystems and human health.

A number of soil microbiological parameters including microbial biomass C and N, basal respiration, microbial community structure and soil enzyme activities have been used as indicators of soil quality (Liao and Xie, 2007). Some researchers held that soil enzymes activities were not necessarily correlated with heavy metal pollution (Tripathy et al., 2014; Zhang et al., 2010), but soil enzymes are involved in redox reactions and nutrient cycling, especially C, N and P recycling, and the determination of heavy metal pollution level through measuring soil enzyme activities is rapid, simple, and inexpensive in comparison with other methods. Moreover, as soil enzyme activities are highly sensitive to soil changes (Jimenez et al., 2002; Lee et al., 2009), enzymes activities have been used as bioindicators for soil quality and environmental

health monitoring in recent years (Hu et al., 2014; Lee et al., 2009).

Some enzymes were chosen as bioindicators for specific heavy metals in polluted environment. Dehydrogenase can catalyze the dehydrogenation of substrate and has been used in Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn polluted areas (Hu et al., 2014; Lee et al., 2002; Shen et al., 2005). Catalase can break down hydrogen peroxide and prevent it from poisoning organisms, and has been used as a bioindicator for As, Cd, Cr, Cu, Hg, Pb and Zn pollution (Cang et al., 2009; Hu et al., 2014; Liang et al., 2014; Xian et al., 2015). Amylase, arylsulphatase, cellulase, β -glucosidase, acetylglucosaminidase and sucrase (for C degradation), protease and urease (for N cycling), and phosphatase (for organic phosphorous compounds transformation) have also been used as bioindicators in certain polluted environments (Ciarkowska et al., 2014; Kuperman and Carreiro, 1997; Mora et al., 2005; Shen et al., 2005). However, the responses of enzymes activities to heavy metals in different soil samples were inconsistent and the selection of enzymes varied in different studies. The particularity in the selection of one enzyme for one specific environment restricted the development of enzyme bioindicator technology. Till now, no enzymes or types of enzymes have been acknowledged as “core enzymes” that could simply and universally indicate the heavy metal pollution degrees of different environments.

Generally, enzymes can be divided into six classes, including oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases, among which oxidoreductases can change the redox state of metal ions to achieve ion detoxification and hydrolases can take part in the nutrition acquisition of both microorganisms and plants. Therefore,

the activities of oxidoreductases (polyphenoloxidase (PPO) and catalase) and hydrolases (amylase, acetylglucosaminidase (NAG), β -glucosidase (BG), cellulose, invertase, protease, urease and acid phosphatase involved in soil C, N and P cycling), concentrations of 8 kinds of heavy metals (As, Cd, Cr, Cu, Hg, Ni, Pb and Zn) in soils and the physicochemical properties of soils were measured in this study. Correlations hypothesis between soil enzymes activities and heavy metals pollution were then proposed based on long-term heavy metal polluted field investigations, which were also verified in microcosm experiments using unpolluted different physiochemical properties background soil. The aims were to (i) investigate the soil properties in an area that suffered from long-term Pb-Zn ore exploitation, (ii) assess the effects of heavy metals on soil properties, (iii) assess the long-term impact of heavy metals on selected soil oxidoreductases and hydrolases activities and evaluate different enzymes activities' sensitivities to heavy metal pollution and (iv) assess the possibility of using oxidoreductases and hydrolases as "core enzyme" bioindicator for heavy metal pollution by microcosm simulation experiments and their potential application value without considering different soil background physicochemical properties.

2. Materials and methods

2.1. Sample site description and soil sample collection in field investigation

Field soil samples were collected from Mengnuo town in Yunnan province of China (located at around 99⁰4'E, 24⁰19' N; Description, Fig. S1 and Table S1 in the Supporting information), which has been affected by Pb-Zn ore exploitation for more than 50 years since the 1950s. Soils from the farmland of site Rs1, which was the

furthest from the lead-sulfur plant, was considered as control. Dry branches, fallen leaves and debris in soil were picked up completely. Samples were then obtained by multipoint (n=3) mixed sampling. Approximately 2.5 kg of 0-30 cm topsoil was collected by shovel at each point. Three replicates of each point were taken. Well-mixed soil samples were put into cleaned ziplock plastic bags and taken to the laboratory in cool boxes. After removing the coarse particles or large materials by using a 2 mm sieve, the samples were then stored at -20°C. Extreme care was taken to avoid sample contamination at every step, and the control (samples at Rs1) used for verification was obtained through the same sampling procedure.

