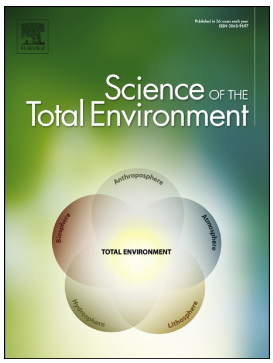

A novel extracellular enzyme stoichiometry method to evaluate soil heavy metal contamination: Evidence derived from microbial metabolic limitation

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A novel extracellular enzyme stoichiometry method to evaluate soil heavy metal contamination: Evidence derived from microbial metabolic limitation

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Abstract

Heavy metal contaminates have become a significant threat to soil ecosystems due to their chronicity and universality in soil. Soil microbial metabolism plays a vital role in biogeochemical cycles and soil functions. However, the response of microbial metabolism to heavy metal contamination in soil remain elusive despite potentially offering important insight into the health and ecological consequences of soil ecosystems under such contamination. This study used extracellular enzyme stoichiometry models to identify the response of microbial metabolism to various heavy metal contaminants, while also revealing potential implications of heavy metal contaminates in soil ecosystems. Results showed that microbial metabolism was restricted by soil carbon (C) and phosphorus (P) within a heavy metal polluted area in Northwest China. Heavy metal stress significantly increased microbial C limitation while decreasing microbial P limitation. However, microbial C and P limitations both responded consistently to different heavy metals (i.e., Cd, Pb, Zn, and Cu). Heavy metals had the greatest effect on microbial C limitation (i.e., 0.720 of the total effects) compared to other soil properties, and soil with the lowest heavy metal concentration exhibited the lowest microbial C limitation, and vice versa. These results indicated that microbial metabolic limitation can robustly and sensitively reflect the degree of heavy metals pollution in soil. Additionally, increased microbial C limitation caused by heavy metal contaminants could potentially escalate C release by promoting soil C decomposition as well as increasing investments in enzyme production and the maintenance of metabolic

processes. Consequently, potential C loss induced by heavy metal pollution on soil ecosystems may be extensive and significant. Generally, our results suggest the usefulness of extracellular enzyme stoichiometry as a new method from which to evaluate heavy metal soil pollution, while microbial metabolic limitation could potentially be a promising indicator.

Key words: Soil heavy metals; Microbial metabolism; Soil C turnover; Biological indicators

1. Introduction

Heavy metal pollution has become a critical issue within many soil ecosystems (Facchinelli et al., 2001; Schloter et al., 2017). Even though heavy metals exist naturally in soil, anthropogenic activities, such as industrialization, agriculture, mining, and urbanization, are by far the greatest source (Bhuiyan et al., 2010; Li et al., 2014; Ali et al., 2017). Heavy metal pollution leads to a deterioration in soil quality and a loss of soil functions (Schloter et al., 2017; Wang et al., 2017), which is harmful to human health, particularly through soil-plant systems and soil-crop-food chains (Wood et al., 2016; Shen et al., 2017). Remediation of heavy metal contaminated soil has thus become an important and extensively studied topic (Zota et al., 2009; Violante et al., 2010; Ju et al., 2019). At the same time, the persistent threat of heavy metal contamination in soil ecosystems has prioritized the development of strict directives for soil protection and pollution assessments, which has recently been promoted by the European Union (EU) (Schloter et al., 2017). Accordingly, the importance of developing reliable, robust, and resilient indicators to monitor heavy metal soil contamination has been emphasized in order to establish early warning and assessment systems.

These indicators should by definition allow for easy measurements while being accurate and sensitive for the purposes for which they were developed. It would also be advantageous if investigative findings reflected the long-term ecological effects of heavy metal contamination on soil ecosystems. Being the most abundant and diverse forms of life on the planet, soil microorganisms play crucial roles in soil functions and

soil health through their metabolic processes (Smith et al., 2015; Beattie et al., 2018). Furthermore, heavy metal contamination can affect the various functions and stability of soil ecosystems because metal ions are in many ways detrimental to soil microorganisms (Kandeler et al., 2000; Jin et al., 2018; Duan et al., 2018). Accordingly, previous studies have attempted to develop biological indicators of soil pollution, mainly focusing on soil microorganisms; and heavy metal contamination has frequently been found to be the impetus in the alteration of microbial community structure through a reduction in biomass and diversity (Beattie et al., 2018; Kasemodel et al., 2019). Cultivation-based studies have in the past provided compelling evidence of such effects on soil microbial communities through the application of various methods (Beattie et al., 2018; Ju et al., 2019). Compared to microbial community structure, microbial activities and metabolic processes are more sensitive to changes in soil and environmental stress (Newman et al., 2010; Guttman et al., 2014). For example, our previous studies observed that the microbial community structure between the rhizosphere and bulk soil were consistent (Cui et al., 2019b), while their metabolic limitations varied considerably within these two microhabitats (Cui et al., 2019c). Furthermore, microbial metabolism drives soil organic matter (SOM) decomposition as well as generating available nitrogen (N) and phosphorus (P), which are vital to biogeochemical cycling and functional soil maintenance (Sinsabaugh et al., 2009; Cui et al., 2018a; Zhang et al., 2019). Therefore, microbial metabolism could potentially be a better indicator than its community structure in reflecting changes in soil functions as well as the impacts of heavy metal

pollution on soil ecosystems.

Extracellular enzymes produced by microorganisms are extremely sensitive to changes in the soil environment, and they are crucial participants in soil nutrient cycles as well as functional sustainability (Sinsabaugh et al., 2009; Duan et al., 2018; Cui et al., 2018a). Over the past two decades, extracellular enzymes have increasingly been used to evaluate environmental pollution caused by heavy metals (Hagmann et al., 2015; Fang et al., 2017). Long et al. (2009) reported that alkaline or acid phosphatase, which play an important role in the decomposition of organic P compounds, can be used as bioindicators of heavy metal pollution. Also, Xian et al. (2015) and Liang et al. (2014) both found that catalase is able to decompose H_2O_2 and protect organisms against damage. Additionally, catalase has also been used as a bioindicator to detect the presence of a variety of heavy metal pollutants. Hu et al. (2014) proposed using dehydrogenase as a catalyst for substrate dehydrogenation, suggesting that this enzyme can be used as another indicator of heavy metal contamination. However, enzymatic activities vary in their response to heavy metals in soil, which depend on differences in soil properties, heavy metal species, and concentrations, consequently leading to inconsistencies in findings as well as incomparability of indicators (Schloter et al., 2017; Duan et al., 2018). Therefore, integrating diverse enzymes that are widely representative of microbial metabolism into a comprehensive indicator is both necessary and promising for the assessment of heavy metal toxicity levels in soil microbes and the ecological effects of heavy metal pollution in soil systems.

Ecoenzymatic stoichiometry is an emerging methodology that incorporates multiple parameters associated with enzyme activities into specific microbial metabolic characteristics (i.e., microbial metabolic limitation) (Sinsabaugh et al., 2009; Moorhead et al., 2016), reflecting the intensity of microbial metabolism as well as the ability of microorganisms to obtain energy and nutrients. This new methodology has been used to assess elemental cycles and energy flow within ecological systems (Tapia-Torres et al., 2015) and to identify microbial response to environment change (Cui et al., 2018a, 2019a). However, no studies have yet been published on ecoenzymatic stoichiometry in association with contaminated environments. Moreover, in heavy metal contaminated soil in particular, little is known about variations in microbial metabolic limitation and associative implications on soil ecosystems. Thus, ecoenzymatic stoichiometry could be used to monitor and assess impacts of heavy metal pollution on soil ecosystems. Additionally, the metabolic limitation of soil microorganisms is both typical and highly variable in diversified ecosystems (Sinsabaugh et al., 2009; Moorhead et al., 2016; Cui et al., 2018a). Our previous studies indicated that vegetation restoration significantly altered microbial metabolic limitations through its effect on soil nutrient availability and their ratios (Cui et al., 2018a). Changes in soil temperature and moisture also markedly affected microbial metabolic limitations in rhizosphere (Cui et al., 2019c). These results indicated that microbial metabolic limitation is highly sensitive to environmental change. Consequently, studying the response of microbial metabolic limitation to heavy metal contamination is paramount for understanding the effects of heavy metal

contamination on soil ecosystems.

