

UNIVERSITÉ D'ORLÉANS

ÉCOLE DOCTORALE

ENERGIE, MATERIAUX, SCIENCES DE L'UNIVERS

Institut des Sciences de la Terre d'Orléans

THÈSE présentée par :

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soutenue le : 20 décembre 2024

pour obtenir le grade de : **Docteur de l'Université d'Orléans**

Discipline/ Spécialité : Sciences de l'Univers

**The bioaugmentation role of wild earthworms in the
phytoremediation of *Sedum plumbizincicola* and**

microbial-mediated mechanisms in metal-contaminated soils

Le rôle de la bioaugmentation des vers de terre sauvages dans la phytoremédiation de
Sedum plumbizincicola

et les mécanismes microbien-médiés dans les sols contaminés par des métaux

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Acknowledgements

During my PhD, there has been the delight of success and the frustration of failure. There are too many people and things worth remembering, I would like to express my gratitude through this article.

This work was completed at Institut des Sciences de la Terre d'Orléans (ISTO), Université d'Orléans and South China Agricultural University. I would like to thank all those who participated in this work. Thanks to my thesis supervisors, **Prof. Mikael MOTELICA HEINO** and **Assoc. Prof. Chi Zhang**. They faithfully followed my work and I would like to thank them for their guidance and for trusting me. I appreciate their enthusiasm and daily availability.

Thanks to **Prof. Ary BRUAND** and **Assoc. Prof. Christian DEFARGE** at Université d'Orléans, **Prof. Jun DAI**, **Assoc. Prof. Yanbiao GUO** and **assistant Xiaofeng LIU** at South China Agricultural University, **Prof. Patrick Lavelle** at Sorbonne Université for their passionate guidance and assistance. Thanks to **Prof. Yan CHEN** in ISTO for his help and kind attention.

Thanks to my colleagues at ISTO, **Meysam GOLMOHAMMADI**, **Léo CHEVRIER**, **Fengfeng ZHANG**, **Pengyuan GAO**, **Xinghua NI**, **Jiashuo LIU**, **Yiyi CHEN**, **Tingting CAO** for their communication and help. Thanks to my colleagues at South China Agricultural University, **Xinyu LI**, **Changhui LIU**, **Dongbin SUN**, **Cevin RWEBUGISA Tibihenda**, **Hesen ZHONG**, **Menghao ZHANG**, **Shuyu HOU**, **Yugong PANG**, **Yiqing CHEN**, and **Siyi CHEN** for their help. Thanks to **Xiangfei ZHU** and **Shaoyun LV** at South China Botanical Garden, Chinese Academy of Sciences, for their help. Thanks to **Caide HUANG** at Tsinghua University, **Shizhong YUE** and **Ruiping WANG** at Dezhou University, for their communication and help. I would like to thank ISTO staff **Marie-France Rouillier** and **Olivier Gaudefroy**, for helping me in various

administrative processes. I also would like to thanks **Julien Catherine**, for helping me with various computer technology issues.

Thanks for the financial support form the China Scholarship Council (CSC), Université d'Orléans and South China Agricultural University. Finally, thanks to my family, for their support, love and everything they do for me. I also thanks to myself.

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Résumé étendu de la thèse en Français

La contamination des sols par les métaux lourds, en particulier dans les zones minières abandonnées, représente un défi environnemental majeur. La phytoremédiation, c'est-à-dire l'utilisation de plantes pour dépolluer les sols contaminés, est apparue comme une solution durable. Cependant, l'efficacité de la phytoremédiation utilisant l'hyperaccumulateur *Sedum plumbizincicola* peut être limitée par une faible production de biomasse et une capacité insuffisante de réapprovisionnement en métaux vers les racines. Dans cette étude, trois catégories écologiques de vers de terre sauvages (*Amyntas cortices*, *Amyntas aspergillum*, *Amyntas robustus* et *Metaphire guillelmi*) ont été introduites dans des sols contaminés par des mines en combinaison avec *Sedum plumbizincicola* afin d'évaluer leurs effets promoteurs de croissance. L'étude visait également à identifier les espèces de vers de terre les plus efficaces dans différents sols contaminés par le cadmium (Cd) et le zinc (Zn) dans des conditions de laboratoire contrôlées pendant une période de 90 jours. Les résultats montrent que : 1) Les vers de terre sauvages favorisent la croissance de *S. plumbizincicola* dans les sols contaminés par des métaux, avec des effets spécifiques aux espèces observés dans l'ordre suivant : *A. cortices* > *A. aspergillum* > *M. guillelmi* ; 2) Les effets de bioaugmentation des vers de terre résultent principalement de l'augmentation de la biomasse de *S. plumbizincicola* et de l'amélioration de la translocation des métaux des racines aux tissus récoltables aériens, augmentant particulièrement la biomasse de ces tissus récoltables ; 3) Un compromis existe entre la minéralisation des nutriments et la résistance au stress environnemental dans différentes catégories écologiques de vers de terre. Sur la base de ces résultats, *A. cortices* et *A. aspergillum* ont été sélectionnés pour des tests supplémentaires, à la fois individuellement et en combinaison, dans le système *Sedum plumbizincicola* afin d'identifier les modèles de remédiation les plus efficaces. Les résultats de ces tests montrent que : 1) Les effets

de bioaugmentation des espèces de vers de terre combinées étaient similaires, voire légèrement inférieurs, à ceux des espèces individuelles ; 2) L'activité des vers de terre a favorisé l'absorption de cadmium et de zinc par *S. plumbizincicola* en augmentant la mobilité et la biodisponibilité des métaux, facilitant le transfert des métaux du sol à la solution du sol, puis aux racines des plantes ; 3) L'analyse statistique multivariée a indiqué que le microbiome du sol (bactéries et champignons) était fortement influencé par l'activité des vers de terre et jouait un rôle essentiel dans le transfert des métaux au sein du système sol contaminé-*S. plumbizincicola*-vers de terre sauvages. Une enquête approfondie sur la communauté microbienne dans les sols en vrac et dans la rhizosphère a été menée pour comprendre l'impact de l'activité des vers de terre sur la composition et la structure microbienne, et pour identifier les principaux prédictors microbiens de l'absorption de Cd et de Zn par *S. plumbizincicola* à différents niveaux de contamination. Les résultats révèlent que : 1) L'activité des vers de terre a significativement augmenté l'abondance des phylums microbiens dominants et la diversité microbienne. En présence de vers de terre, la rhizosphère a montré une augmentation des taxons biomarqueurs de bactéries et de champignons, en particulier dans les sols moyennement et fortement contaminés. Ces taxons étaient principalement associés à des fonctions telles que la décomposition de la matière organique du sol, les cycles du carbone et de l'azote, la survie dans des environnements stressés par les métaux, et la promotion indirecte du transfert des métaux ; 2) Les modèles d'équations structurelles ont montré que l'activité des vers de terre avait un effet positif et indirect sur l'absorption des métaux par *S. plumbizincicola* ; 3) Les microbiotes clés impliqués dans l'amélioration de l'absorption de Cd et de Zn par *S. plumbizincicola* variaient en fonction du niveau de contamination des sols. Plus précisément, dans les sols fortement contaminés, les genres *Fusarium*, *Desulfitobacterium*, *Pseudeurotium*, *Cellulomonas*, *Georgenia*, *Edaphobacter*, *Anaerosporobacter* et *Sorangium* ont été remplacés par

Defluviicoccus, *Methyloceanibacter*, *Microtholunatus*, *Skermanella*, *Aridibacter*, *Myxococcus*, *Intrasporangium*, *Rubrobacter*, *Stenotrophobacter*, *Puia*, et les familles Vicinamibacteraceae et Caldilineaceae. Nous fournissons une base théorique pour comprendre les effets combinés et le potentiel de plusieurs organismes dans la remédiation des sols.

General introduction, hypothesis and objectives

1. Introduction

Metal contamination in soil has become a serious worldwide environmental issue due to anthropogenic activities (Chen et al., 2022). The disused metalliferous mines are considered as the main source of metal contamination of the surrounding agricultural soil in particular (Adnan et al., 2024, Chen et al., 2022). The metals can be accumulated in soil organisms and plants, threaten to human and animal health through food chain. Except for controlling the spread of pollution in abandoned mining areas, it is also urgent issues to be solved metal contamination in surrounding agricultural soil. Limited by the funding and aim of environment protection, phytoremediation is being pursued as cost-effectiveness, eco-friendly and in situ remediation approach. Hyperaccumulator plants are popular due to their ability to uptake large amounts of metals from soil into harvestable tissues such as *Sedum plumbizincicola* (Cd/Zn hyperaccumulation) (Ullah et al., 2015). Regrettably, hyperaccumulator plants are usually shallow root system, followed by slow growth rate, low biomass (Kumar et al., 1995; Hou et al., 2021, Fadzil et al., 2023), and low refilling rate of the soil solution with metals (compared to the phytoextraction rate) (Hou et al., 2021), which is the bottle-neck and challenge of phytoextraction by hyperaccumulator plants. Earthworms are soil macrofauna regarded as “soil ecosystem engineers”, promoting soil organic matter decomposition and significantly influence soil carbon, nitrogen cycles and metal transfer by interactive with soil microorganisms (Jia et al., 2024). Therefore, introducing wild earthworm into is prudence to broken the bottle-neck of phytoextraction in natural soil environment. It is necessary to select the earthworm species of good performance in bioagumentaion for *S. plumbizincicola* due to the different digging and feeding habits of different ecological categories (epigeic, endogeic, anecic). In addition, combined the earthworm species of good performance

may makes greater bioaugmentation effects. In those processes, earthworm could take part in the response to the plant growth, by shaping soil microbiota in a favorable way for the plants (Yang et al., 2024), especially in rhizosphere soil. The interaction pathway of earthworms to *S. plumbizincicola* and the core microbiota in this system are favorable for revealing the underline mechanisms and fully harness the potential of microbial resources in phytoremediation systems. Hence, it is valuable to explore the application of different wild earthworm species and to identify core microbiota within the *S. plumbizincicola* system in mine-contaminated soils.

2. Hypothesis

We hypothesized that inoculation earthworm could promote *S. plumbizincicola* growth and metals extraction efficiency. The phytoremediation efficiencies are influenced by the feeding preference and activities range of different ecological category earthworms and metal stress. In addition, the metals resupply ability in the hotspots soil influenced by roots and earthworms may be different. Additionally, the individually and in combination cultivation modes of different earthworm species facilitate Cd and Zn uptake by *S. plumbizincicola*, and combination cultivation modes better than individually cultivation mode. Furthermore, the underline mechanisms are by driving the soil microbial community structure and diversity, and assistant with *S. plumbizincicola* by promoting the growth and reproduction of some functional microbiota such as soil organic matter decomposition, carbon and nitrogen cycling, survival under metal stress, and indirect promotion of metal transfer.

3. Objectives

The general objective of the thesis concerns the bioaugmentation potential of earthworm activities in *S. plumbizincicola* remediation system.

On the one hand, the effect of different ecological categories of wild earthworms (*Amyntas cortices*, *Amyntas aspergillum*, *Amyntas robustus*, and *Metaphire guillelmi*) on Cd and Zn uptake by *Sedum plumbizincicola*. In this study we aimed to reveal the augmentation potential and identify the most effective earthworm species in different Cd and Zn contaminated soils, and identify the dominate factors.

On the second hand, establish individually and in combination cultivation modes of different earthworm species within the *S. plumbizincicola* system to identify the most effective remediation models and identify the core microbiota in those process under different soil contaminated levels. This part therefore focused on:

(1) The effect of individually and in combination cultivation modes of different earthworm species on Cd and Zn uptake by *S. plumbizincicola*.

(2) The impact of earthworm activity on microbial composition and structure, and the key microbial predictors of Cd and Zn uptake by *S. plumbizincicola* at varying contamination levels.

Chapter I Literature review

1. Soil contaminated with metals

1.1 Overview of soil metal contamination

Soil metal contamination is a serious worldwide and complex environmental issue for both natural and cultivated terrestrial ecosystems. Except for soils with high metals concentration due to the geological background, soil metal concentration is due to atmospheric deposition, waste disposal, and industrial, mining, smelting and agricultural activities (Poggere et al., 2023; Díaz-Morales et al., 2021). Most soils in the world are thus contaminated by multiple potentially toxic trace elements (PTTE). Under anthropogenic activities, some metals and metalloids can be introduced into soil, especially cadmium (Cd), lead (Pb), copper (Cu), zinc (Zn), arsenic (As), mercury (Hg), chromium (Cr), nickel (Ni), antimony (Sb), etc (Qasim and Motelica-Heino et al., 2013; Du and Li et al., 2023; Jia et al., 2020). For example, on the global scale, mining activities have significantly increased the multi-metal (As, Cd, Cr, Cobalt (Co), Cu, Hg, Manganese (Mn), Ni, Pb, and Zn) contamination of soils and the health risk of children and adults in the vicinity (Hou et al., 2023). Kazapoe et al., 2021 investigated the soil metals concentration in the Wassa and Prestea-Huni Valley areas in southwestern Ghana with utilizing a total of 300 soil samples, and founded that 100%, 98%, 50%, and 43% of the soil samples exceeded the standards respectively for Cu, Ba, Co, and As. Moreover, more than half samples were above the Cr, Ni, Zn guideline values. In north China, 376 sampling sites of historical landfill soil were investigated and founded that the concentration of the nine metal elements in the most samples exceeded the maximum allowable concentration for agricultural soils. In addition, the deep soils were severely contaminated with metals, and the potentially processes were leaching and infiltration in the surface soil (Du and Li et al., 2023). In mining area, the investigation of the nationwide 151

mining sites shows that 95% of investigated mines exceeded the screening values and 75% the intervention values in China; the ecological risk of elements As, Cd, Cu, Zn is high around metal mining area located in southern China (Qian et al., 2025).

In terms of metal elements, Cd is of great concern to the environment, because of its toxicity to organisms. It is a nonessential and toxic metal for both humans and plants, and which occurs mostly as an impurity of zinc ore, in sewage sludges, and phosphate fertilizers (Kabata-Pendias, 2010; Satarug et al., 2003). Pb is related to mining and smelting activities, vehicle emissions, fossil fuel burning, etc (Nriagu et al., 1989; Pacyna and Pacyna, 2001). Cu and Zn have biological functions in animal and plants, and are allowed to be added to foods. However, the excessive concentration of Cu and Zn can induce depressive effects and make a damage to organisms. In farming, both elements are excessively used to improve feed conversion ratio, resulting of high Cu and Zn content in livestock manure (Kabata-Pendias and Pendias, 2011). Therefore, the contaminated level and areas of agricultural soil has significantly increased because of livestock manure by using in farming.

In recent years, some soil protection policies were implemented with the increase of sustainable development and environment protection in China, like Soil Pollution Prevention and Control Action Plan in 2016, Soil Environmental Quality Risk Control Standard for Soil Contamination of Agricultural Land (GB 15618-2018) in 2018, Soil Pollution Prevention and Control Law in 2019. Nevertheless, in order to remediate metal contaminated soil and achieve clean production soil contamination must be settled urgently.

1.2 Metal mobility and bioavailability

PTTE such as metals and metalloids exhibit long-distance mobility, high persistence in the environment, bioaccumulation and toxicity to exposed soil organisms (Shen et al., 2024; Zhang et al., 2023). The improper and unavoidable concomitant with production way increased the soil metal contaminated level, which can induce the high ecological risk through the food chain, like “Itai-Itai disease” in Japan caused by people eating rice of contaminated by Cd. The toxicity of trace element to organisms is mainly related to their activity. The activity of PTTE is related to their chemical form, especially soluble metal(loid) species that are preferentially taken up by organisms. Metal(loid)s can be present in a number of chemical forms, and generally exhibit different effects in terms of chemical interaction, mobility, bioavailability and potential toxicity. Metal mobility can be assessed by its ability to migrate between solid and solution phases in the soil environment. The metals bioavailability is assessed by its absorption ability by organisms. While the transfer of PTTE in soil-plant system is dependent on its mobility and bioavailability, mobility and bioavailability of metal(loid) contaminants can be impacted by potential mechanisms such as precipitation and mineral formation, exchange and adsorption by mineral constituents, fixation and release by microbiota, exchange and adsorption by organic matter, formation and decomposition of organic matter, uptake and release by plants (Kabata-Pendias, 2010; Sparks., 2003).