2.2. Microcosm experiment

To verify the applicability of the heavy metal soil contamination hypothesis of relationship between ambient heavy metal and soil enzymes activities inferred from the field investigations described above in other different regions, microcosm experiments were carried out with different soil type and artificial heavy metal contents. Approximately 2.5 kg of 0-20 cm of farmland topsoil at each sampling point (sampling site=9) was collected from agricultural experiment and demonstration stations in Shunyi District of Beijing with no heavy metal pollution history. The details could be seen in the Supporting information. Well-mixed soil was bagged, transported and stored with the coarse particles removed under the same conditions as in the field investigation. To obtain artificial heavy metal contaminated soils, Cd, Pb and Zn ions were dissolved in deionized water and sprayed to 300 g farmland topsoil till the final concentrations reached 0 mg kg⁻¹, 10 mg kg⁻¹, 100 mg kg⁻¹, 1000 mg kg⁻¹,

and 10000 mg kg⁻¹ respectively. The water content of soil was kept at ~15% by adding the evaporated water every other day. Each treatment had 4 replicates. The heavy metal stress was maintained for 30 days at 30°C (Chen et al., 2014a; Hseu, 2006; Yang et al., 2007) at normal day/night light cycle to assess the effects of heavy metal pollution on soil enzyme activities.

2.3. Soil analysis

The physicochemical properties of soil organic matter (SOM), total nitrogen (TN), NH₄⁺-N, NO₃⁻-N, available phosphorus (AP), available potassium (AK) and pH were analyzed. SOM was measured using the potassium bichromate volumetric method-dilution heat method. TN was determined by the semi-micro Kjeldahl method (Page et al., 1982). NH₄⁺-N was measured using the 2 mol/L KCl extraction-indophenol blue spectrophotometric method. NO₃-N was measured using the phenoldisulfonic acid spectrophotometric method. AP was measured using the 0.5 M NaHCO₃ extraction-ammonium molybdate-antimony potassium tartrate and ascorbic acid spectrophotometric method. AK was measured using the NH₄OAc extraction-flame photometry (Cui et al., 2010). The soil pH was measured by twin pH B-212 (Horiba, Kyoto, Japan). In order to reduce the number of samples for heavy metal content measurements, every 3 soil replicates collected from each of the 47 sample sites were air-dried, ground, well-mixed, passed through a 1 mm sieve and treated as one test sample in the analysis of heavy metal contents. Each test sample was measured three times to calculate its mean values. To assess the Hg content, the soils were treated according to the Procedure 5 of dos Santos et al.'s (2005) method.

For As, Cd, Cr, Cu, Ni, Pb and Zn content measurement, the soils were treated according to the Method 2 of Dimpe et al.'s (2014) microwave-assisted digestion procedure. For all the preparation methods, blank solutions were run parallel for verification. The digested solution was analyzed in triplicate by the ICP-OES technique (inductively coupled plasma-optical emission spectrometer, PerkinElmer-Optima 7300 DV, USA). The operating parameters were as follows: 1450 W of RF power, 15 L min⁻¹ of a plasma flow rate, 0.2 L min⁻¹ of an auxiliary gas flow rate, 0.6 L min⁻¹ of a nebulizer flow rate and 1.0 mL min⁻¹ solution uptake flow rate. Sample solutions were introduced into the plasma using a GemTipTM Cross Flow Nebulizer and a Rytan[®] Scott spray chamber. The analytic lines of As, Cd, Cu, Cr, Hg, Ni, Pb and Zn elements were 193.696 nm, 228.802 nm, 324.752 nm, 267.716 nm, 253.652 nm, 231.604 nm, 182.205 nm and 213.857 nm respectively. Calibration standard solutions were prepared by dilution with 2% HNO₃ of a multi-element calibration standard solution (Ultra Scientific, USA, catalog number: IQC-026, Lot n° J00022) that contained 26 elements. Correlation coefficients of the calibration curves were in the range of 0.9986–0.9999. A solution of 2% HNO₃ as blank was used and all reported data were blank corrected. The accuracy of analytical method was valued by analyzing samples prepared by spiking with known amounts of elements to solutions obtained from three samples. The recovery percentage ranged between 91% and 103%.

2.4. Assessment of soil pollution

The soil contamination status was assayed using the contamination factor (CF):

179 $CF = C_s/C_c$.

180 where C_s (mg/kg) is the average concentration of elements in the samples and C_c
181 (mg/kg) is the average concentration of elements in the standards or control or an
182 unpolluted area (Boamponsem et al., 2010). The contamination levels ranged from 1
183 to 6 (0=none, 1=none to moderate, 2=moderate, 3=moderate to strong, 4=strongly
184 polluted, 5=strong to very strong and 6=very strong) (Muller, 1969). CF for each
185 heavy metal at each site was calculated to evaluate the contamination level.

186 For the overall level of soil pollution across the sampling sites, pollution load index
187 (PLI) was determined as (Bhuiyan et al., 2010):

188 $PLI = (C_{f1} \times C_{f2} \times C_{f3} \times \dots \times C_{fn})^{1/n}$

189 Where C_f is the metal contamination factor and n is the number of samples analyzed
190 in this study. Four pollution levels were defined: no pollution ($PLI < 1$), moderate
191 pollution ($1 < PLI < 2$), heavy pollution ($2 < PLI < 3$) and extremely heavy pollution
192 ($3 < PLI$) (Liu et al., 2013). The PLI for each site's overall pollution level was also
193 calculated.