Accordingly, in this study, we established field experimental plots within a series of heavy metal concentration gradients in the largest Zn mining area in Northwest China to identify the response of microbial metabolic limitation to different heavy metal elements and pollution levels while exploring the potential effects of heavy metal pollution on soil ecosystems. We hypothesized that: (1) Heavy metal soil pollution can increase microbial metabolic limitations due to the damaging effects that heavy metals have on soil environments; (2) the response of microbial metabolic limitation can be consistent to different heavy metals because ecoenzymatic stoichiometry reflects the comprehensive metabolic characteristics of microorganisms; and (3) heavy metal pollution in soil can affect soil nutrient turnover rates given that heavy metals can affect both microbial metabolism and activity.

2. Materials and methods

2.1. Field experiment area

The study area was located in Feng County (106°24′-107°07′E, 33°34′-34°18′N), the largest Zn mining area in Northwest China, and this mining area has been accompanied by serious Pb, Cd, and Cu pollutants (Fang et al., 2017). The study area has a warm temperate semiarid climate with an annual rainfall of 612.3 mm and a mean annual temperature of 11.4 ℃. The main soil contaminates in Feng County derive from Zn smelters and processing waste emissions from mining activities over the past 30 years (Shen et al., 2017).

Our study was based on a series of field surveys (from September 2011 to June

2014) which aimed to identify the level of soil pollution within an area of approximately 2.5 km^2 in the Dongling Pb/Zn Smelter (Ltd) (Shen et al., 2017). Accordingly, a five-year phytoremediation trial (from June 2012 to June 2017) in the vicinity of the smelter was conducted. Specifically, six phytoremediation plots were established in the study area under consistent initial soil properties (i.e., consistent heavy metal soil concentrations, soil nutrients, soil physical properties, etc.). The distance of the plots to the smelter was similar as well as their slopes, slopes, gradients, and altitudes. The study site was evenly subdivided into about 30 m^2 plots, and each plot was spaced 0.5 m apart between which a 3 m wide buffer guard was established. Seven phytoremediation treatments (*Lolium* (*Lolium perenne* L.), *Brassica* (*Brassica napus* L.), *Artemisia* (*Artemisia argyi*), *Silphium*, *Taraxacum*, *Populus*, and a control) were established in triplicate under a thoroughly randomized design (Fig. S1). Seeds were evenly sown throughout each sample plot, and redundant seedlings were removed according to the standard of 60 × 40 cm spacing between plants after they first emerged. The biomass of all herbaceous plants (i.e., *Lolium, Brassica, Artemisia, Silphium* and *Taraxacum*) as well as the surface litter of *Populus* were removed from plots during the autumn of each year. Other managerial processes (e.g., fertilization and watering) utilized under conventional field management practices were consistent throughout all treatments.

2.2. Soil and plant sampling

A series of heavy metal concentration gradients subsequently appeared under field experiment conditions as phytoremediation progressed, and this was due to the

differing heavy metal uptake efficiencies of the selected plants. Soil samples were collected in June 2017 from the 0 to 20 cm soil profile using a 5 cm diameter stainless steel corer after removing litter. We randomly established three to four quadrats in each plot before soil and plant sampling. Additionally. six soil cores were collected from each quadrat along an S-shape pattern and then combined into a unified sample. Nine to twelve mixed soil samples were subsequently collected from each treatment. Each soil sample was further divided into two subsamples after removing debris. The first subsamples taken were placed in ice boxes in the field, and then stored at 4 ℃ in the laboratory to determine extracellular enzymatic activities within a period of 15 days. The second subsamples taken were sieved through a 2-mm mesh and air-dried to analyze physical and chemical properties. The core method was used to determine soil bulk density. Plant samples (shoots) were collected from the six treatments (i.e., excluding the control) to determine heavy metal concentrations in plants.

2.3. Analysis of soil physical and chemical properties

The soil moisture was determined by oven drying soils at 110 °C for 48 hours. Soil pH was measured using a compound electrode (InsMark^{TM} IS126, Shanghai, China) in a 1:2.5 mass/volume soil-water suspension. Soil organic carbon (SOC) content was analyzed using potassium dichromate ($K_2Cr_2O_7$) oxidation. Dissolved organic carbon (DOC) was extracted with 0.5 M K_2SO_4, and then measured by a Liquid TOCII analyzer (Elementar, Germany) (Jones and Willett, 2006). Total nitrogen (TN) content was determined using the Kjeldahl method (Bremner and Mulvaney, 1982). Soil NH_4^+-N and NO_3^--N were extracted using 2 M potassium

chloride (KCl), and then were analyzed using a continuous flow autoanalyzer. Available P (Olsen-P) and total phosphorus (TP) were extracted with sodium bicarbonate ($NaHCO_3$) and H_2SO_4-$HClO_4$ (Olsen and Sommers, 1982), respectively, and their specific contents were then analyzed using the molybdenum blue method.

2.4. Determining heavy metal in soil and plants

Soil heavy metal concentrations were analyzed according to the modified Method 3051A (US EPA) (USEPA, 1998). We digested 0.200 g of the soil samples in a 15 ml tri-acid solution (nitric acid (HNO_3), HCl, and perchloric acid ($HClO_4$)) at a volume ratio of 1:3:1. Available heavy metals in soil were extracted with 0.1 M calcium chloride ($CaCl_2$) at a soil ratio of 1:5 (w/v) after shaking for 2 h at 25 °C (Smilde et al., 1992; Houba et al., 1996). Following this, Cu, Cd, Zn, and Pb concentrations were measured using atomic absorption spectrometry (Hitachi, FAAS Z-2000, Japan). The collected plant samples were dried and crushed before being digested in a 10 ml $HClO_4$ and HNO_3 mixture (i.e., at a volume ratio of 1:4). Total heavy metal concentrations for plant samples were quantified using atomic absorption spectrometry.

2.5. Assays of extracellular enzymatic activity (EEA)

The activities of five extracellular enzymes involved in C, N, and P cycling were measured using modified versions of standard fluorometric techniques (Saiya-Cork et al., 2002; Cui et al., 2019a). The detailed experimental methods can be seen in our previous study (Cui et al., 2019a). Finally, the enzyme activities were expressed as nanomoles of substrate released per hour per gram of SOM (nmol g SOM^{-1} h^{-1}; SOM

= 1.724 × SOC).

2.6. Assessment of heavy metal pollution

The single contamination factor ($CF = C_s/C_c$) was used to identify the degree of pollution for each investigated metal. C_s is the average concentration of single heavy metal in the soil sample, and C_c is the average concentration of single heavy metal in the standards (Bhuiyan et al., 2010; Yang et al., 2016). The heavy metal contents in the soil sample were compared with the risk screening values of soil environmental quality risk control standard for soil contamination of agricultural land of China (GB15618-2018). The pollution load index ($PLI = (C_{f1} \times C_{f2} \times C_{f3} \times \cdots \times C_{fn})^{1/n}$) was determined for identifying the overall level of soil pollution at each sampling site, (Bhuiyan et al., 2010). C_f is the heavy metal contamination factor, and n is the number of samples (Yang et al., 2016).

2.7. Calculation of microbial nutrient limitation

Microbial nutrient limitation was quantified by calculating the vector lengths and angles of enzymatic activities for all data. Vector length, representing C limitation, was calculated as the square root of the sum of x^2 and y^2 (Eq. 1), where x represents the relative activity of C versus P-acquiring enzymes, and y represents the relative activity of C versus N-acquiring enzymes (Moorhead et al., 2016). Vector angle, representing N/P limitation, was calculated as the arctangent of the line extending from the plot origin to point (x, y) (Eq. 2). Microbial C limitation increases with the vector length. Vector angles > 45° represent microbial P limitation, and vector angles < 45° represent microbial N limitation. Microbial P limitation increases with the

vector angle, and microbial N limitation decreases with it.

$$Length = \sqrt{(x^2 + y^2)} \quad (1)$$

$$Angle\ (°) = \text{DEGREES (ATAN2 } (x, y)) \quad (2)$$

2.8. Statistical analysis

One-way analysis of variance (ANOVA) was used to determine variations in soil physicochemical properties, heavy metal concentrations in soil and plants, and microbial metabolic limitation among the different treatments, after which means were compared using Tukey's multiple comparisons test. The generalized linear model was adopted to determine the relationships between microbial C limitation and microbial P limitation as well as correlations between microbial metabolic limitation with soil heavy metals. Pearson's chi-squared test was used to determine correlations between microbial metabolic limitation and environmental variables. Significant environmental variables determined by the Pearson's chi-squared test were selected to construct an environmental matrix to conduct variation partitioning analysis (VPA). VPA was used to determine the relative importance of heavy metals, soil physicochemical properties, and plant species in explaining microbial metabolic limitation. Partial least squares path modeling (PLS-PM) further deciphered potential pathways wherein attributes (including soil physicochemical properties and heavy metals) control microbial C and P limitations. All analyses were carried out in R software (v.3.3.2).