The formation of different metal forms is mainly related to the physicochemical reactions processes that occur in the soil and its solution with the metal ion. These processes determine its speciation, including adsorption/ desorption, ion exchange, outer- and inner-sphere surface complexation/chelation, redox, dissolution/precipitation or surface (co-) precipitation reactions (Sparks., 2003). When soil environmental conditions such as pH and redox change, the different

metal forms can be transformed into each other. When the soil environment is altered to promote desorption reactions, a large amount of metal ions became isolated from the bulk phase and relatively non-labile pool for release into soil solution. Therefore, it is crucial to acquire knowledge about the behavior of metal compounds in different abiotic and biotic compartments of soils and to define the mechanisms which condition their reactivity, transfers, their capacity to bioaccumulate and, ultimately, their toxic and ecotoxicological effects.

1.3 The determination methods of metal concentration and forms in soil

The total concentration of metals in soil is determined by microwave pretreatment or electrothermal plate digestion and measured by instrumentals (Table 1). The two different extraction methods of Tessier and BCR can estimate different bioavailability speciation of metals in soils. In descending order of ability to be accessed by plants in Tessier method are the water soluble fraction, exchangeable fraction, bound to carbonates fraction, bound to Fe and Mn oxides fraction, bound to organic matter fraction, residual fraction, while the order in BCR method is acid extraction fraction (included first three fraction in Tessier, most variable because it is sensitive to pH changes), reducible fraction (bounded to Fe-Mn oxides), oxidizable fraction (bounded to organic matter and sulfur, and would be re-released under oxidative conditions (Tessier and Campbell and Bisson et al., 1979; Ure et al., 1993). The organic fraction released in this step is hardly considered very mobile or available, since the metals associated with stable high-molecular-weight humus substances decompose slowly), residual fraction (fixed in primary and secondary minerals, chemically stable biologically inactive, this portion of the metals cannot be re-released to water under normal conditions), respectively. The potential bioavailable contents of metals are also extracted by single chemical extractions (like diethylenetriaminepentaacetic acid (DTPA)) or DGT sampler (Fig. 1 and Table 1.1). DTPA-extractable fraction was considered as the bioavailable

trace metals in the contaminated soil, and it was used as an effective index to assess the bio-toxicity for plants and soil organisms. In addition, the DGT technique can mimic the metal uptake by plants and used a proxy.

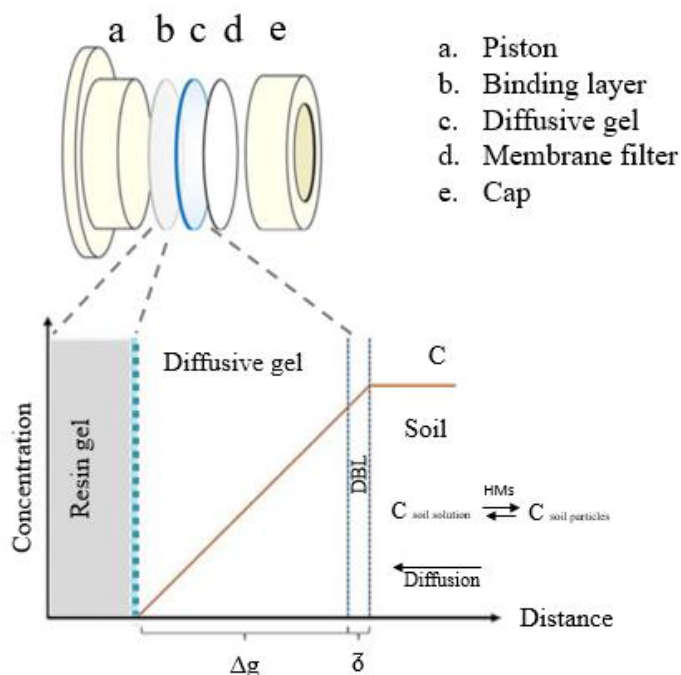


Figure 1 Principle of the DGT sampler (modified from Marrugo-Madrid et al., 2021)

1.4 Soil remediation methods

No matter agricultural clean production, or reusing the abandoned mine area, there is a need to take an efficient action of remediation of contaminated soils, firstly to promote the ecosystem sustainable development. Except for the physical remediation methods (electrokinetic and soil replacement), many remediation approaches for metal contaminated soils were developed and applied to regulate the metals mobility and decrease the ecological risks, based on passivators, conditioners, adsorbent materials, etc. However, those materials can generate secondary contamination risks (e.g. groundwater contamination through metal uncontrolled leaching), and they cannot realize long-term stable effects on metals, and also have high costs (Ali et al., 2020).

Moreover, immobilized metals can be reactive, when the soil environment is changed (Houben and Sonnet, 2015). Ultimately, it makes a great challenge on soil remediation.

Plants have a significant role in the biogeochemical cycling of PTTE in the critical zone. Phytoremediation, a cost-effective approach, is the use of plants to clean up contaminated soil or keep agricultural products clean, and is being pursued as an effective approach for cleanup the contaminated soils. It became a hot research topic in soil remediation field, because it is nondestructive and has no or little secondary contamination risks. Phytoremediation approach includes phytodegradation (degradation of organic contaminants), phytovolatilization, phytoimmobilization, phytostabilization, phytoextraction as five main pathways; as for remediating metal contaminated soils, the last two approaches have been used frequently (Bolan et al., 2014).

2. Phytoextraction

As for phytostabilization, if the soil environment changes, the metals might become mobile again for crops to be taken up (Houben and Sonnet, 2015). But if metals from metal-contaminated soil are removed, it will eliminate the ecological risk permanently. Phytoextraction can realize this goal by employing special plant species to translocate metals from soil into their belowground and aboveground parts (Tibbett et al., 2021). In this process, some accumulator and hyperaccumulator plants which can accumulate large amounts of metals were found and applied to contaminated soils.

2.1 Hyperaccumulator plants

Hyperaccumulator plants are characterized by the hyperaccumulated ability for specific metal elements (Li and Gutajala and Wu, 2018). A hyperaccumulator plant for Cd has around 100

mg·kg⁻¹ Cd in its tissue, compared to a normal level for most plants of 0.1 mg·kg⁻¹ (Kabata-Pendias and Pendias, 2011). Other hyperaccumulator plant for As, Cu, Mn, Zn were also found in the world. In China, some researchers began to test and identify various Cd hyperaccumulators including *Solanum nigrum* (Yang et al., 2004), *Phragmites australis* (Huang and Liao and Wang, 2011), *Viola baoshanensis* (Wu et al., 2010), *Sedum alfredii* (Yang et al., 2002; Deng et al., 2007) and *Sedum plumbizincicola* (Wu and Li and Luo, 2006; Hu et al., 2015). However, there are also some limiting factors for hyperaccumulating metals from soils. The first major limiting factor is shallow root system, followed by slow growth rate, and low biomass (Kumar et al., 1995; Pulford and Watson, 2003); another important factor is the low refilling rate of the soil solution with metals (compared to the phytoextraction rate) (Hou et al., 2021). The above factors result in low remediation efficiency, which is the bottle-neck and challenge of phytoextraction by hyperaccumulator plants.

The metal mobility and bioavailability are two of the key factors of plants extraction. In soil solution, the soluble metals are generally free hydrated ions or complexed ligands, while only small parts of metal in soil pore water can be absorbed freely by plants (McBride et al., 1994; Sauvé Hendershot and Allen, 2000). However, a large number of chemical activators are applied to increase the metal bioavailability, which leads to massive amounts of metal being active in the short term and secondary contamination of surrounding environmental due to the fact that the mobile metals are not absorbed in time by plant roots (Nowack and VanBriesen, 2005).

Metal ions are transported to roots by mass flow and diffusion and are absorbed at rates that depend on their concentration at the root surface (Saleem and Arif and Rashid, 2022). Furthermore, metals concentration at the root surface depends not only on the phytoavailability, but is also influenced by the rate of migration of metals from bulk soil to the root surface; while

the metal concentration at the root surface and the rate of migration of metals from bulk soil to the root surface are not always high (Sufyan et al., 2021). Therefore, the moderate and sustained release of soil metals to soil pore water is important for effective phytoextraction of contaminated soils, but it is still unclear how to realize this. The research gap is the lacking comprehension of the microscopic reaction mechanisms of soil metals transfer processes in natural environment, which restrict the remediation effective and the comprehension to the fate of metals in contaminated soil.

2.2 *Sedum plumbizincicola* (Crassulaceae family)

Sedum plumbizincicola is a new Cd hyperaccumulator found in Pb-Zn mining areas in Zhejiang province, China (Fig. 2) (Wu and Zhou and Bi, 2006). It is capable of extracting Cd and Zn from contaminated soils and accumulating high concentration of the metals in its aboveground parts (Wu et al., 2008). As in particular phytoremediation processes, *Sedum plumbizincicola* also suffers from poor growth and low remediation efficiency.



Fig. 2 *Sedum plumbizincicola* in the original habitat (Wu and Zhou and Bi, 2006)

The root depth of *Sedum plumbizincicola* is 15 cm depth after growing eight months, shallow rooting herb (Fig. 3). Specifically, the roots occurred mainly at 2-15 cm depth and particularly at 2-10 cm depth. The soil Cd concentration decreased mainly within the depth range 2-10 cm (Hu and Zhang and Dong, 2019). In one remediation season, lasting thirty weeks, the

biomass of *Sedum plumbizincicola* is shown approximate “J” growth model (Fig. 4) (Hou, 2021), while the increasing rates of the Cd content and concentration in aboveground parts slowed down or decreased after a period of time. It becomes a practical issue that whether keeps a stable and moderate metals activity rates to improve the remediation efficient and decreases the secondary contaminated risks.

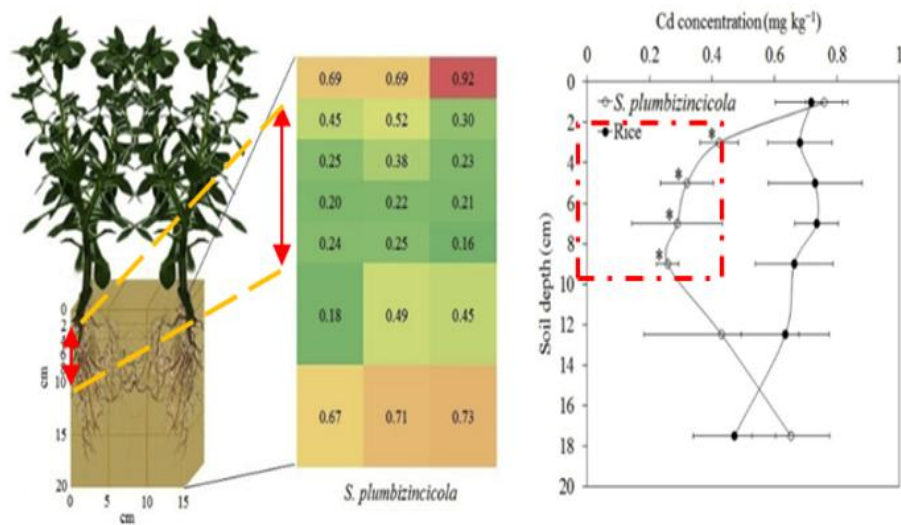


Fig. 3 Plant root and soil Cd distribution at depth 0-20 cm in the soil profile (modified from Hu et al., 2019).

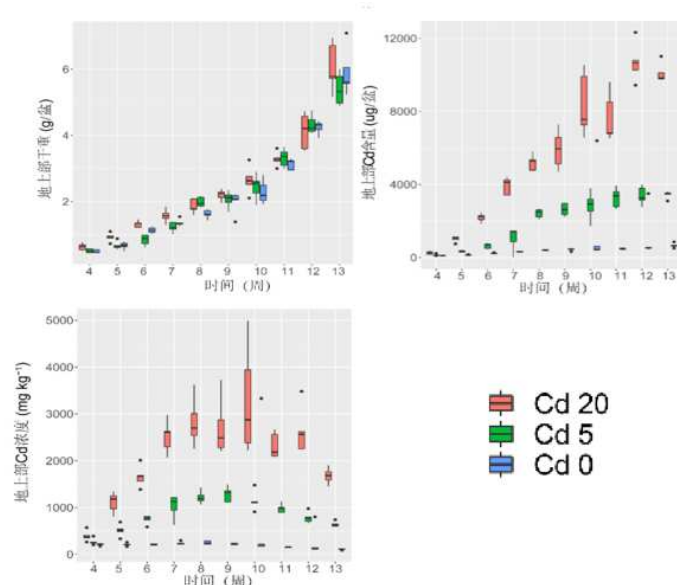


Fig. 4 Shoot biomass, Cd concentration and content in *Sedum plumbizincicola* tissue with time (Hou, D, 2019).

3. The role of soil organisms in phytoremediation

To break the bottle-neck of phytoextraction by hyperaccumulator plants, different remediation methods were employed to get the bioaugmentation effects. This research topic has received widespread attention.

3.1 Microorganisms

Soil microorganisms play a key role in biogeochemical cycling and energy flow in ecosystem, especially in soil environment. A dynamic “underground transport web” exists in soil, related to soil organism- (plant-, fungal- and/or animal-driven). The transport web has strong implications for the occupancy of novel ecological niches and nutrient cycling dynamics (Yang and van Elsas, 2018, Fig. 5). Bacteria stand out as key soil inhabitants and they are the prime players in the soil biogeochemical cycles, such as those of carbon, nitrogen, sulfur, phosphorus and contaminants (metals, organic contaminants).

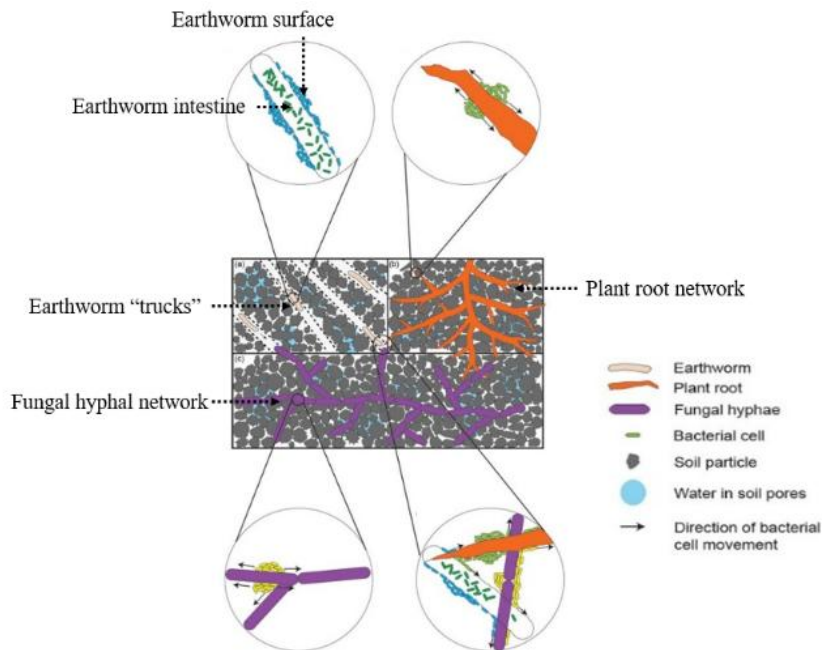


Fig. 5 Underground transport web in soil (modified from Yang and van Elsas, 2018).

Remediation with microorganisms include biosorption, bioaccumulation, biomineralization and biotransformation. Previous studies found that incubating Cd-contaminated soil with microbes in aerobic condition remarkably reduced Cd mobility due to bioaccumulation (Jiang et al., 2009; Kurek and Bollag, 2004). In soil-plant system, Ali and Guo and Li, 2021 showed that the addition of *Streptomyces pactum* (Act 12) can promote wheat growth and increased the uptake of metals in wheat tissues through affecting soil metal mobility and bioavailability. Similarly, Wang et al., 2025 found that native soil bacteria may be involved in the stabilization or absorption of metals by special genes. Therefore, to fully utilize the functions of microorganisms on metals bioavailability, it is necessary to reveal the interaction between organisms and the relationship with metals.