194 2.5. Enzyme activity assays

195 Cellulase (EC 3.2.1.4) activity in soils was determined by estimating the Glucose
196 Equivalents after mixing the soil samples (10 g) with 1.5 ml of methylbenzene, 5 ml
197 of acetate buffer (pH 5.5) and 5 ml of 1% (w/v) carboxy methyl cellulose and
198 incubating for 72 h at 37 °C. The Glucose Equivalents were estimated by the DNS
199 method (Miller, 1969). Cellulase activity was expressed in terms of milligrams of
200 Glucose Equivalents per g of soil per 72 h ($\text{mg glucose g}^{-1} 72 \text{ h}^{-1}$) (Guan et al., 1986).

To measure invertase (EC 3.2.1.26) activity, 10 g of the soils were incubated with 1.5 ml of methylbenzene, 5.0 ml of 20% (w/v) sucrose solution and 5.0 ml of phosphate buffer (pH 5.5) for 24 h at 37 °C. The released glucose was determined by the method described by Shassfer and Somogyi (1933). Invertase activity was expressed in terms of milligrams of Glucose Equivalents per g of soil per 24 h ($\text{mg glucose g}^{-1} 24\text{h}^{-1}$) (Guan et al., 1986). NAG (EC 3.2.1.30), BG (EC 3.2.1.21), PPO (EC 1.14.18.1) and acid phosphatase (EC 3.1.3.2) activities were determined by the micropool plate method under 50 mM sodium acetate buffer (pH 5.0). The activity of enzymes were defined as μmol substrate converted per hour per g dried soil (Allison and Jastrow, 2006; Allison and Vitousek, 2005). Amylase (EC 3.2.1.60) activity was determined by the 3, 5- dinitrosalicylic acid colorimetry and amylase activity was expressed in terms of milligrams of Glucose Equivalents per g of soil per 24 h ($\text{mg glucose g}^{-1} 24\text{h}^{-1}$) (Guan et al., 1986). Protease (EC 3.4.21.14) activity was determined by the ninhydrin colorimetry method and protease activity was expressed in terms of milligrams of $\text{NH}_2\text{-N}$ per g of soil per 24 h ($\text{mg NH}_2\text{-N g}^{-1} 24\text{h}^{-1}$) (Guan et al., 1986). Urease (EC 3.5.1.5) activity was determined by the sodium phenolate-sodium hypochlorite colorimetry method and urease activity was expressed in terms of milligrams of $\text{NH}_3\text{-N}$ per g of soil per 24 h ($\text{mg NH}_3\text{-N g}^{-1} 24\text{h}^{-1}$) (Guan et al., 1986). Catalase (EC 1.11.1.6) activity was determined by the potassium permanganate titration method and catalase activity was expressed in terms of milliliter of KMnO_4 reduction per g of soil per hour ($\text{mL g}^{-1} \text{h}^{-1}$) (Guan et al., 1986).

2.6. Statistical analysis of data

Soil physicochemical properties including SOM, TN, $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$, AP, AK and pH was presented as means $\pm$ standard deviations (n=3). Pearson correlation analyses were performed using the SPSS program. The correlation between soil enzyme activity and heavy metals content were analyzed by the Redundancy Analysis (RDA) lineal ordination method using the R package vegan. NMDS plots were constructed to investigate the similarities between different soil samples using the free software PAST (Copyright Hammer and Harper, <http://folk.uio.no/ohammer/past>). The similarities were measured using the Bray-Curtis index.

3. Results

3.1. The soil properties and heavy metal contents of Yunnan samples

The soil properties were shown in Table S1 in the Supporting information. The contents of As, Cd, Cu, Cr, Hg, Ni, Pb and Zn (Table S1) of the 47 soil samples were compared with those of the class III soil and environment quality criteria of China (GB15618-1995), the threshold values of which were $\text{As} \leq 30 \text{ mg/kg}$, $\text{Cd} \leq 1.0 \text{ mg/kg}$, $\text{Cr} \leq 400 \text{ mg/kg}$, $\text{Cu} \leq 400 \text{ mg/kg}$, $\text{Hg} \leq 1.5 \text{ mg/kg}$, $\text{Ni} \leq 200 \text{ mg/kg}$, $\text{Pb} \leq 500 \text{ mg/kg}$, and $\text{Zn} \leq 500 \text{ mg/kg}$. It was discovered that Cr was not a major heavy metal pollutant and no sites had Cr concentration higher than the class III China soil and environment quality criteria. On the other hand, Cd was the most serious metal pollutant in all the sites except site Rs1. All the 15 Pb polluted soils were coupled with Zn pollution, similar to what reported by other researchers (Zu et al., 2011), one of whom observed strong significant correlation between Pb and Zn contents in topsoil with RTS (ratio of mean trace element in topsoil to subsoil) ≥ 1.0 (Zu et al., 2011). Moreover, all the

areas were polluted to some extent except for Rs1, which was located the farthest (8.67 km straight-line distance) from the Pb-Zn ore mines (Fig. S1).