3. Results

3.1. Differences in soil heavy metals, physicochemical properties, and heavy metal

uptake by plants

Phytoremediation significantly decreased heavy metal concentrations in soil compared to the control ($P < 0.001$; Fig. 1). The highest total and available heavy metal concentrations were measured in the control treatment: Cd (50.8 ± 3.45 mg kg^{-1}), Pb (142 ± 14.7 mg kg^{-1}), Zn (2902 ± 203 mg kg^{-1}), Cu (78.3 ± 8.07 mg kg^{-1}), $CaCl_2$-Cd (6.21 ± 0.81 mg kg^{-1}), and $CaCl_2$-Zn (10.8 ± 0.63 mg kg^{-1}). The lowest total heavy metal concentrations were measured in the *Lolium* treatment: Cd (24.9 ± 4.83 mg kg^{-1}), Pb (81.5 ± 6.66 mg kg^{-1}), Zn (1652 ± 193 mg kg^{-1}), and Cu (34.9 ± 4.35 mg kg^{-1}). All treatments were classified into two groups according to our assessment: heavy pollution (H; $2 < PLI < 3$) associated with the *Lolium* and *Artemisia* treatments and extremely heavy pollution (E; $PLI > 3$) associated with all other treatments (Table 1). Additionally, results showed that the *CF* values of Cd, Pb, Zn, and Cu were 41.6-84.6, 0.480-0.832, 5.51-9.67, and 0.349-0.783, respectively. This suggested that Cd and Zn were the most severe metal pollutants within the sampling area.

Soil physicochemical properties also significantly varied with phytoremediation ($P < 0.05$; Table 2). For example, SOC (12.3 ± 0.88 g kg^{-1}), TN (1.24 ± 0.04 g kg^{-1}), and DOC (113 ± 3.47 mg kg^{-1}) content was highest in the *Taraxacum* treatment, while TN (0.838 ± 0.07 g kg^{-1}), DOC (95.4±8.38 mg kg^{-1}) and Olsen-P (8.50 ± 1.09 mg kg^{-1}) content was the lowest in the *Artemisia* treatment. Additionally, correlation analysis showed that soil physicochemical properties were significantly correlated to heavy metal concentrations except for TP ($P < 0.05$; Fig. 3). For example, pH was significantly and negatively correlated to soil Cd, Pb, Zn, and Cu concentrations.

Heavy metal concentrations (Cd, Pb, Zn, and Cu) within plant bodies significantly differed among the six plant treatments ($P < 0.001$; Fig. S2). The highest concentrations of Cd (191 ± 21.2 mg kg^{-1}), Pb (181 ± 17.4 mg kg^{-1}), Zn (5510 ± 426 mg kg^{-1}), and Cu (34.5 ± 3.74 mg kg^{-1}) were measured in *Lolium*, the second highest concentrations of Cd (74.0 ± 4.25 mg kg^{-1}), Pb (61.4 ± 5.53 mg kg^{-1}), Zn (4380 ± 407 mg kg^{-1}), and Cu (29.1 ± 2.72 mg kg^{-1}) were measured in *Artemisia*; the lowest concentrations of Cd (16.9 ± 1.89 mg kg^{-1}), Pb (21.0 ± 2.59 mg kg^{-1}), Zn (592 ± 50.8 mg kg^{-1}), and Cu (9.12 ± 0.911 mg kg^{-1}) were measured in *Populus*, *Populus*, *Brassica*, and *Brassica*, respectively.

3.2. Microbial metabolic characteristics

Although extracellular enzyme activities significantly changed among the seven treatments, the five enzyme activities exhibited no consistent change among the different treatments (Table S1). For example, the activities of BG, NAG and AP were the highest in the *Populus* treatment, while the activities of CBH and LAP were the highest in the *Lolium* treatment.

Furthermore, characteristics of ecoenzymatic stoichiometry also differed among the treatments (Fig. 2a). Specifically, vector lengths (0.796 to 0.957) and angles (62.9° to 68.2°) differed significantly among the plant treatments ($P < 0.01$; Fig. 2b and c). All data points were above the diagonal line (with vector angles > 45°), indicating strong P limitation for microbial metabolism to occur in heavy metal contaminated soil. Vector lengths and angles (0.796 ± 0.08 and 62.9° ± 4.3°, respectively) were the lowest in the *Lolium* treatment. On the other hand, the vector length (0.957 ± 0.02)

was the highest in the control treatment, which showed that the highest relative C limitation occurred in the control treatment. The vector angle (68.2° ± 3.3°) was the highest in the *Taraxacum* treatment, which showed the strongest P limitation in this treatment. Additionally, linear regression identified significant negative correlations between vector lengths and angles among the treatments ($P < 0.05$; Fig. 2d).

3.3. Effects of heavy metals, physicochemical properties, and plant species on microbial metabolic limitation

Linear regression analysis identified that microbial C limitation was significantly positively correlated to soil Cd, Pb, Zn, Cu, available Cd, and available Zn ($P < 0.01$; Fig. S3). However, microbial P limitation was significantly negatively correlated to all heavy metals mentioned above except for the soil Cu ($P < 0.05$; Fig. S4). Correlation analysis also showed that microbial C limitation was significantly positively correlated to these heavy metals, while microbial P limitation was significantly negatively correlated to these heavy metals except for Cu ($P < 0.05$; Fig. 3). Furthermore, linear regression identified that *PLI* values were significantly positively correlated to microbial C limitation ($P < 0.01$; Fig. 4a), and significantly negatively correlated to microbial P limitation ($P < 0.01$; Fig. 4b).

The VPA showed that soil heavy metals, physicochemical properties, and plant species explained most of the variation found in microbial metabolic limitation ($P < 0.001$; Fig. 5). Specifically, soil heavy metals, physicochemical properties, and plant species explained 44%, 48%, and 7% of variation in microbial C limitation, respectively (Fig. 5a), and explained 36%, 32%, and 21% of variation in microbial P

limitation, respectively (Fig. 5b). With respect to soil heavy metals, Cd contributed the most to variation in microbial metabolic limitation. Soil Cd, Pb, Zn, and Cu explained 45%, 37%, 43%, and 13% of variation in microbial C limitation, respectively (Fig. 5c), and it explained 33%, 11%, 15%, and 8% of variation in microbial P limitation, respectively (Fig. 5d).

3.4. Direct and indirect relationships of soil heavy metals, physicochemical properties, and plant species alongside microbial metabolic limitation

To further decipher the cascading relationships of microbial C and P limitations alongside soil heavy metals and physicochemical properties, PLS-PM was used to identify both the direct and indirect effects of phytoremediation, heavy metals, soil nutrients, and pH on microbial C and P limitations (Fig. 6a). Phytoremediation directly affected soil nutrient content (-0.088 of the direct effects) and soil heavy metal content (0.321 of the direct effects). Furthermore, soil nutrients positively affected DOC (0.493 of the direct effects) and Olsen-P (0.652 of the direct effects), and negatively affected pH (-0.578 of the direct effects). Heavy metals in soil negatively affected pH (-0.170 of the direct effects) and Olsen-P (-0.223 of the direct effects) while positively mediating microbial C limitation (0.721 of the direct effects). Microbial C limitation and Olsen-P further negatively affected microbial P limitation (-0.336 and -0.572 of the direct effects, respectively). Additionally, soil nutrients and heavy metals directly affected microbial P limitation (0.539 and -0.223 of the direct effects, respectively).

Overall, heavy metals had the greatest and most positive effect on microbial C

limitation (0.720 of the total effects), while Olsen-P had the greatest and most negative effect on microbial P limitation (-0.572 of the total effects; Fig. 6b). Both soil heavy metals and microbial C limitation also significantly affected microbial P limitation (-0.348 and -0.336 of the total effects, respectively).