3.2 Soil fauna

Soil fauna activity can reconstruct soil structure, decreasing soil density. For example, the digging and excretion processes of soil fauna can increase soil permeability and pore connectivity, and promote the formation of soil aggregates; those processes have great influence on the supply of water and nutrients to soil organisms and plant (Anitha et al., 2020). As soil passes through the gut, it is exposed to enzymes that break down organic compounds. This process increases the availability of nutrients, ultimately leading to greater plant growth (Zhang et al., 2023). In addition, according to the theory of moderate pastoralism, soil fauna can stimulate plant growth through feeding small amounts of roots (Wang and Li and Hu, 2004). Meanwhile, soil fauna metabolism and the interaction with microorganisms can produce plant hormone-like substances to promote plant growth.

In soil-plant-fauna system, the activities of roots and soil fauna extremely influence the distribution of bacteria. It is mainly due to the active modes of bacterial dispersal (growth, swimming, swarming and twitching mobility) that are limited to the scale of (connected) soil pores or aggregates, while the activities of roots and soil fauna can accelerate the bacterial dispersal through increasing its connection rates. As shown in Fig. 1.9, plant roots, fungal hyphae and soil animals may be in contact with other transport vectors in soil, resulting in overall complex patterns of bacterial dispersal (Yang and van Elsas, 2018). Specifically, specific bacterial types possess capacities to “hitchhike” along with other (vector) organisms (protozoa, nematodes, soil fauna, soil fungi and plant root systems) through the soil. They act either as “trucks” (earthworms, nematodes, protozoa) or as “networks” (fungi, plant roots). The soil microbiome can be strongly modulated at local sites of casting and/or shedding. Plant roots and fungal hyphae both form network structures consisting of interconnected biological surfaces in soil. The ability to attach to

the plant root may have been the key factor that determined the successful bacterial colonization of the root. In particular, the ability to hold on to the root tips, thus, migration along with developing roots, is primordial. In contrast, flagellar dispersion and/or chemotaxis are likely crucial for microbial transport processes on mature roots, where tissues are immobile. Fungal hyphae in soil forming mycelial networks promote the motility of particular bacteria. In addition, bacterial cells may also move to fungal surfaces mediated by other soil organisms (such as other fungal hyphae, plant roots or soil fauna). Overall, the underground transportation network and “truck delivery” can extend the bacterial range of ecological action to microhabitats away from their original sites in soil. Moreover, because of gut conditions of soil fauna, the intestinal digestion processes can regulate the microbial community structure and diversity (Xu et al., 2021), and further impact on plant growth. Therefore, it benefits to introduce “truck delivery” (like earthworms) into the soil heavy metal remediation system of hyperaccumulate plant.

4. Earthworms

4.1 The ecotypes and species of earthworm

Earthworms can be grouped into different ecological categories with different size, feeding habits, physiological trait, soil pore creation, and distribution in the soil profile, etc. In general, earthworms can be separated into four groups, epigeic, endogeic, anecic, and compost (*E. fetida*) (Keitha and Robinson A. Robinsonb, 2012; Zhang et al., 2022). The four ecological types have the different selective feeding habits and burrow systems (Bouché, 1977). In the soil profile, epigeic earthworms exist in the surface of the soil in leaf litter, and the movement is frequent. They tend to feed on and live in the leaf litter, not to make burrows. This species is relatively smaller in size. Endogeic earthworms exist in the soil, making horizontal burrow and ejecting. They appear to move very little and tend to feed and live in the soil. This species is middle in size. Anecic

earthworms exist in the soil, making permanent sub-vertical burrows in soil. They feed on leaves on the soil surface and drag into their burrows. This species are the largest species of earthworms. As for compost earthworms, *E. fetida*, is not common and usually cannot survival in natural soil environment. In the present, in most of studies *E. fetida* was employed as experiment species (Huang et al., 2021). This might not able to get the real state and potential mechanisms in nature. Therefore, it is necessary to include wild earthworms in research.

4.2 The functions of earthworm in ecosystem

As “soil ecosystem engineers”, the bioturbation activities of earthworm, including burrowing, feeding and excretion (casts and surface mucus), can reconstruct soil structure, and accelerate the biogeochemical cycling of nutrients (Lavelle, 1988; van Groenigen and Lubbers and Vos, 2014) and PTTE (Sizmur and Richardson, 2020). Earthworm activities can significantly change soil physical and biochemical properties and habitat conditions (Fig. 6) (Arrazola-Vasquez., et al. 2022). Specifically, the burrowing hole and casts increase soil pH (Wen et al., 2004), aeration, water retention capacity, and organic matter turnover, etc. The surface mucus to facilitated crawling and digging can improve the soil available carbon and nitrogen content (Zhang et al.,

2009). The intestinal digestion of earthworms has great influence on soil organic matter mineralization and stabilization processes (Li et al., 2024).

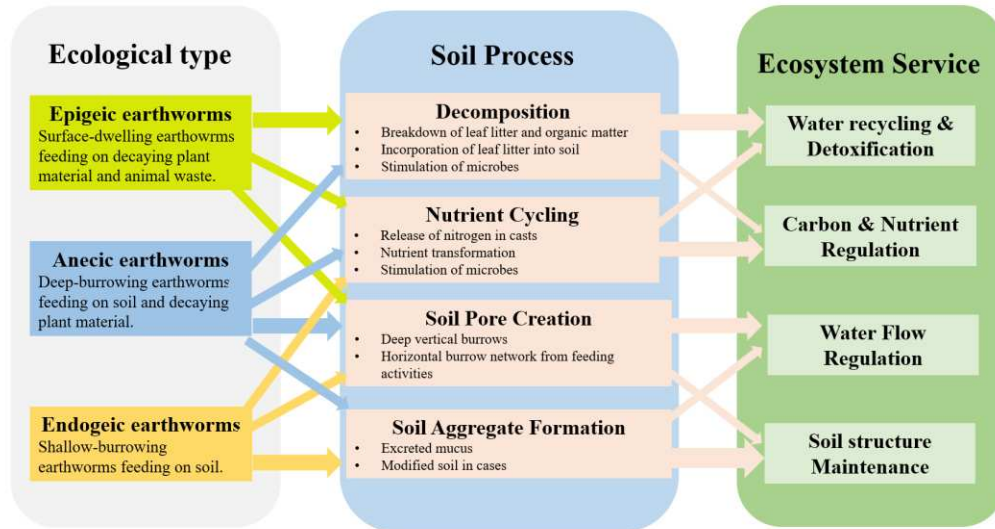


Fig. 6 The ecosystem service of different earthworm ecological types (modified from Keith and Robinson, 2012). The width of arrows represents relative importance.

4.3 The influences of earthworm activities on soil microbial

Earthworm activity has a great influence on the soil microbial community (Fig. 7). Earthworms gut is important habitat for soil microorganisms, and the gut process strongly affects soil microbial biomass, activity, community structure and diversity (Wang et al., 2020). Earthworms tend to feed on rotted leaves or other organic matter. The rotted organic matters have relative rich microorganisms attached. In this case, the feeding activities could affect specific taxa of microorganism, organic matter decomposition and nutrient release. When soil microorganisms pass through the earthworm gut, some species proliferate, while others cannot survive due to specific gut conditions (e.g., abundant soluble organic C, pH value tends to neutral, microbicidal substances and more anaerobic conditions), leading to a decrease in the fungi-to-bacteria ratio (Lavelle et al., 1995). Moreover, earthworm activities can regulate the fungi community structural

and diversity; in particular, it can promote the changes of mycorrhizal fungi directly interacting with roots.

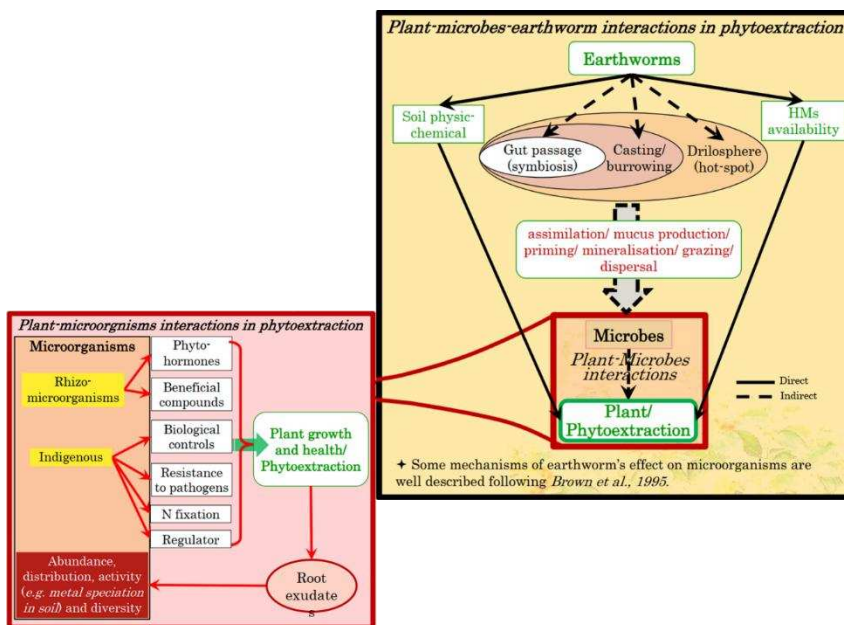


Fig 7 Mechanisms direct and indirect of earthworm's effect on plant and microorganisms in the phytoremediation context (<https://www.intechopen.com/chapters/73253>)

Earthworms could also impact soil microorganisms through modifying the physicochemical properties of their habitats, especially increasing the microbial diversity in drilosphere areas (Wang et al., 2020; Hoeffner et al., 2018). Earthworms redistribute soil organic matter by feeding and mixing soils, which changes the resource accessibility of microbes. As we known, the drilosphere areas enriched carbon and nitrogen contents and elevated rates of nutrient turnover, which have influence on the microbial activities. The surface mucus of earthworm can stimulate the microbial activity. In addition, earthworm act as soil “mixers” and microbial “spreaders”, accelerating the spread and dispersion of microorganisms in the soil and changing the distribution of soil microorganisms.

Previous studies demonstrated that different ecotypes of earthworm shape soil microbial community distinctly (Ferlian et al., 2018; Xiao et al., 2018). Earthworms from different ecotypes affect microbial community through niche partition and resource allocation (Sheehan et al., 2008; Gómez-Brandón et al., 2011). Such species-specific effects also occur in species within the same ecotype due to other factors like feeding preference and body size (Chang and Szlavecz and Buyer, 2016; Hoeffner et al., 2019). Although earthworm species identification has attracted increasing attention, there is limited knowledge about the effects of earthworm species identity on soil microbials. Currently, the impact of earthworms on soil microorganisms has not been determined yet (Geisen et al., 2019).

4.4 The underlying mechanisms of earthworm on soil metal transfer processes

Earthworm can accumulate soil metals by passive diffusion and ingestion. Metals can diffuse into earthworms from soil solution through contact with their body surfaces during earthworm digging in soils, while ingestion activities can introduce metals into earthworms directly and be absorbed by their organs.

During the processes of digging, swallowing, and draining in soils, earthworms produce casts and build a galley system (burrow wall), which greatly accelerate aggregates formation and stabilize formation of macro-porosity (Barois and Lavelle, 1986). This process impact on the soil metals mobility by affecting soil pH and organic matter, CEC, etc (Sizmur and Rhichardson, 2020; Kızılkaya, 2004; Lavelle and Spain, 2001). Earthworm activities can accelerate organic matter decomposition, promoting dissolved organic carbon formation (Wen et al., 2004) and metal releases (Sizmur and Hodson, 2009). Additionally, dissolved organic matter can be complexed with metals and increases metals solubility by active functional group, such as carboxyl, hydroxyl,

carbonyl and methyl. Earthworm mucus consists of low molecular organic matter with lots of active functional groups (Guhra et al., 2020), and also can increase dissolved organic matter content. Zhang et al., (2009) results show that earthworm mucus increased Cd concentration in tomato tissues in hydroponic experiments. Sizmur and Hodson (2009) showed that earthworm gut mucus can increase significantly soil metal mobility. Earthworm gut can secrete massive ammonia. In addition, gut calcium gland of earthworm can secrete CaCO_3 and alkaline excreta containing nitrogen, increasing soil pH (Lavelle and Spain, 2001). Metals can be therefore re-equilibration between organic matter, soil particle and soil solution in intestinal digestion process. Although the properties of earthworm cast can decrease metals mobility, most of studies shows the metals mobility in cast are higher than that in bulk soil (Udovic and Playvc and Lestan, 2007). However, some research shows that earthworm activities can promote the stabilization processes of soil organic matter. This process can facilitate metal transformation to stable forms. In the present, it is still not clear about earthworm effects on soil metal mobility.

Earthworms can influence soil metal activities by affecting microbes within drilosphere areas (burrow wall, cast) (Brown, 1995; Devliegher and Verstraete, 1997; Zirbes et al., 2012). The burrow wall provides the suitable shelters and abundant bioavailable resources for soil microbial communities (Jegou et al., 2001; Lipiec et al., 2015). The gut processes also impact metals availability by regulating microorganisms (Zhang et al., 2018). There are two possible pathways of microorganisms entering the gut and their effects on metal transformation. Specifically, the first way is microorganisms be digested after ingested by earthworms. The tissues of microorganisms are dissolved by gut enzymes, then metals in cells are released and converted to other forms and substances including metals are excreted with cast. The second way is microorganisms being stimulated and proliferation after ingested by earthworms. This process can regulate the physical

and chemical characterizes of gut condition, then metals in gut are conversed to other forms and substances including metals are excreted with cast.

In terms of earthworm species, different species of earthworm have different metal absorption and excretion ability, resulting in different effects on metal transfer in soils (Huang et al., 2022). As unnecessary elements for organisms, Cd has high mobility than Cu and Pb in soils. Therefore, Cd is more easily diffused into earthworm. Even though both Cd and Zn can easily enter earthworm body, the metabolism ability is higher for Cd than that for Zn. This different uptake and elimination ability of earthworm result in different effects on metal cycling.

In conclusion, earthworm activity can change soil metal mobility, but there are conflicting views accounts of the stabilization or activation effects. Limiting the “black-box” research method, the underline mechanisms of earthworm affecting metals cycling is still not clear and mainly rely on speculation to reveal the mechanisms.

5. Combined hyperaccumulator plant with earthworm to remediate the metal-contaminated soils

5.1 The potential of combined hyperaccumulator plant with earthworm to remediate the metal-contaminated soils

The “earthworm induction-phytoremediation” technology is an economic and environment friendly bioremediation measure. Earthworms are known as “soil ecosystem engineers”, and are functionally important due to their capability in regulating nearly all soil biotic and abiotic properties, such as reconstructing soil structure, impacting on organic matter distribution and carbon storage, and in changing microbial activity. The digging behavior and gut digestion process of earthworms have the potential to promote the growth of hyperaccumulator plant roots through

their own metabolism and stimulating soil microorganisms, and replenish metals to rhizosphere area in time. Ultimately, those processes will improve the remediation efficiency and impact on the biogeochemical cycling of trace metals in ecosystem (Sizmur and Richardson, 2020). Wang et al., (2020) investigated the reinforcement effect of compost earthworm *E. fetida* on phytoremediation processes of *Sedum plumbizincicola* in different types of soil; results show that only combined rice straw and earthworm *E. fetida* can enhance the remediation effects, while earthworms alone cannot achieve this aim. As for hyperaccumulate plants, whether wild earthworm bioturbation can further promote transfer of metals to aboveground tissues from roots remains to be investigated. Moreover, the extent and mechanisms of action of the different ecological types of wild earthworm for this process is not clear. Specifically, the bioturbation of earthworms and roots have an influence on the metal species and transformation, particular in rhizosphere area, and overlap area of rhizosphere and drilosphere area. Which organisms and processes play on dominated role is still not very clear.

5.2 Rhizosphere processes, “hotspot” area and influence on ecodynamics of metals in soil-plant-earthworm system

In soil-plant system, the rhizosphere is the hub of metal transfer. The rhizospheric soil, which is the few millimeters of soil covering the roots of plants and influenced by their biological activity, is the connection point between the aboveground and belowground worlds. The accumulation characteristics of metals in plants are not only regulated by genetic mechanisms but also largely affected by the rhizospheric environment. Unlike bulk soils, the rhizosphere is a zone of intense and dynamic system characterized by feedback interactions between soils characteristics, roots processes and dynamics of the associated microbial communities that play an essential role in the transformation of natural organic matter and nutrients (Hiltner 2004; Shi, 2004) and the

cycling of trace metals either directly and indirectly in contaminated soils and play an important role in their uptake by plants. Therefore, revealing the relationship between physicochemical and microorganism characteristics of rhizosphere and heavy metal accumulation characteristics of the hyperaccumulator plants will help to further improve the phytoextraction efficiency.