3.2. Correlation between heavy metals and physicochemical properties

Correlation analysis (Table 1) between heavy metals and pH indicated that most heavy metals were not significantly correlated with soil pH, except that As, Hg and Pb contents were negatively correlated with pH ($P<0.05$), which might be explained that most heavy metals were in active state under neutral to slightly acidic soil pH. The insignificant effects of neutral to slightly acidic soil pH on heavy metals had been reported by other researchers (Bai et al., 2011; Kumpiene et al., 2008; Rennert et al., 2010). In addition, correlation analysis between heavy metals and nitrogen showed that TN affected the concentration of Hg ($P<0.01$), Ni ($P<0.05$), Cu ($P<0.05$), As ($P<0.05$) and Cd ($P<0.05$) the most significantly. NO_3^- -N had significantly positive correlations with the concentration of Cr ($P<0.01$). Cr concentration also had significantly positive correlation with soil AK. No heavy metals showed obvious correlation with soil AP contents. Previously, heavy metals were reported to be significantly correlated with SOM (Bai et al., 2011; Esmaeili et al., 2014; Takamatsu et al., 2010). However, the results in this study were exactly the opposite, which might be attributed to the fact that the SOM in this study (the average value $< 2.41\%$ in Table S1) was much lower than those in previous studies and fertilizers needed to be applied to guarantee crop growth.

3.3. Spatial distribution of heavy metals

Samples could be divided into four groups based on the NMDS analysis of the

concentrations of As, Cd, Cu, Cr, Hg, Ni, Pb and Zn (Fig. 1). The total heavy metal concentration of As, Cd, Cu, Cr, Hg, Ni, Pb and Zn in group I to IV were respectively 391-1900.9 mg/kg (light pollution), 4223.8-13615.8 mg/kg (medium pollution), 33527.9-34653.4 mg/kg (high pollution) and 134492.4-235576.9 mg/kg (extreme pollution), which were above the criteria of Soriano et al. (2012) who classified the polluted soils into four groups of light, medium, high and extreme pollution, which were below 1501 mg/kg, 1501-3003 mg/kg, 3003-11550 mg/kg, and above 11550 mg/kg respectively. Therefore, the soils in this study were more seriously polluted.

Table S2 showed that Pb, Cd and Zn were the primary pollutants and were very high in more than half of the samples ($C_f > 4$). 13 of the sites had high As pollution ($C_f > 4$). And sample Bf5 and Bf6 were extremely polluted by almost all the metals, which was explainable as the sample site of Bf5 was next to the waste liquid pool of the Pb-S factory and Bf6 was collected from the mine dump tip. PLI results showed that there were only one unpolluted site (field control-Rs1), 25 moderately polluted sites, 5 highly polluted sites and 16 extremely polluted sites.

3.4. Heavy metal correlation analysis

Correlation among heavy metals (Table S3) showed that all the metals had significant positive relationship with each other except for the Ni-Pb and Cd-Pb pairs. As, Cu and Hg contents were positively correlated with other 7 metals at $P < 0.01$. Wei et al. (2009) also reported that As and Cu contents were positively correlated with Cd, Cr, Cu(As), Pb and Zn contents from Pb-Zn smelters, mines, and the tailing dam in Shuikoushan of Changning County, Hunan Province in central south of China. The

strong positive correlation among the heavy metals indicated their common mining origins.

3.5. Soil enzyme activities analysis

As was shown in Table S4, PPO activity was detected in all the samples except for Dm5 and varied significantly from sample to sample. Catalase activity was commonly seen in almost all samples except for Bf5. Acid phosphatase activity was the second commonest enzyme, vastly different in different samples as well. In C transformation, cellulase activity was not detected in most samples or showed very low activity when detected. In N transformation, the activity of urease was higher than that of protease. Soil enzymes activities could be used as ecological health indicators for terrestrial ecosystems under heavy metal pollution.

3.6. Soil enzyme activities correlation matrix

As shown in Fig. 2, PPO activity was found positively correlated with AK, pH, TOC, TN and AP contents in soil ($P<0.05$) in this study. Amylase activity was positively correlated with pH, TOC, TN and AP ($P<0.05$), contrary to Jusselme et al. (2013) who discovered that amylase activity had significantly negative correlation with soil organic matter, TN, AP and AK. Catalase activity had strong and weak positive correlations with pH and TN respectively. NAG activity was negatively correlated with pH, which coincided with Kuperman and Carreiro's (1997) results, but was in contradiction with Xu et al's (2015) discoveries. BG activity was positively correlated with SOM, consistent with what was reported by other researchers (Jusselme et al., 2013; Kuperman and Carreiro, 1997; Xu et al., 2015). Acid

phosphatase activity was found positively correlated with AK, which might be attributed to the different soil types and environmental conditions. Invertase activity had positive correlation with AK. Whereas, cellulose, protease and urease activities were all found to have no significant correlation with soil properties. Similar to the results of many other studies, pH was the relatively most important environmental factor for PPO, NAG, amylase and catalase activities in this study.