4. Discussion

4.1. Distinct effects of soil heavy metals on microbial C and P limitations

To gain a comprehensive understanding of microbial metabolic characteristics, we integrated multiple metabolic processes associated with microbial communities using extracellular enzyme stoichiometry modeling. For soil contaminated with heavy metals, our results firstly revealed that heavy metal pollution significantly affected microbial metabolic limitation (Figs. 3, S3 and S4), explaining most of the variation in microbial metabolic limitation (Figs. 5 and 6). Significant correlations between *PLI* and microbial C and P limitations further confirmed the significant effects that heavy metal pollution has on microbial metabolism ($P < 0.01$; Fig. 4). Previous studies indicated that soil microorganisms are highly sensitive to stress caused by heavy metals because these contaminates significantly affect their growth and metabolism through functional disturbances, protein denaturation, or the complete destruction of cell membranes (Kandeler et al., 2000; Jin et al., 2018; Xu et al., 2018a). Microorganisms, being mostly prokaryotic, also participate in changing the valence of heavy metals and redox reactions, thereby altering their associative activities (Gavrilescu, 2004; Jin et al., 2018). Additionally, heavy metal ions bind to cell surfaces not only through electrostatic interaction and complexation but also through

ion exchanges on the cell surface (Xu et al., 2018a). Therefore, there are close interactions between heavy metal ions and soil microorganisms, and heavy metals can significantly alter microbial metabolic limitation as this study has shown (Figs. 3-6).

Specifically, heavy metal pollution positively affected microbial C limitation (Figs. 3, 4a, and S3). Microbial C limitation (0.796-0.957; Fig. 2) was apparently higher in heavy metal contaminated soil compared to uncontaminated soil (the microbial C limitation generally was 0.415-0.786) (Cui et al., 2019a). These findings indicated that heavy metal contamination can stimulate microbial C metabolism. Additionally, heavy metals in soil had the greatest positive total effects on microbial C limitation compared to other environmental factors (Fig. 6b), which further indicated that heavy metals play a significant role in microbial C metabolism. Microbes in heavy metal contaminated soil will accumulate heavy metal ions in their intracellular regions and cytoderms (Kandeler et al., 2000; Jin et al., 2018). The primary mechanisms of accumulation are adsorption, which do not typically depend upon energy metabolism but impede electron and matter transport and absorption processes, which greatly depend on energy (C sources) metabolism (Wang et al., 2001). These two processes will consume additional C sources to cope with the toxic effects of heavy metals. A previous study also indicated that soil microbes under toxic stress will consume more substrates for energy production and the synthesis of redox active-compounds (Bore et al., 2017). These redox active-compounds are responsible for the extracellular disposal of electrons that bypass inhibited electron transport chains within cells. Consequently, the increase in microbial C limitation measured in

heavy metal contaminated soil is primarily attributable to microbial alleviation mechanisms under heavy metal toxicity.

However, negative effects of heavy metal stress on microbial P limitation were identified (Figs. 3, 4b, 5b and S4). Unlike C metabolism, which is primarily involved in energy production through electron transfer, P metabolism is mainly involved in the synthesis of matter through ions (Sinsabaugh et al., 2009; Zhu et al., 2017). There could therefore be three main reasons for the negative effects that heavy metals have on microbial P limitation. Firstly, heavy metal ions can increase the release of phosphate radicals by competing adsorption sites and pH regulation, thereby increasing available P for microbes. This mechanism could be supported by the negative correlation between heavy metals and pH (Fig. 3). Secondly, increased C metabolism induced by heavy metal stress can release more available P from organic matter. As our PLS-PM results show (Fig. 5a), microbial C limitation had a significant negative effect on P limitation, which was also confirmed by our previous study (Cui et al., 2019c). Thirdly, heavy metal stress significantly inhibits microbial growth and proliferation (Yergeau et al., 2014), thus decreasing their need for P. Furthermore, due to the structure of the cell surface and differential principality, heavy metals that come into contacting with cell walls and mucus layers can both be adsorbed and the absorption can occur easily. Many ions on the cell surface, such as N, O, S, and P, can be complexed with heavy metal ions as the coordination number of atoms (Jin et al., 2018). Additionally, phosphoric acid anions and carboxyl anionic groups on microbial cell wall surfaces are negatively charged. Conversely, most heavy metal surfaces carry

a cationic group, which will interact with the cell wall, allowing heavy metal ions to bind or pass through the cell membrane (Sarret et al., 1998). As a result, such processes decrease microbial P requirements as microbes absorb heavy metal ions or adsorb a significant amount of them.

Furthermore, we observed composite effects of multiple heavy metals on microbial metabolic limitation (Fig. 5c and 5d). This suggested that the effects of a single heavy metal cannot truly reflect the response of microbial metabolism to heavy metal contamination, particularly in soil systems contaminated by multiple heavy metal contaminates, which is more typical throughout the world (Lessard et al., 2014). Our results also showed consistent effects of different heavy metals on microbial C limitation (positive effects) or P limitation (negative effects) (Figs. S3 and S4), which was also confirmed by the correlation between the *PLI* and microbial metabolic limitation (Fig. 4). Therefore, microbial metabolic limitation could be successfully used as an indicator in evaluating the effects of compound heavy metal pollution in soil systems. However, it is important to note that we only identified the effects of plant species as a single variable on microbial metabolic limitation (Figs. 3, 5 and 6). Our results are therefore inadequate in reflecting the various effects of these plants (such as plant secretions) on microbial metabolic limitation. It would therefore also be necessary in further studies to analyze root exudates from each treatment to compare their differences, and then decipher their own effects on microbial metabolic limitation.

4.2. Microbial metabolic limitation assessment for soil remediation of compound

heavy metal contamination

Compared to the other five sample plots, the *Lolium* plot had the lowest Cd, Pb, Zn, and Cu concentrations (similar to the *PLI*) after our five-year phytoremediation experiment concluded (Fig. 1). Enzymatic stoichiometry also revealed that the *Lolium* treatment had the lowest microbial C limitation (Fig. 2b and c). As our results indicate, the lowest microbial C limitation is indicative of the lowest heavy metal pollution and toxicity levels. Thus, microbial metabolic limitation can accurately reflect heavy metal pollution in soil. Furthermore, the highest microbial C limitation is indictive of the highest *PLI* in the control treatment, and this treatment also had both the highest total heavy metal concentration and the highest available heavy metal concentration (Table 1; Figs. 1 and 2). On the other hand, the sensitivity of microbial P limitation was not as good as the sensitivity of microbial C limitation (Fig. 4). This further suggested that microbial C limitation could be a more appropriate indicator in assessing soil heavy metal pollution compared to microbial P limitation because such characteristics are key in establishing indicators to assess the nature of deleterious changes and thus reflect soil functional degradation (Schloter et al., 2017).

During our phytoremediation experiment, the lowest total heavy metal concentration, *PLI*, and pollution grade (from E to H) consistently indicated that the *Lolium* treatment removed more heavy metals from soil compared to the other treatments (Table 1 and Fig. 1). The high heavy metal removal efficiency of the *Lolium* treatment could be attributable to the high heavy metal uptake and biomass of *Lolium* (Wood et al., 2016). The simulated experiments that we conducted in our

previous study found that heavy metal concentrations and total uptake (e.g., Pb, Cd, and Zn) in *Lolium* were both higher compared to alfalfa (Cui et al., 2018b). Results from our present study further indicated that all heavy metal concentrations in *Lolium* components were significantly higher than all the other plant species used in our field experiments (Fig. S2). Furthermore, *Lolium*, being a gramineous species, is characterized by rapid growth and appreciable biomass (Thomas et al., 1996), and this species also has been widely reported to be a suitable pasture species for the phytoremediation of heavy metal contaminated soil (Meng et al., 2011; Cui et al., 2018b). At the same time, the uptake efficiency of heavy metals in *Lolium* is higher than other species under compounded heavy metal contamination, such as Cd, Pb, Zn, and Cu (Fig. S2; Cui et al., 2018b). *Lolium* is therefore highly recommended for the remediation of soil contaminated by multiple heavy metals.