The main forms of metals that can be absorbed by plants are 1) hydrated, ionized, free ionic species, 2) soluble inorganic ion pair species, including metal hydroxyl species, and 3) organometallic chelates mainly of low molecular weight (Tack, 2010). Conversely, neither adsorbed Cd onto soil colloids nor insoluble complexes suspended and precipitated in soil solution are capable of being taken up by plants (Huang et al., 2010). As for metal transfer from soil solids to plants, it involves five necessary steps, which are desorption or dissolution from the solid (Houben and Sonnet, 2015), diffusion and convection through solution, sorption or precipitation at a new site, absorption by plant root, translocation to plant organs (Fig. 1.14). Those processes occur continuously and intensively in the rhizosphere area. In addition, the high refilling rate of soil solution with metals from soil particles and the desorbed metals migrating from the bulk soil to the rhizosphere before being taken up by plant are the key steps in phytoextraction and critical to lifting the main limiting factors of remediation. While earthworms have the functions of activating and expanding the soil beneficial microflora and accelerate the biogeochemical cycling of elements, so that introducing it into soil-hyperaccumulator plants system might improve the remediation efficiency. In soil-earthworm-hyperaccumulator plant system, between the soil roots, microorganisms and fauna strong interactions occur in the surface of plant root (Fig. 8), and the different microzone are formed by plant roots and earthworm activities. This microzone of rhizosphere, drilosphere (cast and burrow wall) and overlap areas are called “hotspot” area. Earthworm burrow wall and casts enriched in organic matter, various available nutrient, which can

promote soil microorganisms and plant growth. Therefore, the functional domains or hotspots area become the critical areas for metal transfer and transformation due to root exudates and soil microbial interactions.

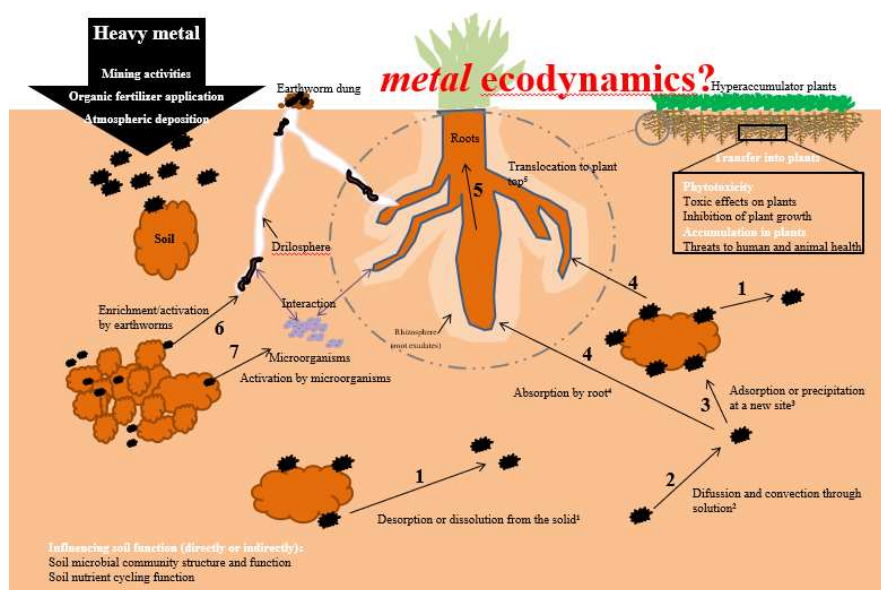


Fig. 8 The “hotspot” area of metal transfer in soil-plant-earthworm system (Some soil processes reference to Kabata-Pendias and Pendias, 2011)

The metal transport processes are influenced by abiotic (e.g. pH, organic matter, redox potential, cation exchange capacity, DOC, soil texture, etc.) and biotic (e.g. root exudates, macrofauna and microbes) factors (Lv et al., 2016). In hotspots area, the plant exudates 30 - 40% carbon of photosynthetic products to rhizosphere to support some microorganisms. Those microorganisms can mineralization soil nutrient for plants as feedback. Soil microorganisms play key roles in the biogeochemical transformation of metals, while plant roots and earthworm can regulate and improve the composition, diversity and functions of microbial communities. There is evidence that the bioturbation of plant roots and earthworm can shape the soil microbial communities by their activities and its metabolites, such as *Actinobacteria*, *bacteroidetes*, and TM7 were especially abundant in rhizospheres under high Cd stress (Hou et al., 2018). In spite of

past decades have witnessed increasing studies of bacterial and fungal communities within earthworm-mediated soil hotspots (Aira et al., 2022; Hoeffner et al., 2018), but few studies focus on the rhizosphere processes within earthworm-hyperaccumulator plants co-mediated. Consequently, we thought to further explore the effects of hyperaccumulator plant and earthworm activity in metals contaminated soil on the soil microbial community and determine if the changes observed were also associated with changes in soil metabolites. In conclusion, future investigations are required to obtain insight into the effects of the complex interaction between plant, soil, earthworm and microbial activity in contaminated soil on ecodynamic of metals, especially in rhizospheric area.

5.3 The aims of this research

The study of rhizosphere/drilosphere-related processes on mobility, availability and toxicity of metals in rhizosphere/drilosphere soils, based, in particular, on the role of the root-microorganism-earthworm associations in their transformation of, is a major challenge to understand the ecodynamics of these contaminants in the context of phytoremediation of polluted soils. Thus, in the present study, the main thrust of my work is to study the speciation, the reactivity, and the transfer of Cd in the rhizosphere/drilosphere overlap of contaminated soils into hyperaccumulator plants. Metal ecodynamics (characterization, speciation, mobility) under plant root and earthworm bioturbation process will be studied to assess the contribution of soil microbial communities to these processes and the significance of rhizospheric/drilospheric effects. This research will offer new conceptual approaches to better assess and predict their fate and better assess and predict their fate and behavior in soil-hyperaccumulator plant-microorganism-earthworm system.

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Chapter II: Bioaugmentation effects of three ecological categories of wild earthworms on phytoextraction of *Sedum plumbizincicola* in Cd and Zn contaminated soils of South China

Résumés: La contamination des sols par les métaux et les métalloïdes, en particulier dans les zones minières, pose un grand défi environnemental. Les mines métallifères sont considérées comme la principale source de contamination métallique des sols agricoles environnants en particulier (Adnan et al., 2024, Chen et al., 2022). Il est urgent de remédier aux sols pollués par les métaux lourds, c'est pourquoi certaines techniques de remédiation in situ ont été bien développées, comme la phytoremédiation (Lavanya et al., 2024). La phytoremédiation, c'est-à-dire l'utilisation de plantes pour assainir les sols contaminés, est apparue comme une solution durable. Cependant, l'efficacité de la phytoremédiation de l'hyperaccumulateur *Sedum plumbizincicola* est limitée par sa faible biomasse et sa production lente de biomasse, ainsi que par sa faible capacité de réapprovisionnement des racines en métaux. Les vers de terre sont considérés comme des « ingénieurs de l'écosystème du sol », car ils peuvent reconstruire la structure du sol, modifier les propriétés chimiques du sol et accélérer le cycle biogéochimique des métaux traces et des nutriments (Van Groenigen et al., 2019). Utiliser le ver de terre pour coopérer avec l'hyperaccumulateur ne consiste pas seulement à éliminer les métaux des sols contaminés in situ mais, plus important encore, à restaurer la capacité des sols à fonctionner ou à fonctionner selon leur potentiel. Cependant, il y a un manque de recherche sur l'efficacité de la bioaugmentation de *S. plumbizincicola* par différentes espèces de vers de terre sauvages appartenant à différentes catégories écologiques. Le co-système des vers de terre et des plantes hyperaccumulatrices a suscité de nombreuses inquiétudes, mais le mécanisme des effets des vers de terre sur

l'accumulation des métaux dans les plantes hyperaccumulatrices est toujours controversé. Il s'agit de l'augmentation de la croissance des plantes due aux nutriments du sol, de l'amélioration de la mobilité des métaux due aux facteurs environnementaux du sol ou de la modification de la capacité d'adsorption des plantes et de la résistance au stress. Ici, trois catégories écologiques de vers de terre sauvages (*Amyntas cortices*, *Amyntas aspergillum*, *Amyntas robustus* et *Metaphire guillelmi*) ont été introduites dans un système de remédiation composé de sols miniers et de *Sedum plumbizincicola* hyperaccumulateur à des niveaux de contamination par le Cd et le Zn faibles, moyens et élevés, dans des conditions de laboratoire contrôlées pendant 90 jours. Nous avons étudié les états physiologiques et la concentration de métaux dans différents tissus de *S. plumbizincicola* ainsi que la disponibilité des métaux pour évaluer les effets de bioaugmentation du ver de terre sauvage. Les résultats montrent que l'introduction de vers de terre a significativement favorisé la croissance vigoureuse de *Sedum plumbizincicola*, améliorant à la fois sa robustesse et l'efficacité de la phytoextraction du sol. En détail, la biomasse des différents tissus était plus élevée dans l'inoculation de *S. plumbizincicola* avec les vers de terre *A. cortices*, *A. aspergillum* et *M. guillelmi* que dans la culture seule de *S. plumbizincicola*, la biomasse la plus élevée de la partie aérienne et des racines atteignant $1,17 \pm 0,34$ g DW/plante et $0,13 \pm 0,07$ g DW/plante par l'inoculation d'*A. cortices* et d'*A. aspergillum* dans un sol moyennement contaminé, respectivement. La concentration de Cd et de Zn dans les tissus aériens récoltables a augmenté de 5,95%-76,7% et de 7,49%-20,8%, respectivement. La quantité de Cd et de Zn absorbée s'est avérée significativement plus élevée dans l'inoculation de *S. plumbizincicola* avec les vers de terre *A. cortices*, *A. aspergillum* et *M. guillelmi* que dans l'inoculation avec *A. robustus* et la seule culture de *S. plumbizincicola*. En outre, la quantité la plus élevée d'absorption de Cd a atteint $177 \pm 40,2$ et $142 \pm 19,5$ µg/1,5 kg de sol avec la participation d'*A. cortices* et *M. guillelmi*, respectivement ; et la

quantité la plus élevée d'absorption de Zn a atteint $13,4 \pm 3,66$ mg/1,5 kg de sol avec la participation d'*A. cortices*. Les vers de terre, en particulier les *A. cortices* épigés et les *A. aspergillum* et *M. guillelmi* anéciques, ont augmenté la minéralisation des nutriments du sol et la biodisponibilité des métaux, en favorisant le transfert des métaux des racines vers les tissus aériens, et les facteurs de transfert du Cd et du Zn ont augmenté d'un maximum de 98,3 % et 110 %, respectivement. En outre, les différentes espèces de vers de terre appartenant aux mêmes catégories écologiques ont montré des effets différents sur la biodisponibilité des métaux dans la zone des hotspots. L'analyse de la forêt aléatoire a montré que les facteurs dominants d'amélioration de l'efficacité de la phytoremédiation étaient la biomasse aérienne, suivie des facteurs de transfert et du phosphore disponible, puis de l'azote alcali-hydrolysable. **En conclusion, les** vers de terre sauvages, en particulier ceux des catégories écologiques épigée et anécique, peuvent effectivement améliorer la phytoextraction des métaux par *Sedum plumbizincicola*, avec des effets spécifiques à l'espèce sur l'efficacité de la remédiation. L'étude donne un aperçu de l'utilisation combinée de plantes hyperaccumulatrices et de vers de terre pour l'assainissement des sols pollués par plusieurs métaux, offrant ainsi une stratégie prometteuse pour la restauration de l'environnement.

Bioaugmentation effects of three ecological categories of wild earthworms on phytoextraction of *Sedum plumbizincicola* in Cd and Zn contaminated soils of South China

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Highlights:

- Wild earthworms can promote *S. plumbizincicola* growth, and shows species-specific augmentation effects, in the order of *A. cortices* > *A. aspergillum* > *M. guillelmi*.
- The bioaugmentation effects of earthworms dominated by increasing the biomass of *S. Plumbizincicola*, followed by the metals transfer capacity from roots to harvestable aboveground tissues and available phosphorus.
- The trade-off of earthworm on nutrient mineralization and resistant environment stress capacity exists in different ecological categories.

Abstract: Soil contamination with metals and metalloids, particularly in mining areas, poses a great environmental challenge. Phytoremediation, the use of plants to remediate contaminated soils, has emerged as a sustainable solution. However, the phytoremediation efficiency of hyperaccumulator *Sedum plumbizincicola* is limited by its low biomass and slow biomass production and also low metal resupply capacity to roots. Here, three ecological categories of wild earthworms (*Amyntas cortices*, *Amyntas aspergillum*, *Amyntas robustus*, and *Metaphire guillelmi*) were introduced in *Sedum plumbizincicola* remediation system in different Cd and Zn contaminated mining soils under controlled laboratory condition for 90 days. We investigated the physiological states and metals concentration in different tissues of *S. plumbizincicola* and metals availability to assess the bioaugmentation effects of wild earthworm. Result show that the introduction of earthworms significantly promoted the vigorous growth of *Sedum plumbizincicola*, enhancing both its robustness and soil phytoextraction efficiency. In details, the biomass of different tissues was high in inoculation *S. plumbizincicola* with earthworm *A. cortices*, *A. aspergillum*, and *M. guillelmi* than only cultural *S. plumbizincicola*, the highest biomass of the aboveground and roots reaching 1.17 ± 0.34 g DW/plant and 0.13 ± 0.07 g DW/plant by inoculation *A. cortices* and *A. aspergillum* in medium contaminated soil, respectively. The Cd and Zn concentration in harvestable aboveground tissues increased by within 5.95%-76.7% and 7.49%-20.8%, respectively. Cd and Zn uptake amount shown significantly high in inoculation *S. plumbizincicola* with earthworm *A. cortices*, *A. aspergillum* and *M. guillelmi* than inoculation with *A. robustus* and only cultural *S. plumbizincicola*. Moreover, the highest Cd uptake amount reached at 177 ± 40.2 and 142 ± 19.5 $\mu\text{g}/1.5$ kg soil with the participation of *A. cortices* and *M. guillelmi*, respectively; and the highest Zn uptake amount reached at 13.4 ± 3.66 mg/1.5 kg soil with the participation of *A. cortices*. Earthworms, particularly epigeic *A. cortices* and anecic *A. aspergillum*

and *M. guillelmi*, increased soil nutrient mineralization and metal bioavailability, promoting the transfer of metals from roots to aboveground tissues, and the transfer factors of Cd and Zn increasing by a maximum of 98.3% and 110%, respectively. Furthermore, the different earthworm species belonging to same ecological categories shown different effect on metal bioavailability in hotspots area. The random forest analysis shown that the dominant factors of improving phytoremediation efficiency was the aboveground biomass, followed by the transfer factors and available phosphorus, and then alkali-hydrolysable nitrogen. **In conclusion**, wild earthworms, particularly those in epigeic and anecic ecological categories, can effectively enhance the phytoextraction of metals by *S. plumbizincicola*, with species-specific effects on remediation efficiency. The study provides insights into the combined use of hyperaccumulator plants and earthworms for the remediation of multi-metal polluted soils, offering a promising strategy for environmental restoration.

Keywords: Phytoremediation; Cd and Zn, earthworm ecological category; *Sedum plumbizincicola*; Diffusive gradients in thin-films (DGT);

1. Introduction

Soil metal contamination had become worldwide issues (Chen et al., 2022). The metalliferous mines are considered as the main source of metal contamination of the surrounding agricultural soil in particular (Adnan et al., 2024, Chen et al., 2022). It is urgent to remediate soils polluted by heavy metals, therefore some in situ remediation techniques have been developed well, such as phytoremediation (Lavanya et al., 2024). Phytoremediation, using plants to remediate environmental media, is being pursued as an effective approach for cleanup of contaminated soils (Lavanya et al., 2024). Among these methods, the use of hyperaccumulator plants increasing metal transportation from soil to the aboveground tissues of plants have become a cost-effective, eco-

friendly and in situ remediation approach (Ullah et al., 2015). Regrettably, hyperaccumulator plants are usually low biomass and have also slow rates of biomass production (Fadzil et al., 2023). For *Sedum plumbizincicola* (*S. plumbizincicola*), a cadmium/zinc (Cd/Zn) native hyperaccumulator in China, similar limitations occur (Wu et al., 2006; Wu et al., 2013; Cui et al., 2016). In addition, the low refilling rate of the soil solution with metals from soils compared to the phytoextraction rate which is potentially much higher is also the main limiting factors of phytoremediation efficiency. Nowadays, combining with multi-bioremediation approach has been employed to enhance remediation efficiency, such as chemical activators (Wu et al., 2007), derived activator - agricultural waste solution (Liu et al., 2022), endophytic bacteria and plant growth bacteria (Ma et al., 2016; Ullah et al., 2015), and earthworms (Jusselme et al., 2013; Das et al., 2018; Zhang et al., 2023; Zhang et al., 2024). However, there is a lack of research on the bioaugmentation efficiency of *S. plumbizincicola* by different species of wild earthworms belonging to different ecological categories until now.