A redundancy analysis (RDA) ordination of the 47 soil samples was conducted to test the effects of different heavy metals on different soil enzyme activities (Fig. 3). The lengths of the arrows indicate the relative importance of each heavy metal to soil enzyme activities. It could be seen that enzymes activities in the samples of the light pollution group showed no obvious correlation with different heavy metals. Along with the heavy metal pollution increase, the correlation between metals and enzymes activities was more obvious. PPO, catalase, amylase and phosphatase activities were correlated with heavy metal contents. Other enzymes activities had no correlation with heavy metals.

In the case of pearson correlation analysis between heavy metals and soil enzymes activities (Fig. 4), it was revealed that PPO activity was the highest sensitive to heavy metals, compared with catalase, amylase and acid phosphatase activities, and showed significantly positive correlation with almost all the metals ($P<0.05$) except for Cr contents. Catalase activity, the second sensitive enzyme activity, was found to have positive correlation with As, Cd, Ni, Pb and Zn contents ($P<0.05$). Amylase activity was found to have positive correlation with As, Cd, Pb and Zn contents ($P<0.05$). As

is depicted in Fig. 4, acid phosphatase activity showed positive correlation with only As, Pb and Zn contents ($P<0.05$) though it had been used as a bioindicator for soil heavy metal pollution due to its significantly positive or negative correlation with As, Cd, Cu, Pb and Zn contents (Hu et al., 2014; Lee et al., 2002; Ye et al., 2015). Urease activity had been used as bioindicators in many other researches (Hu et al., 2014; Liang et al., 2014; Majera et al., 2002; Tripathy et al., 2014), but in this study, the effects of heavy metals on urease activity was insignificant. NAG, invertase, cellulase and protease activities demonstrated no significant correlation with heavy metals, which was also different from previous reports. It might be caused by the differences in soil physicochemical properties, environmental influences and pollution history.

3.7. Microcosm verification experiments

According to the pollution situations at the sample sites and the investigation results depicted in Fig. 3 and Fig. 4, the correlation between three heavy metals (Cd, Pb and Zn) contents and six soil enzymes (PPO, catalase, amylase, acid phosphatase, BG, and urease) activities were chosen to be tested in microcosms experiments. Though some heavy metals in low concentration could increase certain enzyme activities (Cd and Zn for amylase, acid phosphatase, catalase and PPO; and Pb for BG), enzyme activity assessment (Fig. 5) showed that the overall activities of these six enzymes decreased along with the increase of Cd, Pb and Zn concentrations, consistent with the findings of previous studies (Pan and Yu, 2011). The only exception was that higher catalase activities were coupled with higher Pb concentration.

The microcosm pearson correlation analysis (Fig. 6) showed that the responses of enzymes activities to heavy metals were very different from the above Pb-Zn ore field investigation results. PPO, amylase and acid phosphatase activities had no significant correlations with Cd, Pb and Zn contents, though they were found to have significant correlations with certain heavy metals after long-term pollution in the above field investigations. However, urease (one kind of hydrolase) and catalase (one kind of oxidoreductases) activities had significant correlations ($P<0.05$) with the above three metals in short-term simulation experiments using different soils under different conditions.

4. Discussion

4.1 Cause analysis of soil pollution in mining areas

From Table S1, it could be seen that all the sample sites around the Pb-Zn ore were polluted to some extent, except for the farthest control sample site-Rs1. Therefore, mining could be considered as the most important source for heavy metal pollution in this studied area. Many other researches considered mining as one of most significant sources of heavy metal pollution in soils, especially in China (Li et al., 2014). Runoff or frequent flooding is another reason for soil pollution. In Vietnam, frequent flooding during the rainy seasons led to the collapse and malfunction of dikes constructed around mines and consequently heavy metal pollution in low streams and farmland areas (Kien et al., 2010). Zuazo et al. (2011) also reported that in the coastal areas of Granada and Malaga, agricultural runoff was the major reason for heavy metal pollution in the agroecosystem. Air also plays an important role in long distance

heavy metal pollution in soils (Soriano et al., 2012; Zhang et al., 2011). Heavy metals are emitted into the atmosphere as aerosols through human activities (Soriano et al., 2012) or just as wind-blown dust from mine tailings (Candeias et al., 2014). Metal deposition is an important contributor to soil contamination and transfer of metals from the atmosphere into soil is a significant part of their biogeochemical cycles (Cizmecioğlu and Muezzinoglu, 2008). Therefore, though mining activities are considered as typical local point source pollution, researches on soil heavy metal pollution in mining areas should also consider the influence of human activity, runoff and wind to have more accurate findings.