4.3. The potential contribution of heavy metal contamination to soil C cycling

The potential effects of heavy metal contamination in soil ecosystems could be significant considering the influence of heavy metals on microbial metabolic limitation. Heavy metals increased microbial C limitation through their effect on metabolism processes. Moreover, C limitation represents a high potential for SOM decomposition due to high C-acquiring enzyme activities. Thus, microbial C limitation can strengthen SOM decomposition, providing more bio-available C sources and nutrients to satisfy their own requirements (Sinsabaugh et al., 2009; Moorhead et al., 2012). Furthermore, SOM compounds that require a variety of enzymes to degrade may decrease conversion efficiency to new biomass because the

production of heterotrophic microorganisms is used to make enzymes that secrete into the environment (Manzoni et al., 2012). Additionally, various substrates require different metabolic pathways to complete assimilation by microorganisms, which may lead to a wide range of respiration rates per unit C assimilated (Pawvan et al., 2005). As a result, these processes would cause a microbial metabolism shift from growth and synthetic metabolism to maintenance respiration and an increased investment in the production of enzymes as decomposition progresses (Xu et al., 2018a), which could considerably decrease the C assimilation efficiency of microorganisms.

Xu et al. (2018b) also reported a reduction in microbial C use efficiency (CUE) in heavy metal contaminated soil. Specifically, microbial CUE values were 0.35, 0.29, and 0.31 in Cd, Pb, and Cd + Pb spiked soil, respectively, while it was 0.41 in uncontaminated soil (Xu et al., 2018b). A more recent study by Xu et al. (2019) found that the microbial CUE was repressed as heavy metal concentrations increased in a laboratory incubation experiment, which was due to the fact that a higher microbial quotient (qCO_2) in contaminated soil results in a higher energy demand with less microbially immobilized C as microbial biomass C. These findings further indicated that a higher portion of assimilated C was allocated to alleviate heavy metal toxicity rather than being used by microorganisms (Lehmann et al., 2011). Consequently, these microbial metabolism processes would greatly increase soil C loss in soil ecosystems contaminated by heavy metals.

5. Conclusions

Our study provided new insight into the development of a promising indicator by

which to monitor and assess heavy metal contamination as well as increase our understanding of the effects of heavy metal pollution in soil ecosystems. This study revealed that heavy metal stress significantly increased microbial C limitation while decreasing microbial P limitation. The various responses of microbial metabolism to heavy metals could be an important adaptive mechanism of microorganisms to polluted environments. Importantly, an increase in microbial C limitation within heavy metal polluted soil could increase soil C loss by promoting soil C decomposition and altering microbial metabolic processes. Accordingly, the potential consequences of heavy metal pollution on soil C cycling in terrestrial ecosystems may not be wholly negligible. Furthermore, the response of both microbial C and P limitations was consistent under different types of heavy metals, which suggested that extracellular enzyme stoichiometry can successfully be used as a new methodology to evaluate heavy metal contamination soil. Further studies should extend our current understanding of soil metabolic limitation mechanisms induced by heavy metal stress as well as the microbial metabolism response to other environmental pollutants, such as polycyclic aromatic hydrocarbon and microplastics.

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Supplemental information

Supplementary information provides additional tables and figures that illustrate changes in extracellular enzyme activities and heavy metal concentrations in plant bodies among our treatments; identifying relationships between microbial metabolic limitations and soil heavy metals through linear regression analysis.

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Figure captions:

Fig. 1 Changes in heavy metal concentrations in soil among the different plant treatments.

Fig. 2 Ecoenzymatic stoichiometry of the relative proportions of C to N acquisition versus C to P acquisition (a), the variation of the vector angle and length (b and c) and their relationships (d).

Fig. 3 Heat map of correlations among soil physicochemical properties, heavy metals in soil, and microbial nutrient limitations.

Fig. 4 Relationships of the *PLI* (pollution load index) with microbial C limitation (a) and P limitation (b).

Fig. 5 The effects of heavy metals, soil properties and plant species on microbial metabolic limitation.

Fig. 6 Cascading relationships of microbial C and P limitations with heavy metals in soil and soil physicochemical properties.

Table 1

Heavy metal pollution assessment by the metals contamination factor (*CF*) and the level of heavy metal pollution (*PLI*) among the different plant treatments.

Treats	*CF*				*PLI*	Grade
	Cd	Pb	Zn	Cu		
Lolium	41.6	0.480	5.51	0.349	2.48	H
Brassica	56.6	0.603	6.70	0.689	3.53	E
Artemisia	43.8	0.541	5.79	0.375	2.67	H
Silphium	60.8	0.759	8.97	0.414	3.62	E
Taraxacum	62.6	0.591	7.09	0.477	3.29	E
Populus	51.9	0.581	6.85	0.467	3.08	E
Control	84.6	0.832	9.67	0.783	4.80	E

Note: N is no pollution, M is moderate pollution, H is heavy pollution, E is extremely heavy pollution.

Table 2

Changes in soil physicochemical properties among the different plant treatments.

Treats	SOC ($g\ kg^{-2}$)	TN ($g\ kg^{-2}$)	TP ($g\ kg^{-2}$)	DOC ($mg\ kg^{-2}$)	TAN ($mg\ kg^{-2}$)	Olsen-P ($mg\ kg^{-2}$)	Bulk density ($g\ cm^{-3}$)	Moisture	pH
Lolium	9.27±0.87 c	0.934±0.12 cd	0.934±0.12 a	112±11.2 a	49.9±5.67 a	24.6±6.17 ab	1.36±0.06 bc	0.185±0.02 d	8.09±0.02 b
Brassica	10.7±1.26 b	1.15±0.07 ab	0.942±0.03 a	106±5.62 ab	53.6±11.0 a	21.5±2.70 b	1.26±0.16 c	0.163±0.01 e	7.97±0.08 c
Artemisia	9.5±0.75 bc	0.838±0.07 d	0.805±0.04 b	95.4±8.38 c	54.0±2.91 a	8.50±1.09 d	1.36±0.10 bc	0.197±0.01 cd	8.18±0.02 a
Silphium	9.62±0.80 bc	0.979±0.06 c	0.968±0.10 a	107±10.2 ab	48.8±3.35 a	28.1±2.89 a	1.55±0.06 a	0.205±0.01 bc	8.09±0.03 b
Taraxacum	12.3±0.88 a	1.24±0.04 a	0.904±0.05 a	113±3.47 a	39.9±2.35 b	27.2±2.05 a	1.36±0.04 bc	0.236±0.02 a	7.96±0.02 c
Populus	9.21±0.73 c	0.956±0.01 c	0.764±0.02 b	96.8±9.75 bc	57.0±6.56 a	7.64±0.87 d	1.43±0.05 ab	0.214±0.01 b	8.11±0.02 b
Control	9.87±0.99 bc	1.08±0.09 b	0.785±0.08 b	97.8±6.08 bc	53.2±4.21 a	15.7±1.05 c	1.37±0.16 bc	0.230±0.01 a	8.10±0.02 b

Note: Values are the means ± standard error ($n > 9$). Different letters indicate significant differences ($P < 0.05$) among the treatments based on one-way ANOVA followed by Tukey's test. SOC, soil organic C; TN, soil total N; TP, soil total P; DOC, soil dissolved organic C; TAN, NO_3^--N + NH_4^+-N.