Earthworms are ubiquitous soil macrofauna with the largest biomass and considered as “soil ecosystem engineers”, as they can reconstruct soil structure, change soil chemical properties and also accelerate the biogeochemical cycling of trace metals and nutrients (Van Groenigen et al., 2019). In metal-contaminated soil, previous studies have shown that earthworm activities can increase phytoremediation efficiency by improving soil properties, activating and expanding soil beneficial microflora, and changing metals mobility and bioavailability (Jusselme et al., 2013; Kaur et al., 2019). Using earthworm to cooperate with hyperaccumulator is not only to remove metals from contaminated soils in situ but, more importantly, to restore the capacity of soils to function or perform according to their potential. In hyperaccumulator plant-earthworm-metals contaminated soil system, plant roots and earthworm activity can create different hotspot areas,

such as rhizosphere, drilosphere (earthworm cast, burrow wall), and overlap areas (Jacquiod et al., 2020). In those area exists stronger interaction between abiota and biota than for bulk soil, and intense matter and energy exchange (Medina-Sauza et al. 2022). Most importantly, those interaction have greatly influence on metal activities and distribution in soils (Jacquiod et al., 2020; Song et al., 2024). Those processes also determine the phytoremediation efficiency by influencing metals resupply ability to plants. Hence, analyzing the effects of roots and earthworm activities on soil metals resupply ability is critical to elaborate the underline mechanisms of earthworm on those processes, and to break the bottleneck of phytoremediation. The co-system of earthworm and hyperaccumulator plants caused widespread concern, however the mechanism of earthworm effects on metal accumulation in hyperaccumulator plants were still controversial on the increasing of plant growth caused by soil nutrients, the improvement of metal mobility caused by soil environmental factors or the change of plant adsorption capacity and stress resistance.

Moreover, the choice of earthworm is the key to the application of hyperaccumulator plant-earthworm-heavy metals contaminated soil system. Previously, *Eisenia fetida* (*E. fetida*) has been an international standard species in researches on and the toxicity tests of organic, inorganic pollutants and the interior test for bioremediating contaminated soils and vermicomposting (Robinson et al., 2021; Huang et al., 2021; Liu et al., 2020; Zhou et al., 2021). Wang et al., (2020) combined *S. plumbizincicola* and *E. fetida* and exogenous organic matter to remediate Cd contaminated soils and achieved the bioaugmentation effects. However, because *E. fetida* prefer to feed on manure and does not long term exist in natural soil system (Nahmani and Hodson and Black, 2007), it is necessary to study remediation effects and mechanisms of wild and geological earthworm species in soil phytoextraction. In the natural ecosystem, due to different feeding preference and activities characteristics of earthworms, they are traditionally classified into three

ecological categories, namely epigeic, endogeic, and anecic (Bouche et al., 1977; Keith and Robinson, 2012). The effects of different ecological categories earthworms on the phytoremediation processes needs to be analyzed and elaborated.

Based on literature review, we hypothesized that inoculation earthworm could promote *S. plumbizincicola* growth and increase metals phytoextraction efficiency. The phytoremediation efficiency is influenced by the injection preference and activities range and survival strategies of different ecological categories earthworm and metal stress. In addition, the metal resupply ability in hotspots soil strongly influenced by roots and earthworms may be different between different earthworm species. To test this hypothesis, the present study collected four species of wild earthworm belonging to three ecological categories (*Amyntas cortices* (*A. cortices*), *Amyntas robustus* (*A. robustus*), *Amyntas aspergillum* (*A. aspergillum*), and *Metaphire guillelmi* (*M. guillelmi*), to co-remediate metal contamination soils with *S. plumbizincicola*. Additionally, some studies have employed the diffusive gradients in thin-films (DGT) device to estimate soil metal re-supply ability (Li et al., 2014; Zhu et al., 2023). While this process depended on the resupply flux of the labile metal pool of the soil solid phase to be released into the solution (Bashar et al., 2016). Therefore, the analysis of soil DGT-metals concentration and the resupply flux in different hotspot soils were employed to provide a more direct insight the action method and the influence degree of different earthworm species have on metals activity. The specific research objectives were 1) to explore the combined effects of *S. plumbizincicola* and different species of wild earthworm belonging to different ecological categories to improve the phytoremediation efficiency in Cd and Zn contaminated soil through plant physiological indicators and Cd and Zn accumulation evaluation; 2) to analyze the effect of earthworm species and metals stress on the remediation efficiency, and to elaborate the underline mechanisms of different earthworm species in those

processes; 3) to clarify Cd and Zn bioavailability in the hotspot soils affected by *S. plumbizincicola* and different species of earthworm. This study can provide a theoretical basis for insight into the combined effects of hyperaccumulator *S. plumbizincicola* and wild earthworms on the soil remediation polluted by multiple-metals.

2. Materials and Methods

2.1 Experiment materials

Three Cd contaminated level soils were collected from the Renhua mining area (25° 05' 47.1" -25° 05' 59.5" N, 113°39' 59.8" -19.2" E), Guangzhou city, Guangdong province, China. The soil type is paddy soil. The three soils were low Cd contaminated soil, medium Cd contaminated soil and high Cd contaminated soil, namely L, M, and H, respectively. Rice straw passing 2 mm sieve was employed as exogenous organic fertilizer and food for plant and earthworm. The basic properties of soil and rice straw are shown in Table 1.

S. plumbizincicola, a hyperaccumulator plant, was employed to realize metals extraction from soil. In addition, three ecological category wild earthworms, epigeic *A. cortices*, endogeic *A. robustus*, anecic *A. aspergillum*, and anecic *M. guillelmi*, were collected to co-remediate metal contaminated soils with *S. plumbizincicola*. In details, earthworm *A. cortices*, *A. robustus* and *A. aspergillum* were collected at tea garden from Guangdong province, China. Earthworm *M. guillelmi* was collected at farmland from Jiangsu province, China. Those four species are wild earthworm, and native species of China.

Table 1 Basic properties and metal concentrations of soil and rice straw

	pH (H ₂ O)	Clay (%)	OC (g/kg)	TN (g/kg)	C:N	Cd (mg/kg)	Zn (mg/kg)	Cu (mg/kg)	Pb (mg/kg)	DTPA-Cd (mg/kg)	DTPA-Zn (mg/kg)
Soil I (L)	6.13±0.13	27.7±0.90	27.5±0.36	2.20±0.05	13.0±0.45	0.53±0.08	254±53.5	22.2±0.19	96.2±0.53	0.39±0.09	15.9±0.59
Soil II (M)	5.84±0.28	32.7±3.66	25.8±0.67	2.33±0.04	11.1±0.45	0.66±0.06	247±47.4	27.5±0.14	94.9±4.31	0.47±0.12	21.9±0.21
Soil III (H)	5.87±0.21	22.2±0.58	26.4±0.55	2.30±0.09	11.5±0.51	1.06±0.19	277±53.7	15.9±0.68	79.4±2.39	0.73±0.07	18.7±0.38
Rice straw			242±3.03	9.37±0.14	25.8±0.68	0.01±0.00	6.72±1.55	1.24±0.90	3.25±0.26		
Threshold for soil metals ^a			5.5<pH≤6.5			0.4	200	50	100		
			6.5<pH≤7.5			0.6	250	100	140		

Note: The uppercase letter L, M and H represent clean soil, low Cd concentration soil and high Cd concentration soil, respectively; OC represents organic carbon; TN represents total nitrogen; The ratio of C:N represents organic carbon content/total nitrogen content; CEC represents cation exchange capacity. Threshold ^a represents the risk control standard for soil metal contamination of agricultural land (GB 15618-2018).

2.2 Experiment design

There were three contaminated level soils and six treatments for each soil, with five replicated per treatment, 90 pots in total. The treatments for each soil were set up as follows: 1) without plants and earthworms (CK); 2) only planting *S. plumbizincicola* seedlings (P); 3) planting *S. plumbizincicola* seedlings and inoculation with earthworm *A. cortices* (PE1); 4) planting *S. plumbizincicola* seedlings and inoculation with earthworm *A. aspergillum* (PE2); 5) planting *S. plumbizincicola* seedlings and inoculation with earthworm *A. robustus* (PE3); 6) planting *S. plumbizincicola* seedlings and inoculation with earthworm *M. guillelmi* (PE4). Each pot contained 1.5 kg of air-dried soil sieved to 2 mm. The pot has an upper diameter × bottom diameter × height of 176 × 143 × 185 mm, with micro-holes at the bottom. The bulk density of soil was 1.14 g/cm³. All treatments were equilibrating and aged at 70% of maximum soil water holding capacity for one month after thoroughly mixing soil with 5% rice straw. Then, four *S. plumbizincicola* seedlings (~8 cm height) were planted into each pot. After one month for seedling roots stabilization, 15 ± 1g of healthy clitellate adult earthworms were inoculated in PE treatments, approximately 0.54 ±

0.16 g • individual⁻¹ for *A. cortices*, 1.35 ± 0.30 g • individual⁻¹ for *A. robustus*, 5.16 ± 0.10 g • individual⁻¹ for *A. aspergillum*, 2.39 ± 0.21 g • individual⁻¹ for *M. guillelm*. To prevent earthworms from escaping, the top and bottom of each pot were covered with a fine nylon mesh with a 1 mm pore diameter. Soil moisture in each pot was maintained at ~70% of soil maximum water holding capacity (MWHC) and the temperature was maintained at 25 °C. Day/night=16:8.

After 90 days of cultivation, the surviving seedlings were counted and harvested. The leaf chlorophyll, height, stem thickness and fresh weight of plants was measured and weighted. The plant tissue was oven dry to constant weight, then separated aboveground and root tissues, and then weighted. Earthworms were hand-picked, and then counted and weighted during sampling. The bulk soil of each pot and the hotspot micro-area (rhizosphere soil, drilosphere soil (earthworm burrow wall (1-2 mm) and belowground casts)) soil of treatments with earthworm *A. aspergillum* and *M. guillelm* were collected. Then, the soil samples were thoroughly air-dried and sieved for the analysis of soil properties. It needs to be illustrated that the rhizosphere soil in PE treatment is same as the overlap area of actual rhizosphere and drilosphere, collected ~ 60 g soil from this area. Moreover, the sampling area of rhizosphere soil was lightly lenient than for 1-3 mm to get enough soil sample weight, but still trying to sample near the root system. The hotspot soils are sampling from different parts in the pot and trying to avoid sampling area overlap.

2.3 Soil properties

Soil pH was measured using a glass electrode in a 1:2.5 (w/v) soil/water suspension. Soil clay, soil organic matter (SOC), total nitrogen (TN) and alkali-hydrolysable nitrogen (AN) content were analyzed by the pipette (Lu, 1999), dichromate oxidation (Sparks, 1996), Kjeldahl (Brookes et al., 1985) and alkali N-proliferation (Cornfield, 1960) methods, respectively. dissolved organic matter (DOC) content was extracted with deionized water (1:5) and determined (Dai et al., 2004).

Available phosphorus. Soil respiration rate was determined by measuring CO₂ evolution in a week incubation (Zibilsk, 1994).

2.4 Total metal content in rice straw, plant tissues and soils, and DTPA extractable metal content in soils

Total Cd, Pb, Cu, Zn in the soils were extracted by microwave digestion with aqua regia (concentrated HNO₃: concentrated HCl (v/v) = 1:3). Total Cd, Pb, Cu, Zn in the rice straw and total Cd and Zn in the plant tissues were extracted by microwave digestion with concentrated HNO₃. The diethylenetriamine pentaacetic acid (DTPA)-extractable fraction was regarded as bioavailable metals and toxic. DTPA-Cd and DTPA-Zn were extracted with DTPA extractant at a Soil:DTPA extractant ratio of 5:25 (g·mL⁻¹), which included a solution of 5 Mm DTPA and 10 mM CaCl₂. The extracted suspensions were determined for total metals using AAS and DTPA-extractable contents using ICP-MS. GBW07455 (GSS-26) was employed as certified reference material for soil analyses, and the measured concentrations were within 85%-105% of the reported certified concentration for Cd and Zn. GSBZ5001-88 ESS was employed as certified reference material for rice straw and plant tissue analyses, and the measured concentration were within 95%-110% of the reported certified concentration for Cd and Zn. Quality control measures included three repetitions for each soil and three blanks for each batch to ensure the quality of the analysis.

2.5 DGT extractable metal content in hotspot micro-area soils

The type of DGT device is DGT passive samplers for deployment in soil Chelex-100. Moreover, the binding resin is Iminodiacetate for measuring metals (cationic). Before DGT deployment, amounts of 50 g air-dried soil with sieved 2 mm was wetted to 80% maximum water holding capacity (MWHC) and mixed well to obtain a soil slurry, and left to equilibrate for 48 h in containers covered with polypropylene film to avoid water evaporation. Subsequently, soils

were wetted to 100% MWHC. Then, the DGT devices with Chelex-100 as resin gel and agarose crosslinked polyacrylamide diffusive gel (DGT Research, Lancaster, UK) were coated gently onto the exposure window of the DGT with soil paste then carefully inserted to ensure complete contact with the soil slurry at room temperature ($22 \pm 1^\circ\text{C}$) for 48 h. Upon retrieval, the filter surface of DGT devices were cleaned with ultrapure water jet to remove the coherent soil particles. The resin binding gel was transferred into tubes to elute metals in 5 mL of 1 mol/L HNO_3 for 24 h prior ($22 \pm 1^\circ\text{C}$) to analysis (Senila et al., 2024).

The eluate was analyzed by ICP-MS to determine the retained concentrations (C_e) of metals used further to calculate the concentration of the labile fraction in soil solution (C_{DGT}), Using Eq. (1) (Zhang and Davison, 2015).

$$C_{\text{DGT}} = \frac{C_e * \Delta g (V_e + V_{\text{gel}})}{f_e * D * A * t} \quad (1)$$

where C_e is the metal concentration in eluate determined by ICP-MS, (ng/mL), V_e the volume of 1 mol·L⁻¹ HNO_3 solution used as eluent (5 mL), V_{gel} is the volume of the binding gel (0.15 mL); f_e is the factor of elution recommended by the manufacturer (0.85); Δg is the thickness of the diffusion layer (0.094 cm=0.080 cm diffusive gel + 0.014 cm filter membrane); A is the physical exposure area surface (3.14 cm²); t is the deployment time for uptake (172800 s) and D is the diffusion coefficient of the analyte through the diffusion layer gel (cm²·s⁻¹), provided by producer, like Zn = 5.60×10^{-6} , Cd = 5.61×10^{-6} (at 22 °C). Immediately after DGT retrieval, the soil slurries were centrifugated for 10 min at 3000 rpm to obtain the soil solution, which was filtered using 0.45 µm polyethersulfone filter, acidulated with 65% HNO_3 ultrapure and measured for metals concentration by ICP-MS (C_{soln}). The concentration was used as reference to estimate the index of resupply (R) definite as the fraction between C_{DGT} and C_{soln} (Eq. (1)).

$$R = \frac{C_{DGT}}{C_{soln}} \quad (1)$$

The R-value indicates the replenishment of the metal in the soil solution at the DGT-biota interface. An $R < 0.1$ stands for a low resupply in soil solution and reduced mobilization of metals from the solid phase; $0.8 > R > 0.1$ resupply is intermediary; An $R > 0.8$ indicates a fast and sustained supply capacity from the soil solid phase.