4.2 Effects of soil physicochemical properties on heavy metal content in soils

SOM is a key determinant of soil fertility and environmental quality as it is an important plant and microbial nutrient source. Some researchers have proved that SOM could act as a major sink for heavy metals (i.e., Cd, Cr, Cu, Ni, Pb and Zn) due to its strong complex capacity for metallic contaminants (Bai et al., 2011). But relationship between SOM and heavy metal ions was not always the same in different samples (Bai et al., 2011; Esmaili et al., 2014; Takamatsu et al., 2010). Fu et al. (2014) investigated the heavy metal pollution in the waters and sediments of Jialu River, and discovered that nitrogen had significant correlation with many metals, for example, TN with As content, and NO_3^- -N with Hg and Zn contents in the aquatic system. In the sediments, TN and As content ($P<0.05$), TN and Cu content ($P<0.05$), and TN and Ni content ($P<0.05$) also showed significantly positive correlation. As N is typically of biological origin (metabolism and decomposition of organisms) (Fu et

al., 2014), its correlations with heavy metals in this study might be explained by the fact that soil nitrogen was the essential nutrient for microorganisms or plants activities (Ye et al., 2015). Then microorganisms and plants' activities changed the physicochemical properties of the soil. Consequently, N indirectly led to the changes of heavy metal contents in soils. P and K are fundamental macronutrients that affect all aspects of plant growth, environmental sustainability, human health and well-being (Zhang et al., 2015a). Actually, K constitutes about 2.1–2.3% of the earth's crust and soil K reserves are generally large. However, 3/4 of the paddy soils in China and 2/3 of the wheat belt in Southern Australia were reported to be deficient in K availability (Zorb et al., 2014). In addition, pH affects the existence state of heavy metals in soil. Although the relationships between heavy metal ions and P, K and pH has been extensively studied before (Bai et al., 2011; Chapman et al., 2013; Kumpiene et al., 2008; Rennert et al., 2010), these relationships could not be extensively applied to different mining areas and regions. The same is true with the correlations among different heavy metals. Therefore, it could be concluded that though soil physicochemical properties played a role in soil heavy metals deposition, their effects were variable under different conditions and was not a suitable indicator for heavy metal pollution.

4.3 Assessment of heavy metal pollution

In this study, the mean heavy metal contents in soils were found to be in the order of Zn> Pb> Cr> As> Ni> Cu> Cd > Hg. In comparison, other similar Pb-Zn mineralized and polluted zones, such as the Pb-Zn sulfide bearing mineralized zone of

421 Indian plate in the Pazang and Lahor sites, had heavy metal concentrations in the
 422 order of $Zn > Cu > Cr > Pb > Ni > Cd$ (Muhammad et al., 2011). It could be seen that
 423 the heavy metal contents in soils were different even in different mining areas of the
 424 same metals. The soil heavy metal contents were also different in mining regions of
 425 other metals. For instance, in a gold mining area located at N $34^{\circ}23'$ – $34^{\circ}40'$ and E
 426 $110^{\circ}09'$ – $110^{\circ}25'$, the concentrations of metals in soil were in the order of $Hg > Cd >$
 427 $Pb > Cu > As > Cr > Zn$ (Wu et al., 2010). In a copper mining area located in Turkey's
 428 eastern Black Sea region, the metal concentrations were in the order of $Cu > Zn > Cr >$
 429 $Ni > As > Pb$ (Koz et al., 2012). Li et al. (2014) reported that in 72 examined mines
 430 of China, the heavy metal levels of contaminated soil were generally in the order of
 431 $Zn > Pb > Cu > As > Ni > Cr > Cd > Hg$. In 3 Iranian mine soils, 16 Spanish mine soils,
 432 70 South Korean mine soils, 3 Vietnamese mine soils and 5 Indian mine soils, the
 433 heavy metal concentrations were in the order of $Pb > Zn > As > Cu > Hg > Cd$, $Pb > Zn >$
 434 $As > Cu > Cr > Hg > Ni > Cd$, $Zn > Pb > Cu > As > Ni > Cd > Hg$, $Zn > Pb > As > Ni > Cr >$
 435 $Cu > Cd$, and $Cr > Ni > Zn > Pb > Cu > As > Cd$ respectively. Moreover, Soonthornnonda
 436 et al. (2008) presented an exponential wash-off rate equation based on the linear
 437 build-up of pollutant mass and applied it to residential and open land areas, trying to
 438 explain the differences in the content of each metal in soils. A transport coefficient
 439 was defined and used by Revitt et al. (2014) to predict the removal efficiency of
 440 metals from a surface, with a heavy metal concentration in the order of $Pb > Zn > Cu >$
 441 $Ni > Hg > Cd$ in the soils, but its applicability needed to be tested. In contrast, PLI
 442 was a simple and useful means to assess the overall level of heavy metal pollution.

Therefore, it could be concluded that it was of no significant value to compare the pollution levels of each heavy metal, but the overall pollution level evaluation might be of referential value for ecosystem restoration.