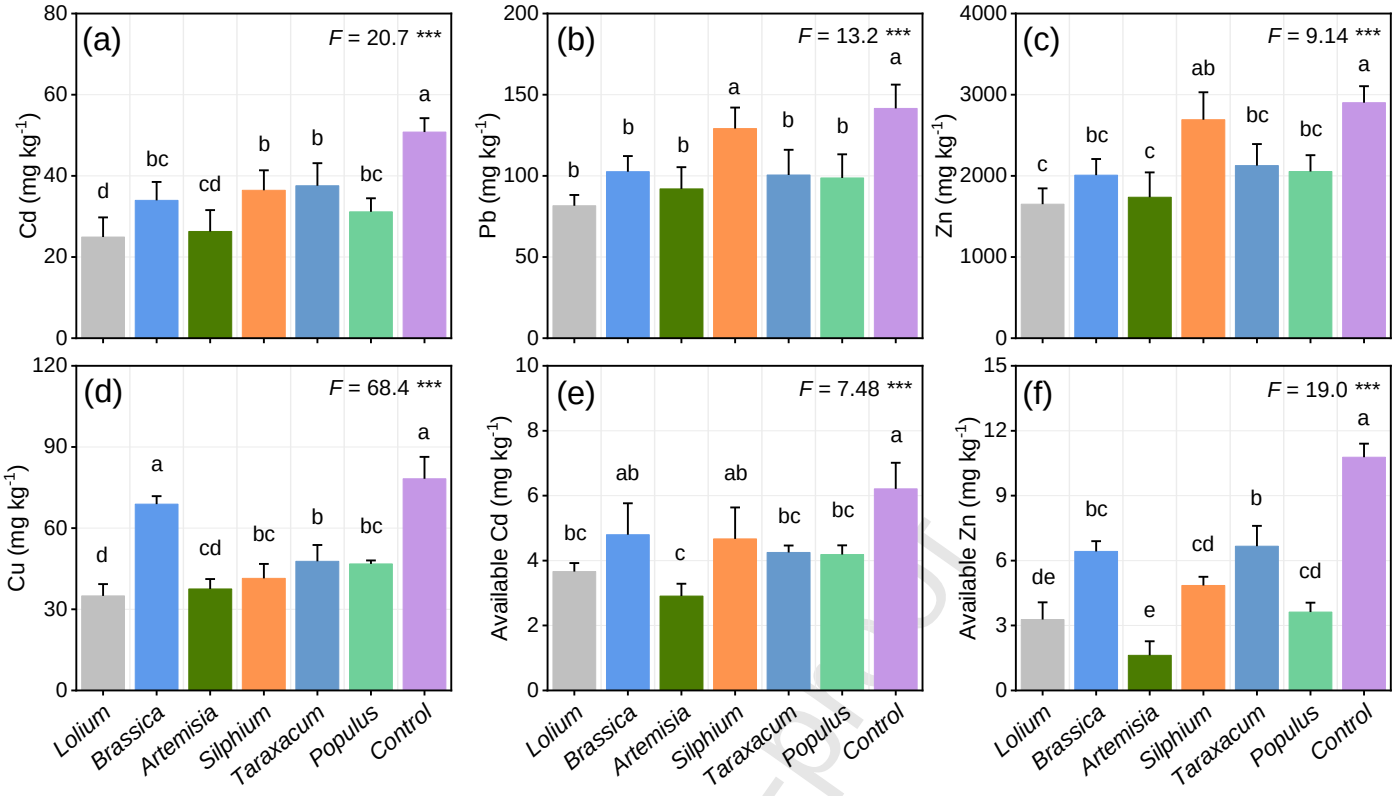


Fig. 1 Changes in heavy metal concentrations in soil among the different plant treatments. Values are the means ± standard error (n > 9). Different letters indicate significant differences (P < 0.001) among the treatments based on one-way ANOVA followed by Tukey's test.

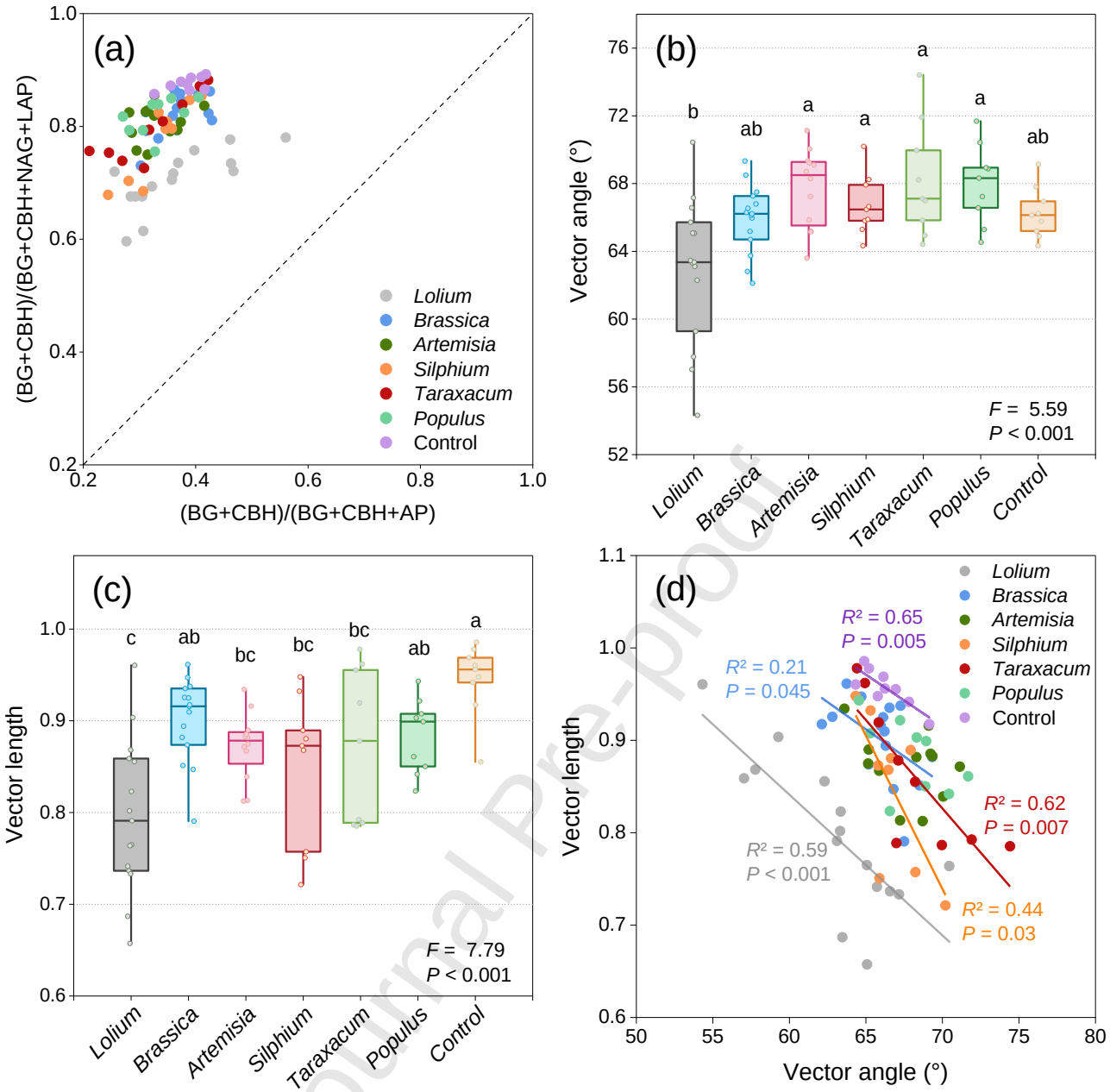


Fig. 2 Ecoenzymatic stoichiometry of the relative proportions of C to N acquisition versus C to P acquisition (a), the variation of the vector angle and length (b and c) and their relationships (d). (A): BG, β-1,4-glucosidase; CBH, β-D-cellobiosidase; NAG, β-1,4-N-acetylglucosaminidase; LAP, L-leucine aminopeptidase; AP, alkaline phosphatase; vector length represents soil C limitation for microbes, vector angle represents soil N/P limitation for microbes. (B and C): Values are the means ± standard error (n > 9). Different letters indicate significant differences ($P < 0.05$) among the treatments based on one-way ANOVA followed by Tukey's test. (D): Linear-regression analysis to identify the relationships of microbial C

limitation with microbial N/P limitation in different treatments.