2.6 Data analysis

Data were analyzed and plotted with SPSS statistical software (Version 24.0), Origin 9.0, R (Version 4.2.0). Duncan's test AND variance (ANOVA) analysis were performed. Data were presented as the mean $\pm$ SD. Transfer factor (TF) = metal concentration in aboveground tissue ($\text{mg} \cdot \text{kg}^{-1}$) / metal concentration in root tissue ($\text{mg} \cdot \text{kg}^{-1}$). Principal components analysis (PCA) was conducted analyze the relationships of plant physiological items, metals concentration in plant tissues, as well as soil properties and DTPA-metals concentration; The analysis was performed in R version 4.2.0 using the “ade4” packages. To determine the integrated differences in soil properties, plant growth and Cd and Zn in soil and plant tissues across the different earthworm species treatments, principal coordinates analysis (PCoA) was performed using the “phyloseq” package in R (version 4.2.0) based on the Bray-Curtis dissimilarity (Qiu et al., 2023). Random Forest analysis was performed with the “randomForest” package to identify the main factors of the Cd and Zn uptake amounts of *S. plumbizincicola*.

3. Results

3.1 Earthworm survival rate and biomass increase

After 90 days, except for L-PE4 and H-PE3, the average survival rate of four earthworm species was higher than 80% (Table 2). Earthworm biomass was increased, except for M-PE4.

Specifically, the survival rate of *A. cortices*, *A. aspergillum*, *A. robustus*, and *M. guillelmi* was 76.7%~100%, 66.7%~100%, 57.1%~100%, 14.3%~100%, respectively; Additionally, the biomass of four earthworm species increased by 1.66%~51.1%, 55.5%~145%, 97.4%~132%, -81.8%~64.1%, respectively.

Table 2 Survival rate and biomass increase of four earthworm species at the 90th day

Soil	Treatments	Survival rate (%)	Mean biomass increase (%)
L	PE1	94.5 ± 4.77 aA	44.3 ± 6.21bA
	PE2	88.9 ± 19.3 aA	78.8 ± 21.2 aB
	PE3	96.7 ± 5.77 aA	110 ± 3.78 aA
	PE4	84.1 ± 16.7 aA	35.0 ± 26.8 bAB
M	PE1	83.2 ± 5.82 bB	12.8 ± 16.7 bB
	PE2	100 ± 0 aA	104 ± 22.1 aAB
	PE3	100 ± 0 aA	123 ± 14.2 aA
	PE4	46.0 ± 27.9 bB	-29.5 ± 48.4 bB
H	PE1	95.0 ± 0 aA	32.3 ± 12.6 bAB
	PE2	100 ± 0 aA	129 ± 14.4 aA
	PE3	65.2 ± 12.9 bB	123 ± 24.8 aA
	PE4	100 ± 0 aA	48.1 ± 16.4 bA

Note: Results are expressed as the mean value ± SD (n=3). The uppercase letters L, M, and H represents the soil L, soil M, and soil H. PE1, PE2, PE3, and PE4 represents the treatments with *Sedum plumbizincicola* + *A. cortices*, *Sedum plumbizincicola* + *A. aspergillum*, *Sedum plumbizincicola* + *A. robustus*, *Sedum plumbizincicola* + *M. guillelmi*. Different lowercase letters indicate significantly differences ($P < 0.05$) between different treatments. Different uppercase letters indicate significantly differences ($P < 0.05$) between different soils.

3.2 The physiological response of *S. plumbizincicola* under earthworm activities

The physiological characterizes of *S. plumbizincicola* are shown in Fig. 1. The earthworm activities significantly increased the leaf chlorophyll, stem thickness, plant height, and biomass (P

<0.05) (Fig. 1 a, b, and c). Moreover, the plant biomass showed well response from increasing leaf chlorophyll for each treatment (Fig.1 a and b). The plant biomass in M soils was roughly twice to three times higher than in L and H soils. In terms of different plant tissues, the aboveground and root biomass were significantly increased under earthworm activities, and roots were greatly affected ($P<0.05$) (Fig.1 c). The aboveground biomass in PE1, PE2, and PE4 treatments was high, and reaching 1.17 ± 0.34 g DW/plant in M-PE1 treatment. The root biomass showed same trend under different earthworm species activities in three contaminated soils, ranked as $PE2 > PE4 \approx PE1 > PE3$, and reaching 0.13 ± 0.07 g DW/plant in M-PE2 treatment.

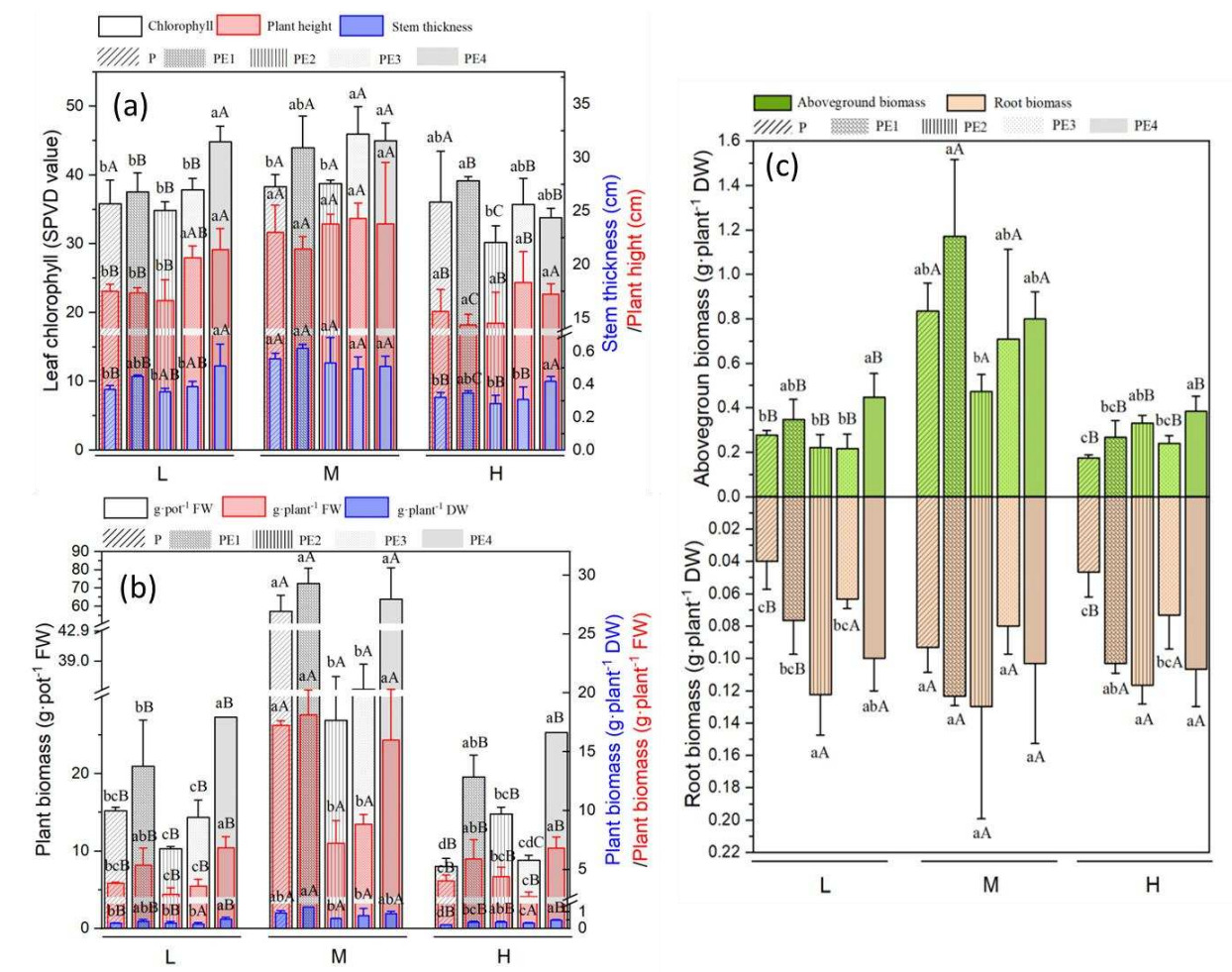


Fig. 1 The physical characteristics of *Sedum plumbizincicola* in different treatments, (a) the basic physical characteristic, (b) the plant biomass, (c) the different organ tissues biomass. Results are expressed as the mean value $\pm$ SD (n=3). The uppercase letters L, M, and H represents the soil L, soil M, and soil H. PE1, PE2, PE3, and PE4 represents the treatments with *Sedum plumbizincicola* + *A. cortices*, *Sedum plumbizincicola* + *A. aspergillum*, *Sedum plumbizincicola* + *A. robustus*, *Sedum plumbizincicola* + *M. guillelm*. Different lowercase letters indicate significantly differences ($P < 0.05$) between different treatments. Different uppercase letters indicate significantly differences ($P < 0.05$) between different soils.

3.3 Phytoremediation efficiency under earthworm bioaugmentation

The metals concentration in different tissues of *S. plumbizincicola* are shown in Fig. 2. The Cd concentration in the aboveground tissues in treatments be observed significantly higher than that in the P treatment in each soil ($P < 0.05$), increased Cd concentration in L-PE1 (4.70 ± 0.81 mg/kg), L-PE2 (4.17 ± 0.22 mg/kg), L-PE4 (5.00 ± 0.17 mg/kg), M-PE1 (36.3 ± 2.37 mg/kg), M-PE4 (41.7 ± 0.47 mg/kg), H-PE1 (9.08 ± 2.45 mg/kg) by 66.1%, 47.3%, 76.7%, 22.7%, 41.1%, and 5.95%, respectively. As for Zn concentration in the aboveground tissues, there were no significantly increase in PE treatments ($P > 0.05$); notably, the high Zn concentration of PE treatments in the aboveground tissues in each soil reached 741 ± 293 , 778 ± 40.3 , 2720 ± 141 mg/kg and 2705 ± 126 mg/kg in L-PE1, L-PE3, M-PE1 and M-PE4, increasing by 15.1%, 20.8%, 8.08% and 7.49%, respectively. Metal concentrations in plant tissues were reversed with the root biomass (Fig. 2 c and d, Fig. 1 c). In terms of soil contaminated levels, the Cd concentration in aboveground tissues and roots was not increased with soil contaminated levels, but showed $M > H > C$ ($P < 0.05$) (Fig. 2 a and c). The Zn concentration showed similar trend with Cd concentration in three soils, and showed $M > H \approx C$ ($P < 0.05$) (Fig. 2 b and d).

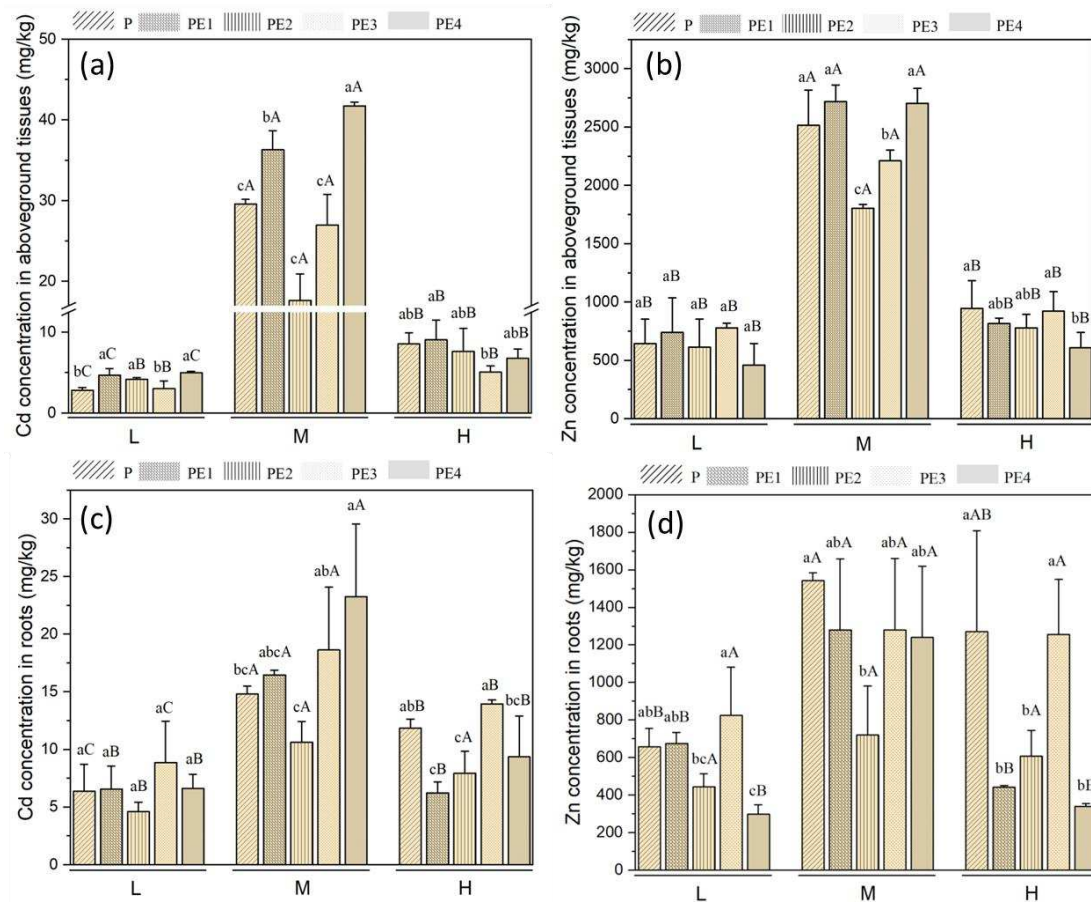


Fig. 2 Cd and Zn concentration in different tissues of *Sedum plumbizincicola*, Cd concentration in (a) aboveground tissues and in (c) roots, Zn concentration in (b) aboveground tissues and in (d) roots. Results are expressed as the mean value $\pm$ SD ($n=3$). The uppercase letters L, M, and H represents the soil L, soil M, and soil H. PE1, PE2, PE3, and PE4 represents the treatments with *Sedum plumbizincicola* + *A. cortices*, *Sedum plumbizincicola* + *A. aspergillum*, *Sedum plumbizincicola* + *A. robustus*, *Sedum plumbizincicola* + *M. guillelm*. Different lowercase letters indicate significantly differences ($P<0.05$) between different treatments. Different uppercase letters indicate significantly differences ($P<0.05$) between different soils.

The Cd and Zn uptake by *S. plumbizincicola*, with or without earthworm activity, is shown in Fig. 3. The Cd uptake content in PE treatment was higher than P treatment ($P<0.05$), except for M-PE2, M-PE3 (Fig. 3 a). Specifically, in L soils, the Cd uptake amount by *S. plumbizincicola* were 8.52 ± 2.29 , 5.78 ± 0.97 , 4.70 ± 1.37 , and 11.5 ± 2.07 $\mu\text{g}/\text{pot}$ in PE1, PE2, PE3, and PE4

treatment, increased by 110%, 42.7%, 16.0%, and 184%, respectively; in M soils, the Cd uptake amount were 177 ± 40.2 and 142 ± 19.5 $\mu\text{g}/\text{pot}$ in PE1 and PE4 treatment, increased by 69.2% and 36.2%, respectively; in H soils, the Cd uptake amount were 11.9 ± 1.00 , 13.6 ± 3.40 , 9.03 ± 2.42 , and 14.6 ± 3.54 $\mu\text{g}/\text{pot}$ in PE1, PE2, PE3, and PE4 treatment, increased by 46.8%, 68.8%, 11.9%, and 81.0%, respectively. However, compared to P treatments, the Zn concentration did not significantly increase in PE treatment for the three soils ($P > 0.05$), except for M-PE1 (13.4 ± 3.66 mg/pot) ($P < 0.05$) (Fig. 3 b). Nevertheless, the Zn uptake amount in PE treatments still showed an increasing trend. No matter Cd and Zn, the *S. plumbizincicola* absorbed more metals from M soils, and the Cd and Zn uptake amounts under earthworm activity ranked as $\text{PE1} > \text{PE4} > \text{PE3} > \text{PE2}$ (Fig. 3 a and b), while the Cd uptake amount ranked as $\text{PE4} > \text{PE1} > \text{PE2} > \text{PE3}$ in L soils and ranked as $\text{PE4} > \text{PE2} > \text{PE1} > \text{PE3}$ in H soils.