4.4 Effects of soil physicochemical properties on soil enzymes activities

Hu et al. (2014) noted that soil physicochemical properties played only a minor role on soil enzyme activities, while other researchers held a different opinion. In this study, the relationships between soil physicochemical properties and soil enzymes activities were found to be different from many other studies. For instance, the relationships between amylase activity and SOM, TN and AP, catalase activity and pH and TN, NAG activity and pH, acid phosphatase activity and AK, invertase activity and AK, all disagreed with the results of Jusselme et al.(2013), Liang et al. (2014) and García-Gil et al. (2000), Xu et al. (2015) and Li et al. (2009), Kuperman and Carreiro (1997), and Cang et al. (2009). Comparatively, pH was the most significant environmental factor for soil enzyme activities, as it could affect the dissociation state of the enzyme active sites and enzyme stability. However, its effects varied in different environment conditions. The reasons for these differences were as follows: (i) the methodologies for enzyme activity measurements in different studies were not entirely the same; (ii) soil physicochemical properties were different in different studies. Therefore, it could be concluded that the relationships between environmental factors and soil enzyme activities were not universally applicable.

4.5 Correlation analysis of heavy metals and soil enzyme activities

Correlation between soil enzymes activities and heavy metals had been reported by

many other researchers (Hu et al., 2014; Lee et al., 2009; Liang et al., 2014). Although many kinds of enzymes activities had been used to assess the level of heavy metal pollution in recent years, such as amylase, cellulase, invertase, NAG and BG (involved in C recycling), urease, protease and nitrate reductase (involved in N recycling), acid/alkaline phosphatase (involved in P transformation), catalase and dehydrogenase (involved in metal oxido-reduction), there had been no reports on PPO's response to long time heavy metals. PPO activity was found to be sensitive to long-term heavy metal pollution in this study.

For the correlation between catalase activity and heavy metals under similarly low SOM condition, the response of catalase activity to heavy metals coincided with Xian et al.'s (2014) findings that catalase activity was positively correlated with soil Cd and Pb contents within the 5th ring road of Beijing, but was different from Hu et al.'s (2014) results that catalase activity had significantly negative correlation with Cd, Cu, Ni, Pb, Zn contents, and from Liang et al.'s (2014) results that agricultural irrigation by wastewater from pharmaceutical, chemical, textile and electronic factories led to the accumulation of As, Cr, Cu, Zn contents and other heavy metals in the soils and catalase activity in the polluted soils had significantly negative correlation with these four metals. The above discrepancies of relationships between soil enzymes activities and heavy metals might be caused by the different lengths of pollution time and the long-term heavy metal remediation effects of catalase. The effects of soil physicochemical properties on correlations between enzymes activities and metal pollution still needed to be investigated. Amylase, BG, NAG, invertase, cellulase, acid

phosphatase, protease and urease activities also showed similar or different correlations with certain metals, in comparison with other researches' results. In a word, it could be seen that till now, there had been no uniform standards in soil enzyme selection, which made it difficult for researchers to use soil enzymes activities to accurately assess heavy metal pollution levels in specific areas. But according to published papers (Alvarenga et al., 2012; Hu et al., 2014; Lee et al., 2009; Majera et al., 2002; Pérez-de-Moaa et al., 2006), all the enzymes that had been used as indicator for heavy metal pollution could be classified into two major types of enzymes, oxidoreductases and hydrolases. Our results showed that oxidoreductases (PPO and catalase) activities were more sensitive than hydrolases (amylase, BG, cellulose, invertase, NAG, phosphatase, protease and urease) activities to heavy metal pollution. Only oxidoreductases could directly change ion valence and get involved in heavy metal detoxification. Other enzymes mainly participate in the metabolic activities of microorganisms and plants. Therefore, based on the above investigations, analysis and comparative studies, this paper proposes a hypothesis that oxidoreductases, not a specific enzyme activity like that in previous studies, could be used as a "core enzyme" bioindicator for heavy metal pollution level evaluation.

To verify this hypothesis, microcosm experiments using different kinds of soils were further conducted. Fig. 5 showed that one kind of oxidoreductases-catalase activity was still sensitive to soil heavy metal pollution and increased along with Pb concentration. Though Chen et al. (2014b) and Zhang et al. (2015b) found that soil catalase activity was increased by Pb under certain conditions, there had been no

509 reports that catalase activity would increase along with Pb concentration increase.

510 Catalase breaks down H_2O_2 and prevents it from poisoning organisms. The

511 accumulation of H_2O_2 in the roots of *Vicia faba* was observed during its early stage of

512 heavy-metal exposure to Pb (Pourrut et al., 2008) and H_2O_2 increases in root tissues

513 had also been detected after long-term exposure (14 days) to Cd in pea plants

514 (Lopez-Orenes et al., 2014). Therefore, it could be concluded that the increase in

515 catalase activity might be related to soil microbial detoxification. From the microcosm

516 experiments and analyses, it could be concluded that the hypothesis was basically

517 applicable, i.e., oxidoreductases (no matter which specific oxidoreductase it was)

518 might be considered as a “core bioindicator enzyme” for heavy metal pollution as it

519 was closely related to heavy metal detoxification under different soil conditions.

520 There were no doubts that other oxidoreductases still needed to be tested and verified

521 in future studies except for catalase and PPO used in this study. Though this

522 hypothesis also needed to be verified in different contamination areas and through

523 long-term microcosm experiments as it was based on field investigations of only

524 heavily contaminated and nutrient-poor soils and short-term microcosm experiments,

525 this finding would be helpful for future soil enzyme selection in pollution areas.