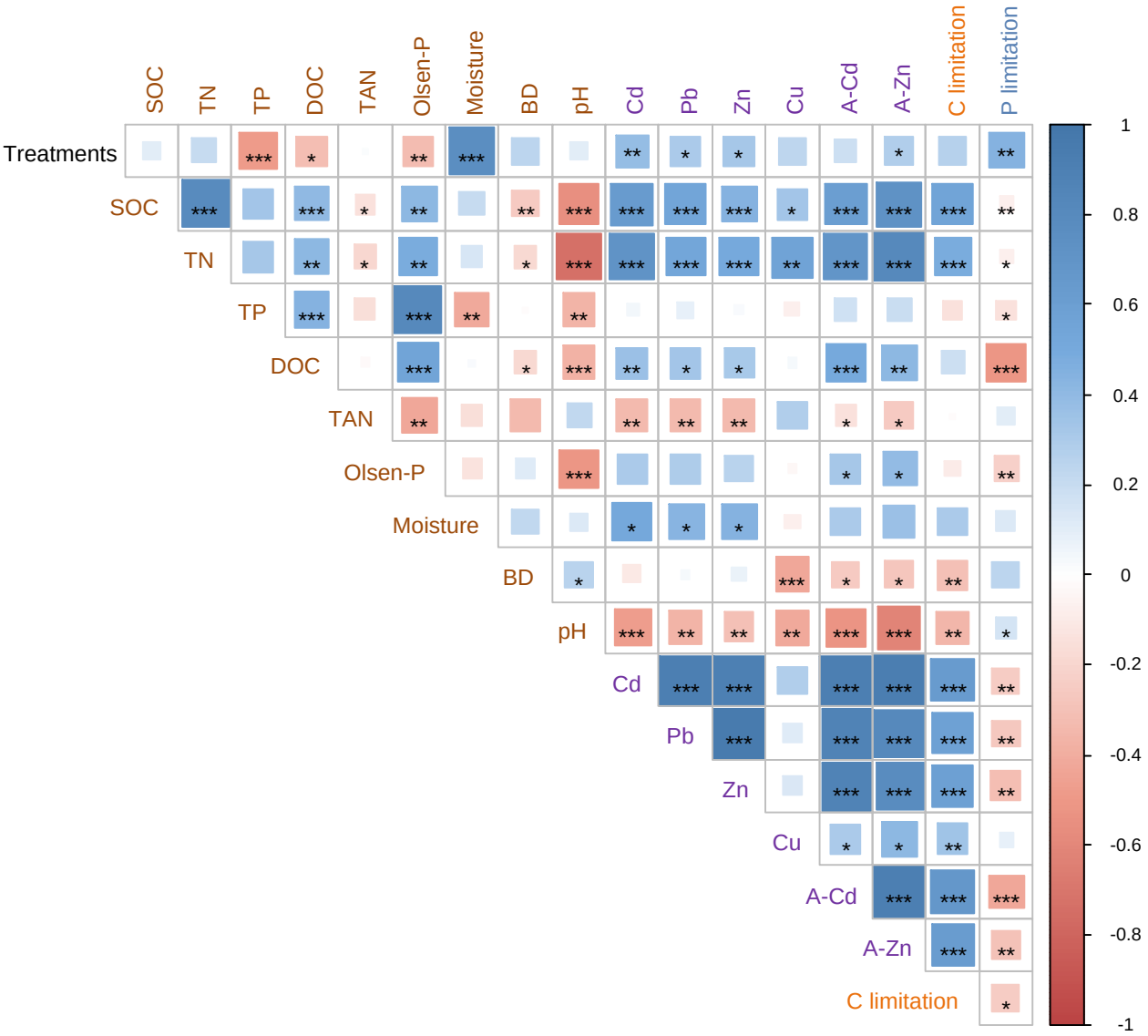


Fig. 3 Heat map of correlations among soil physicochemical properties, heavy metals in soil, and microbial nutrient limitations. SOC, soil organic C; TN, soil total N; TP, soil total P; DOC, soil dissolved organic C; TAN, NO_3^--N + NH_4^+-N; A-Cd, $CaCl_2$-Cd; A-Zn, $CaCl_2$-Zn. * Correlation is significant at $P < 0.05$ (two-tailed); ** Correlation is significant at $P < 0.01$ (two-tailed); *** Correlation is significant at $P < 0.001$ (two-tailed).

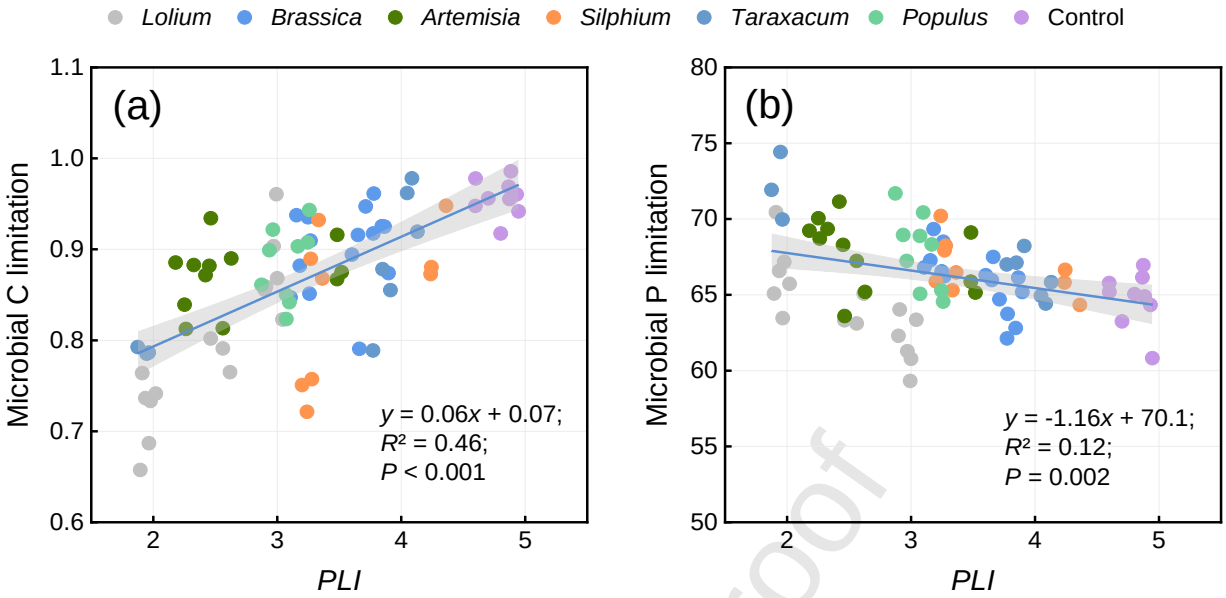


Fig. 4 Relationships of the *PLI* (pollution load index) with microbial C limitation (a) and P limitation (b). Solid lines indicate the model fits between the microbial C or P limitation and the *PLI*, and grey areas are the 95% confidence intervals of the models.