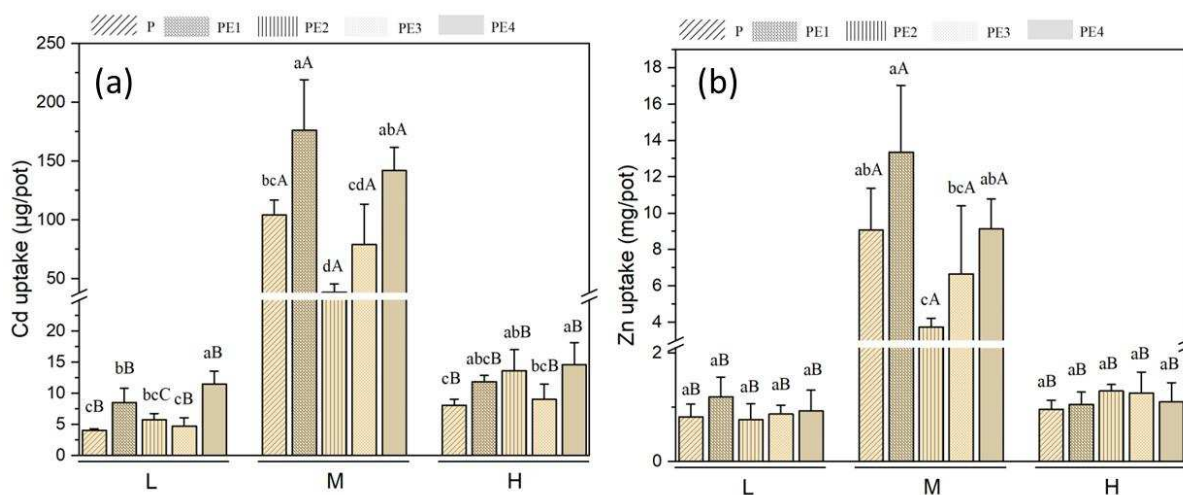


Fig.3 The Cd and Zn uptake by *Sedum plumbizincicola* in different treatments, (a) Cd uptake amount, (b) Zn uptake amount. Results are expressed as the mean value $\pm$ SD (n=3). The uppercase letters L, M, and H represents the soil L, soil M, and soil H. PE1, PE2, PE3, and PE4 represents the treatments with *Sedum plumbizincicola* + *A. cortices*, *Sedum plumbizincicola* + *A. aspergillum*, *Sedum plumbizincicola* + *A. robustus*, *Sedum plumbizincicola* +

M. guillelm. Different lowercase letters indicate significantly differences ($P < 0.05$) between different treatments. Different uppercase letters indicate significantly differences ($P < 0.05$) between different soils.

The transfer factor of *S. plumbizincicola* in different treatments are shown in Fig. 4. In general, the trend of transfer factor-Cd and transfer factor-Zn was consistent with those of the corresponding root biomass. Specifically, except for PE3, Cd and Zn transfer factor were increased in other PE treatments, and significantly promote the metals transfer from roots to aboveground tissues in L and H soils ($P < 0.05$); besides, the overall performance ranked as PE1 > PE2 > PE4 (Fig. 4 a and b). The transfer factors of Cd and Zn increased by a maximum of 98.3% and 110%, respectively, in H-PE1 treatment.

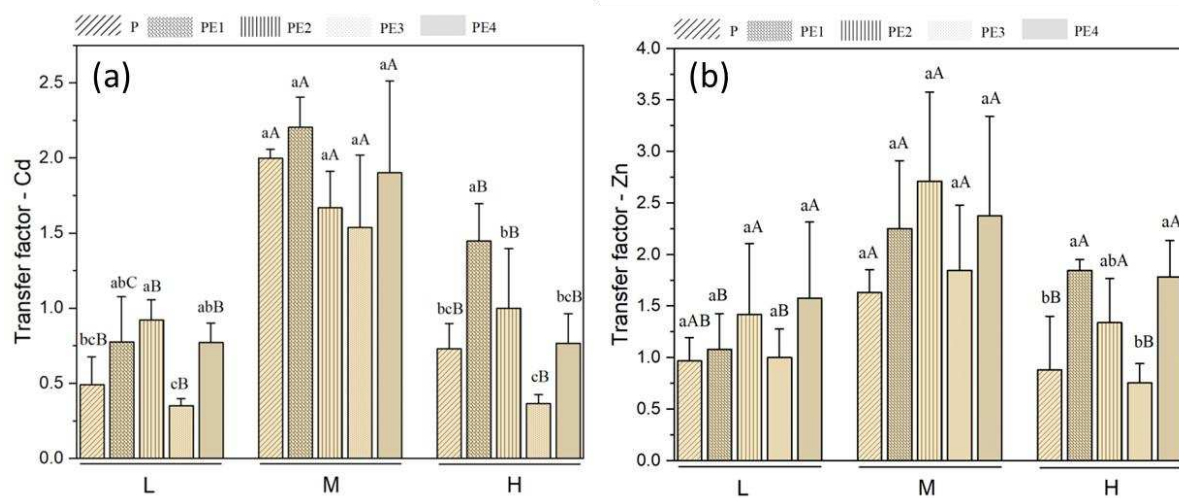


Fig. 4 Transfer factor of *Sedum plumbizincicola* in different treatments, (a) Transfer factor - Cd, (b) Transfer factor-Zn. Results are expressed as the mean value $\pm$ SD (n=3). The uppercase letters L, M, and H represents the soil L, soil M, and soil H. PE1, PE2, PE3, and PE4 represents the treatments with *Sedum plumbizincicola* + *A. cortices*, *Sedum plumbizincicola* + *A. aspergillum*, *Sedum plumbizincicola* + *A. robustus*, *Sedum plumbizincicola* + *M. guillelm*. Different lowercase letters indicate significantly differences ($P < 0.05$) between different treatments. Different uppercase letters indicate significantly differences ($P < 0.05$) between different soils.

3.4 Variations of soil properties after phytoextraction

The soil properties after phytoremediation are shown in Table 3. The soils pH in PE treatment was lower than that in P treatment, especially in L and M soils, the pH significantly decreasing in PE1, PE2, and PE4 treatments ($P < 0.05$). As for EC, the PE1 and PE2 treatments were significantly increased in three contaminated soils ($P < 0.05$); while the PE4 treatments were decreased soil EC. Earthworm activities decreased soil organic carbon, especially in M soils. The SOC and TN content were decreased in the PE treatments, especially significantly decreasing in M soils, showing $P > PE1 > PE2 > PE3 > PE4$. Among the different PE treatments, PE4 had a strong effect on SOC and TN, and significantly reducing its content in M and H soils ($P < 0.05$). There had no significant effect on the C:N ratio ($P > 0.05$); In H soils, PE2 reduced the C:N ratio, and this ratio in PE1, PE3 and PE4 treatments shows reverse trend with PE2.

Compared to P treatment, treatments with earthworms significantly increased DOC content ($P < 0.05$). The DOC content in PE1 treatments in three contaminated soils show increased with the degree of contamination, reaching 101 ± 7.21 mg/kg in H soil. However, PE3 only significantly increased DOC content in L soil. Both PE2 and PE4 treatments shows higher SOC content than P treatment in three soils. The trend of AN content had consistent with TN content in L and H soils, while had reverse with TN content in M soil, such as in M-PE2 (262 ± 5.35 mg/kg) and M-PE3 (240 ± 4.04 mg/kg) treatments. The treatments with earthworms in L soil were increased as for AP content, especially PE1 and PE3 treatments. However, the AP content was reduced in PE treatment in M and H soils. The soil respiration rate also showed different trends between L, H soils and M soils. In L soil, compare to P treatment, the soil respiration rate was decreased by 12.4 %, 7.45%, 4.97%, and 15.5% in PE1, PE2, PE3, and PE4 treatments, respectively; in H soil, it was decreased

by 27.9%, 8.17%, 17.3%, 15.4% in PE1, PE2, PE3, and PE4 treatments, respectively; while in M soil, it was increased by 17.2% and 40.6% in PE3 and PE4 treatments, respectively.

Table 3 Soil chemical and biological properties after 90 days

Soil	Treatments	pH	EC ($\mu\text{S}\cdot\text{cm}^{-1}$)	SOC ($\text{g}\cdot\text{kg}^{-1}$)	TN ($\text{g}\cdot\text{kg}^{-1}$)	C:N	DOC ($\text{mg}\cdot\text{kg}^{-1}$)	AN ($\text{mg}\cdot\text{kg}^{-1}$)	AP ($\text{mg}\cdot\text{kg}^{-1}$)	Soil respiration rate ($\text{mgC}\cdot\text{CO}_2\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$)
L	P	6.71 $\pm$ 0.12 aA	75.7 $\pm$ 4.24 bcB	28.7 $\pm$ 0.46 aA	2.38 $\pm$ 0.08 abB	12.1 $\pm$ 0.24 aA	59.2 $\pm$ 3.66 cA	204 $\pm$ 5.35 aB	7.48 $\pm$ 0.58 bB	161 $\pm$ 9.90 aB
	PE1	6.52 $\pm$ 0.03 bA	239 $\pm$ 17.8 aB	28.5 $\pm$ 0.77 aA	2.34 $\pm$ 0.09 bA	12.2 $\pm$ 0.56 aA	57.1 $\pm$ 0 cB	186 $\pm$ 7.29 bC	10.6 $\pm$ 0.39 aB	141 $\pm$ 4.70 bcA
	PE2	6.56 $\pm$ 0.02 bA	232 $\pm$ 8.92 aB	28.9 $\pm$ 0.44 aA	2.54 $\pm$ 0.12 aA	12.5 $\pm$ 0.35 aA	90.5 $\pm$ 10.8 bA	190 $\pm$ 5.35 bC	8.53 $\pm$ 0.37 bB	149 $\pm$ 12.3 abcB
	PE3	6.64 $\pm$ 0.10 abB	85.5 $\pm$ 10.4 bA	29.0 $\pm$ 0.82 aAB	2.39 $\pm$ 0.08 abA	12.1 $\pm$ 0.07 aA	105 $\pm$ 7.21 aA	184 $\pm$ 4.04 bC	10.1 $\pm$ 1.11 aB	153 $\pm$ 4.70 abA
	PE4	6.27 $\pm$ 0.06 cA	50.9 $\pm$ 20.2 cAB	28.6 $\pm$ 0.39 aA	2.27 $\pm$ 0.04 bAB	12.6 $\pm$ 0.17 aA	81.5 $\pm$ 3.53 bAB	187 $\pm$ 4.04 bC	8.11 $\pm$ 0.94 bB	136 $\pm$ 1.78 cB
M	P	6.48 $\pm$ 0.32 aA	69.5 $\pm$ 11.3 cB	30.1 $\pm$ 0.72 aA	2.65 $\pm$ 0.04 aA	11.4 $\pm$ 0.42 aA	31.7 $\pm$ 0 cB	227 $\pm$ 8.08 cA	7.87 $\pm$ 0.32 aB	128 $\pm$ 3.08 cC
	PE1	5.77 $\pm$ 0.07 bB	223 $\pm$ 31.2 aB	29.0 $\pm$ 0.46 abA	2.48 $\pm$ 0.05 bA	11.7 $\pm$ 0.43 aA	45.1 $\pm$ 23.1 bcB	221 $\pm$ 7.29 cA	7.27 $\pm$ 0.57 abC	129 $\pm$ 3.56 cB
	PE2	5.76 $\pm$ 0.06 bC	122 $\pm$ 11.5 bC	28.6 $\pm$ 1.52 abA	2.46 $\pm$ 0.07 bcA	11.7 $\pm$ 0.79 aA	65.5 $\pm$ 10.8 abB	262 $\pm$ 5.35 aA	6.09 $\pm$ 0.32 bC	107 $\pm$ 10.8 dC
	PE3	6.58 $\pm$ 0.15 aA	59.1 $\pm$ 14.9 cA	27.6 $\pm$ 0.66 bcB	2.38 $\pm$ 0.03 bcA	11.6 $\pm$ 0.21 aB	28.1 $\pm$ 10.8 cC	240 $\pm$ 4.04 bA	6.59 $\pm$ 0.40 bC	150 $\pm$ 9.90 bA
	PE4	6.38 $\pm$ 0.11 aA	27.0 $\pm$ 9.33 dB	26.5 $\pm$ 0.56 cB	2.37 $\pm$ 0.06 cA	11.2 $\pm$ 0.30 aB	71.3 $\pm$ 9.33 aB	208 $\pm$ 5.35 dA	6.61 $\pm$ 1.20 bB	180 $\pm$ 9.24 aA
H	P	6.56 $\pm$ 0.39 aA	123 $\pm$ 32.0 cA	29.2 $\pm$ 1.88 abA	2.47 $\pm$ 0.01 abB	11.8 $\pm$ 0.79 abA	57.1 $\pm$ 12.7 bcA	215 $\pm$ 7.00 aAB	19.8 $\pm$ 0.35 aA	208 $\pm$ 3.08 aA
	PE1	6.48 $\pm$ 0.11 aA	343 $\pm$ 42.7 aA	29.5 $\pm$ 1.29 aA	2.36 $\pm$ 0.05 cA	12.5 $\pm$ 0.35 aA	101 $\pm$ 7.21 aA	205 $\pm$ 6.06 bB	19.6 $\pm$ 0.62 aA	150 $\pm$ 7.75 cA
	PE2	6.36 $\pm$ 0.06 aB	278 $\pm$ 19.1 bA	29.1 $\pm$ 0.36 abA	2.52 $\pm$ 0.03 aA	11.6 $\pm$ 0.06 bA	63.5 $\pm$ 7.21 bB	205 $\pm$ 3.50 bB	19.3 $\pm$ 1.21 aA	191 $\pm$ 3.56 abA
	PE3	6.20 $\pm$ 0.15 aB	71.4 $\pm$ 14.2 cA	29.8 $\pm$ 0.83 aA	2.40 $\pm$ 0.02 bcA	12.4 $\pm$ 0.23 aA	46.8 $\pm$ 0 cB	205 $\pm$ 4.04 bB	16.7 $\pm$ 1.35 bA	172 $\pm$ 21.6 bA
	PE4	6.45 $\pm$ 0.15 aA	80.2 $\pm$ 17.8 cA	27.2 $\pm$ 0.99 bB	2.20 $\pm$ 0.07 dB	12.3 $\pm$ 0.24 abA	93.7 $\pm$ 9.33 aA	197 $\pm$ 5.35 bB	15.2 $\pm$ 1.12 bA	176 $\pm$ 9.90 bA

Note: Results are expressed as the mean value $\pm$ SD (n=3). The uppercase letters L, M, and H represents the soil L, soil M, and soil H. PE1, PE2, PE3, and PE4 represents the treatments with *Sedum plumbizincicola* + *A. cortices*, *Sedum plumbizincicola* + *A. aspergillum*, *Sedum plumbizincicola* + *A. robustus*, *Sedum plumbizincicola* + *M. guillelm*. Different lowercase letters indicate significantly differences ($P < 0.05$) between different treatments. Different uppercase letters indicate significantly differences ($P < 0.05$) between different soils.

3.5 Different forms of Cd and Zn in soil

The concentration of DTPA-Cd and DTPA-Zn in soil after phytoremediation are shown in Table 4. The DTPA-metals concentration was significantly increased with the degree of contamination ($P < 0.05$). Additionally, the soil DTPA-metals concentration of the treatments with earthworms was different with P treatment, especially for increasing DTPA-Zn concentration in M and H soils ($P < 0.05$). In three contaminated soils, the DTPA-Cd and DTPA-Zn concentration

in PE2 treatment was highest, and followed by PE3. While the DTPA-Cd concentration in PE4 treatment was similar with that in PE1 treatment, and were lower than other PE treatments.

Table 4 DTPA-metals concentration in soil after phytoremediation

Contaminated level	Treatments	DTPA-Cd (mg·kg ⁻¹)	DTPA-Zn (mg·kg ⁻¹)
L	P	0.45±0.00 aB	8.76±0.32 abB
	PE1	0.36±0.09 aB	8.21±0.62 bB
	PE2	0.51±0.16 aB	10.4±0.77 aC
	PE3	0.39±0.03 aB	8.84±0.76 abC
	PE4	0.32±0.06 aB	7.44±1.28 bC
M	P	0.37±0.01 bC	10.6±1.52 bB
	PE1	0.34±0.05 bB	12.7±1.84 abB
	PE2	0.49±0.04 aB	14.8±1.77 aB
	PE3	0.41±0.02 abB	14.6±1.01 aB
	PE4	0.36±0.02 bB	12.4±1.06 abB
H	P	0.67±0.04 aA	20.9±2.66 bcA
	PE1	0.69±0.16 aA	17.5±1.83 cA
	PE2	0.81±0.11 aA	28.2±2.36 aA
	PE3	0.70±0.14 aA	23.5±0.79 abA
	PE4	0.57±0.09 aA	22.3±2.48 bcA

Note: Results are expressed as the mean value ± SD (n=3). The uppercase letters L, M, and H represents the soil L, soil M, and soil H. PE1, PE2, PE3, and PE4 represents the treatments with *Sedum plumbizincicola* + *A. cortices*, *Sedum plumbizincicola* + *A. aspergillum*, *Sedum plumbizincicola* + *A. robustus*, *Sedum plumbizincicola* + *M. guillelm*. Different lowercase letters indicate significantly differences ($P < 0.05$) between different treatments. Different uppercase letters indicate significantly differences ($P < 0.05$) between different soils.