526 Moreover, Urease (for N recycle), amylase (for C recycle) and acid phosphatase (for P

527 recycle) could be used as auxiliary enzymes to improve the correlation accuracy

528 between enzymes activities and different heavy metals in different heavy metal

529 pollution environments. However, this hypothesis could only achieve qualitative

530 characterization of heavy metal polluted soils by comparing enzyme activity changes

between the polluted areas and unpolluted areas, and quantitative standards to assess heavy metal contamination degree through the numeric values of soil enzymes activities was yet to be established. Statistical analysis of heavy metal content and enzyme activities data in multi regions need to be further carried out to make quantitative description of enzyme activities possible.

5. Conclusions

Long-term Pb-Zn ore mining in Mengnuo town of Yunnan province, China had led to a point source heavy metal pollution, and the pollution load index indicated that Pb, Cd, Zn and As were the primary metal pollutants in this region. Field investigations showed that PPO, amylase, acid phosphatase and catalase activities could be considered as bioindicators for multiple Pb, Cd, Zn and As contamination. Oxidoreductases could be considered as a “core enzyme” bioindicator for heavy metal pollution. Hydrolases (involving in C, N, P and S recycling) activity could be used as supplements to improve the accuracy in correlation analysis. But this hypothesis was based on the investigations and analysis results in only one heavily contaminated and nutrient-poor mining area and short-term microcosm simulation experiments, the applicability of the hypothesis still needed to be verified and evaluated in other different mining areas.

Acknowledgements

This work was supported by the China Geological Survey Grant (no.12120113015200) and National Science Foundation of China (no. 31270533).

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Figure captions:

Fig. 1. Non-metric multidimensional scaling (NMDS) analysis of 47 soil samples based on heavy metal concentrations in Pb-Zn mining area. Heavy metal concentration was mg/kg; I, light pollution; II, medium pollution; III, high pollution; IV, extreme pollution.

Fig. 2. Heat map of correlation between physicochemical property and soil enzyme specific activities in soil samples (n= 47) based on pearson correlation coefficients. Red, strong positive correlation; yellow, weak correlation; green, strong negative correlation; *, significant correlations (at $P < 0.05$).

Fig. 3. The redundancy analysis (RDA) with 47 soil samples as affected by heavy metal, based on soil total enzymes specific activities analysis. The length of the arrows indicated the relative importance of each heavy metal on soil total enzymes activities.

Fig. 4. Heat map of correlation between heavy metal and soil enzyme specific activities in soil samples (n=47) based on pearson correlation coefficients. Red, strong positive correlation; yellow, weak correlation; green, strong negative correlation; *, significant correlations (at $P < 0.05$).

Fig. 5. The effects of different heavy metal contents on soil enzymes specific

activities under microcosm experiments.

5a) The influence of heavy metals on soil amylase activity ($\text{mg glucose g}^{-1}24\text{h}^{-1}$)

5b) The influence of heavy metals on soil β -glucosidase activity ($\mu\text{mol g}^{-1}\text{h}^{-1}$)

5c) The influence of heavy metals on soil urease activity ($\text{mg NH}_3\text{-N g}^{-1}24\text{h}^{-1}$)

5d) The influence of heavy metals on soil phosphatase activity ($\mu\text{mol g}^{-1}\text{h}^{-1}$)

5e) The influence of heavy metals on soil catalase activity ($\text{mL g}^{-1}\text{h}^{-1}$)

5f) The influence of heavy metals on soil polyphenoloxidase activity ($\mu\text{mol g}^{-1}\text{h}^{-1}$)

Fig. 6. Heat map of correlation between heavy metal and soil enzyme specific activities in soil samples ($n=60$) of microcosm experiment based on pearson correlation coefficients. Red, strong positive correlation; yellow, weak correlation; green, strong negative correlation; *, significant correlations (at $P < 0.05$).

Fig. 1

Fig. 2

Fig. 3

Fig. 4

5a)

5b)

5c)

5d)

5e)

5f)

Fig. 5

Fig. 6

Table 1 Correlation between heavy metals and physicochemical properties of soil samples.

	SOM	TN	NH ₄ ⁺ -N	NO ₃ ⁻ -N	AP	AK	pH
Cr	0.006	0.071	0.021	0.373**	-0.061	0.387**	-0.029
Ni	-0.249	-0.289*	-0.034	-0.098	0.003	-0.156	0.119
Cu	-0.265	-0.369*	-0.052	-0.112	-0.003	-0.208	-0.033
Zn	-0.193	-0.253	-0.022	-0.136	0.012	-0.204	0.072
As	-0.112	-0.320*	-0.057	-0.13	0.041	-0.213	-0.313*
Cd	-0.257	-0.296*	-0.031	-0.093	-0.015	-0.168	0.103
Hg	-0.184	-0.379**	-0.07	-0.131	0.026	-0.241	-0.299*
Pb	-0.005	-0.2	-0.034	-0.129	0.059	-0.195	-0.332*

* $P < 0.05$; ** $P < 0.01$