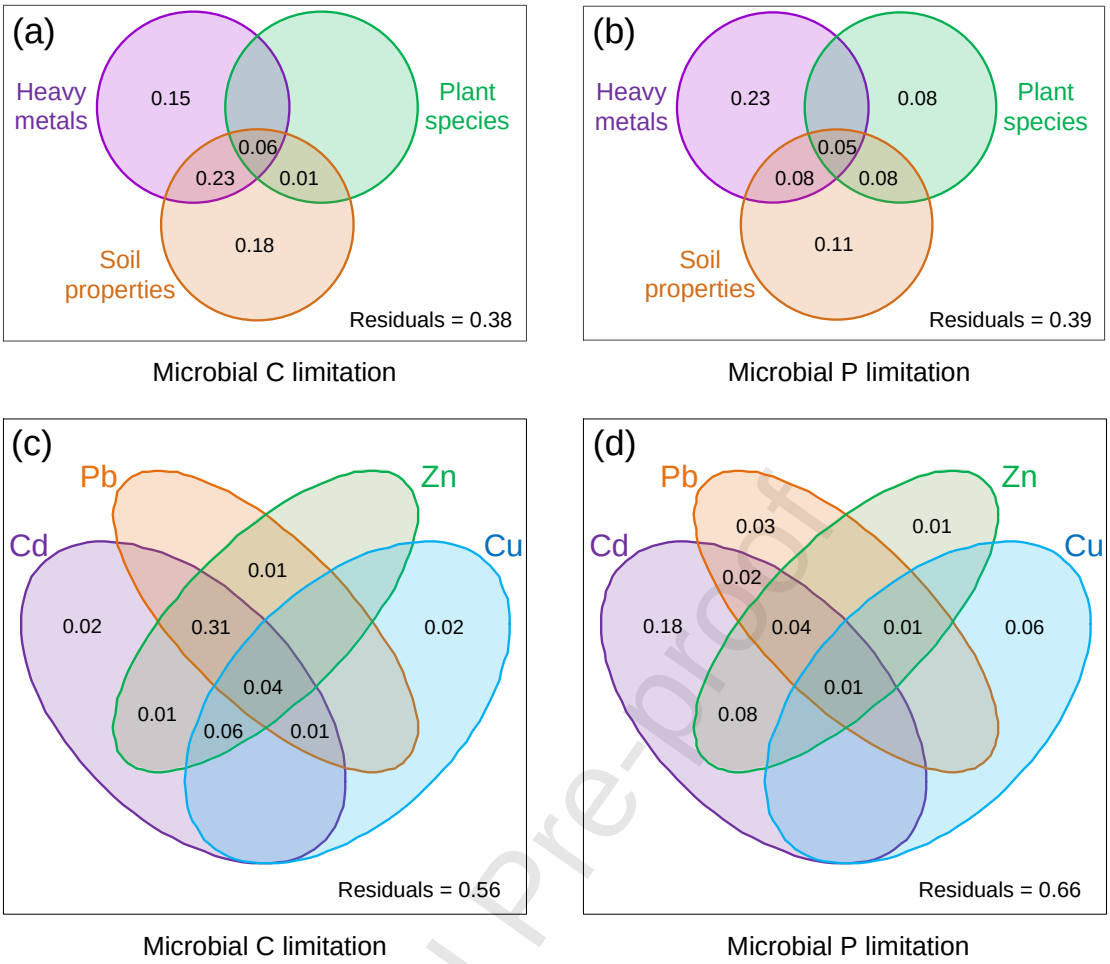


Fig. 5 The effects of heavy metals, soil properties and plant species on microbial metabolic limitation. The percentages of variance in microbial metabolic limitation explained by soil heavy metals, soil physicochemical properties and plant species (A and B) were determined by variation partitioning analysis (VPA). Further, the percentages of variance in microbial metabolic limitation explained by soil Cd, Pb, Zn and Cu contents (C and D). Soil heavy metals include Cd, Pb, Zn, Cu, $CaCl_2$-Cd, and $CaCl_2$-Zn. Soil properties include SOC, TN, TP, DOC, Olsen-P, bulk density, and pH.

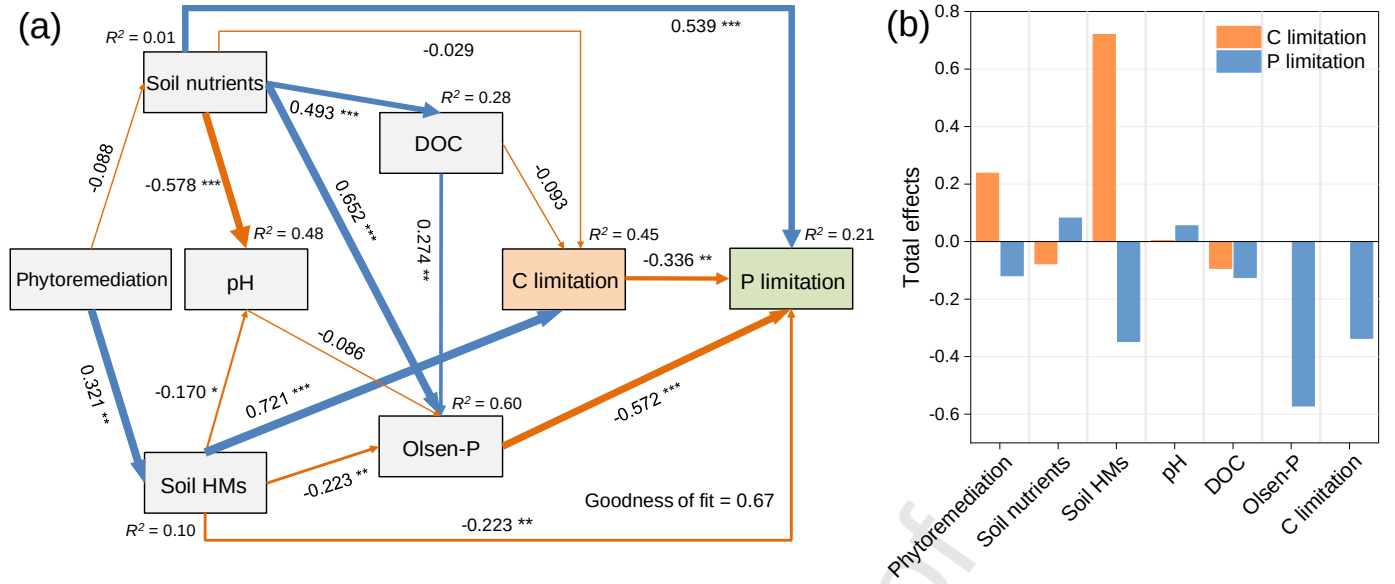
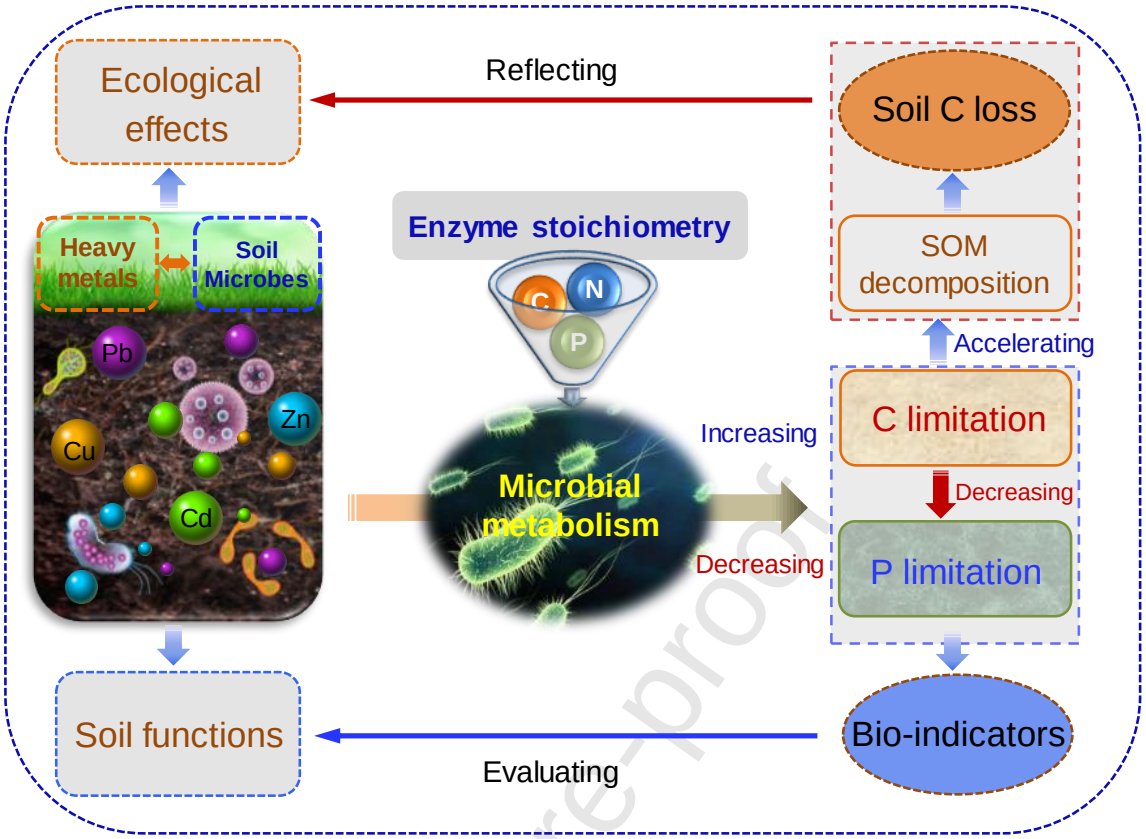


Fig. 6 Cascading relationships of microbial C and P limitations with heavy metals in soil and soil physicochemical properties. Partial least squares path modelling (PLS-PM) disentangling major pathways of the influences of plant treatments (phytoremediation), soil heavy metals and soil physicochemical properties on microbial metabolic limitations. Blue and yellow arrows indicate positive and negative flows of causality ($P < 0.05$), respectively. Numbers on the arrow indicate significant standardized path coefficients. R^2 indicates the variance of dependent variable explained by the model. Soil HMs include Cd, Pb, Zn, Cu, $CaCl_2$-Cd, and $CaCl_2$-Zn. Soil nutrients include SOC, TN, and TP.

Graphical abstract



Highlights

- Heavy metal stress increased microbial C limitation while decreasing P limitation
- Microbial C and P limitations both had consistent response to various heavy metals
- Heavy metal pollution could increase soil C loss by affecting microbial metabolism
- Enzyme stoichiometry was a new method in evaluating heavy metal soil contamination
- Microbial metabolic limitation, particularly C limitation, was a promising indicator