3.6 The effect of earthworm activities on physiological and metals phytoextraction capability of *Sedum plumbizincicola*, soil properties and DTPA-metals

Principal Component Analysis was carried out to identify the overall trends in *S. plumbizincicola*, soil properties and metals activities in response to different ecological categories

and species earthworm's activities in metal contaminated soils (Fig. 5). In PCA analysis (Fig. 5 a and b), Axis 1 (41.1% variance explained) was primarily associated with Leaf chlorophyll, Plant height, Stem thickness, Plant biomass FW, Above biomass DW, Uptake Cd, Uptake Zn, and Soil respiration rate. Axis 2 (15.2% variance explained) was mainly linked to TF-Cd, pH, EC, AN, TN, DTPA-Cd, DTPA-Zn, and AP. Axis 3 (9.2% variance explained) was mainly related to Root biomass DW, TF-Zn, TF-Zn, DOC, and SOC. The soils of inoculation with earthworms were significantly differentiated from the soil of only inoculation with *S. plumbizincicola* (Fig. 5 c and d) ($P < 0.001$ and $P < 0.003$). Principle coordinate analysis was employed to understand the integrated differences of *S. plumbizincicola* combined with different earthworm species in soil properties, plant growth and Cd and Zn in soil and plant tissues in low, medium and high contaminated soils (Fig. 5 e-g). The points of PE1, PE2 and PE4 were clearly separated from P treatment in three contaminated soils. Random Forest regression model was employed to identify the main factors of the Cd and Zn uptake amounts of *S. plumbizincicola* (Fig. 5 h-i). The above biomass DW, TF-Cd, AP, leaf chlorophyll and pH were significantly influence on the Cd uptake amount ($P < 0.05$). The above biomass DW, AP, AN, C:N, EC, Leaf chlorophyll and DTPA-Zn were significantly influence on the Zn uptake amount. Furthermore, the aboveground biomass ranked first, followed by TF-Cd and AP, and then AN.

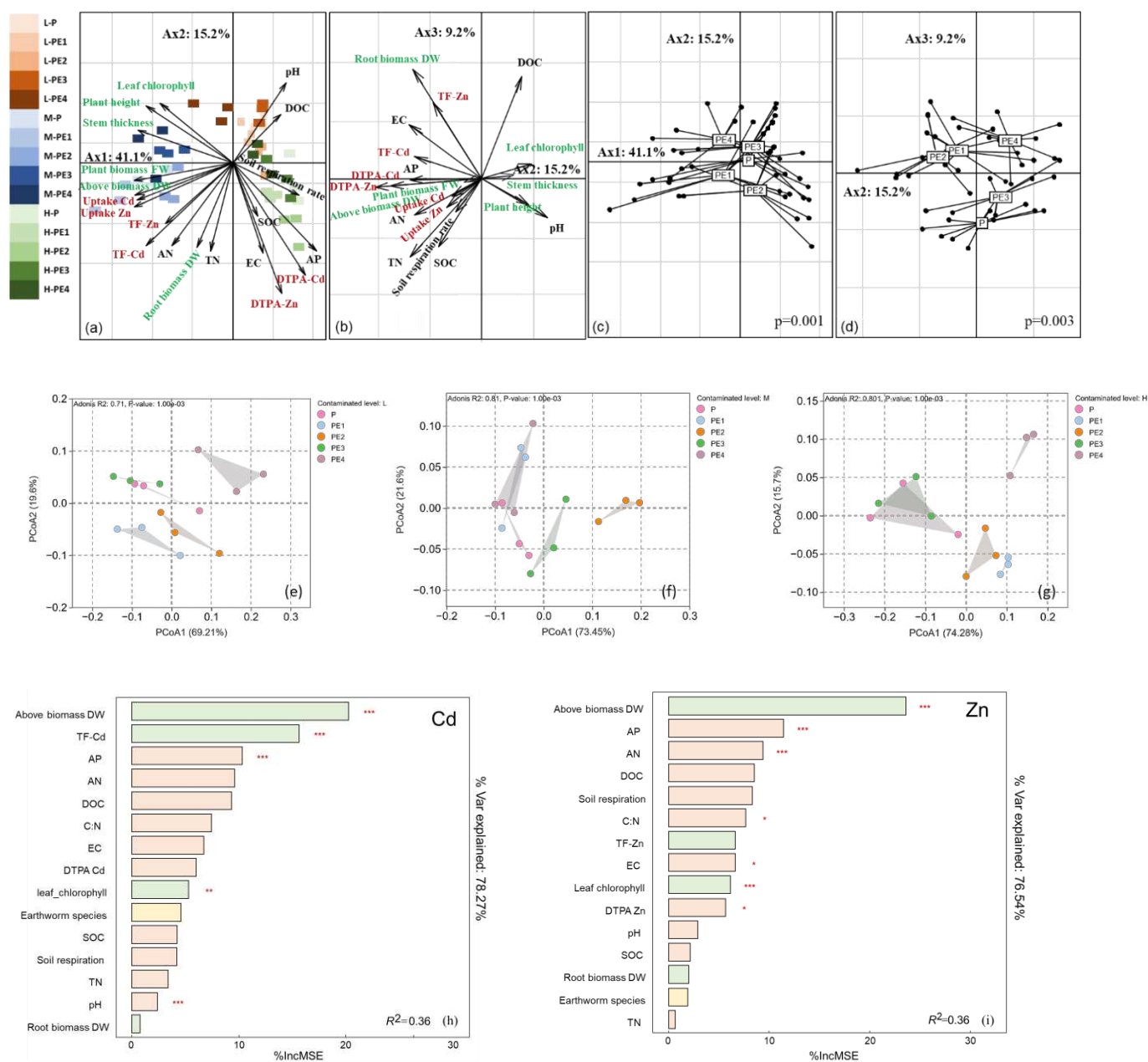


Fig. 5 Principal component analysis of the effect of earthworm activity on physiological and metals phytoextraction capability of *S. plumbizincicola*, soil properties and DTPA-HMs in metals contaminated soil in fractal F1, F2 and F3 planes (a-b). Projection of experimental points according to treatments with or without earthworm in metals contaminated soil in fractal F1, F2 and F3 planes (c-d). Principle coordinate analysis plot with Bray-Curtis dissimilarity among soil properties, plant growth and Cd and Zn in soil and plant in different treatments in low, medium, and high contaminated soils (e-g). Random Forest regression model shows the main factors of the Cd and Zn uptake amounts of *S. plumbizincicola* (h-i).

3.7 DGT-metals concentration of different hotspot soil components

To further analysis the difference between PE2 and PE4 in M and H soils, DGT-metals concentrations and the partition coefficient in hotspots soil were determined and calculated (Fig. 6 and Fig. 7). In general, the rhizosphere soil and drilosphere soils (cast and burrow wall) in P and PE treatments had higher C_{DGT-Cd} and C_{DGT-Zn} than that in the CK treatment, especially in M soils (Fig. 6). In M soils, the C_{DGT-Cd} and C_{DGT-Zn} in different hotspots soil of the PE2 and PE4 treatments showed a significantly difference, and the trends also different ($P < 0.05$); Specifically, the C_{DGT-Cd} and C_{DGT-Zn} in different hotspots soil in M soil ranked as Burrow wall $>$ Cast $>$ Rhizo (overlap area) in PE2 treatment, and ranked as Rhizo (overlap area) $>$ Cast $>$ Burrow wall in PE4 treatment; however, the DGT-metals concentration treatment showed differences in H soil, and ranked as Rhizo (overlap area) $>$ Cast $\approx$ Burrow wall in PE2 treatment, and ranked as Rhizo (overlap area) $>$ Burrow wall $>$ Cast in PE4 treatment. The index of resupply of Cd and Zn elements in M and H soils were consistently with $C_{DGT-metals}$ (Fig. 7). The R-Cd in each hotspots soil was greater than 0.8 in both soils (Fig. 7 a). The R-Zn was between 0.09 to 0.53. In addition, the Earthworm cast and burrow wall significantly increased the R-Cd and R-Zn, such as PE2-Cast, PE2-Burrow wall ($P < 0.05$), and PE4-Rhizo in M soil (Fig. 7 a), and PE2-Rhizo, PE2-Cast, and PE4-Rhizo in H soil (Fig. 7 b). Meanwhile, compare to CK treatment, the R-Cd was not significantly increased in H soil ($P > 0.05$), but it increased by 61.3%, 43.5%, and 59.5% in PE2-Rhizo, PE2-Cast, and PE2-Burrow wall, and increased by 47.6%, 25.6%, and 17.9% in PE4-Rhizo, PE2-Cast, and PE2-Burrow wall, respectively (Fig. 7 a).

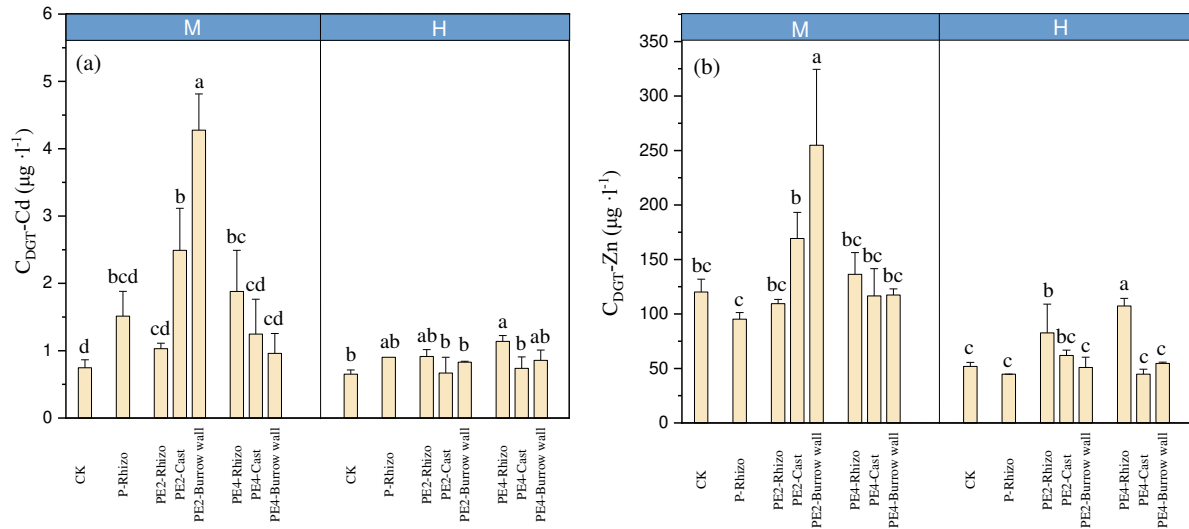


Fig. 6 DGT-metals concentration in hotspot soil, (a) DGT-Cd, (b) DGT-Zn. The uppercase letters M and H represent the M and H soils, respectively. CK, control; P-Rhizo, the rhizosphere soil in P treatment; PE2-Rhizo and PE4-Rhizo, the rhizosphere soil in PE2 and PE4 treatments, also represent the overlap area of rhizosphere and drilosphere area; PE2-Cast and PE4-Cast, the earthworm cast in PE2 and PE4 treatments; PE2-Burrow wall and PE4-Burrow wall, the earthworm burrow wall in in PE2 and PE4 treatments. The lowercase letters indicate significantly difference ($P < 0.05$) between different hotspots soil in same soils.

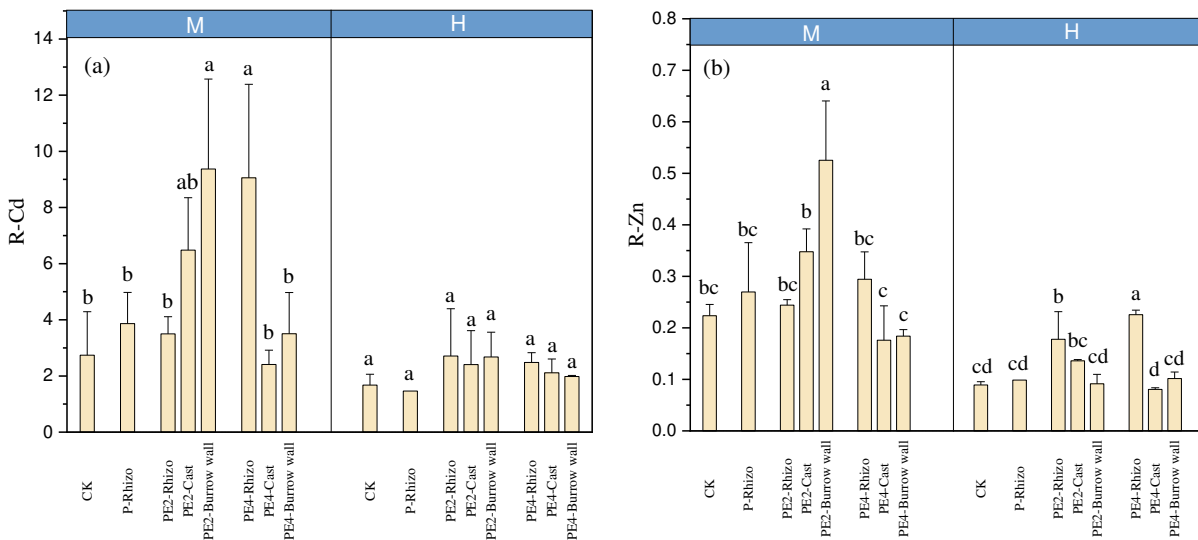


Fig. 7 The index of resupply in hotspots soil, (a) R-Cd, (b) R-Zn. The uppercase letters M and H represent the M and H soils, respectively. CK, control; P-Rhizo, the rhizosphere soil in P treatment; PE2-Rhizo and PE4-Rhizo, the rhizosphere soil in PE2 and PE4 treatments, also represent the overlap area of rhizosphere and drilosphere area; PE2-

Cast and PE4-Cast, the earthworm cast in PE2 and PE4 treatments; PE2-Burrow wall and PE4-Burrow wall, the earthworm burrow wall in in PE2 and PE4 treatments. The lowercase letters indicate significantly difference ($P < 0.05$) between different hotspots soil in same soils.

4. Discussion

4.1 Earthworm survival, growth and species-specific effects on growth and remediation efficiency of *S. plumbizincicola* under different contaminated levels

The high survival rate and biomass of four wild earthworm species after 90 days indicates its ability to tolerate metal pollution stresses and adapt to this phytoremediation system. This high tolerance ability of wild earthworms to environmental stress align with a previous study (Zhang et al., 2022), and this combined remediation model may be promising in the field. According to the well performance of earthworm *A. robustus* and *M. guillelmi* in other treatments and soils, the decrease of survival rate and biomass of these two species in medium and high contaminated soils may be due to escape from the pot (Table 2).

The inoculation of wild earthworms shows growth-promoting effects on *S. plumbizincicola*, such as increasing its photosynthesis capacity, root development and nutrient absorption capacity, and adaptability to environmental stress (Fig. 1). The response of plant to earthworm activities was consistent with Kaur et al., (2019) and Tibihenda et al., (2022). Those positive effect may be attributed to the improvement of soil porosity, nutrient availability influenced by earthworm activity and intense fine root colonization of soil casts (Tibihenda et al., 2022; Decaens and Jiménez and Lavelle, 2001), and the moderate pastoralism effect on plant roots stimulating the plant growth (Brussaard., 1999; Manuel., 2000). From the final remediation effect, the earthworm activity improved the soil remediation efficiency of *S. plumbizincicola*, especially the epigeic *A. cortices* and the anecic *A. aspergillum* and anecic *M. guillelmi* (Fig. 1 b and c, Fig. 4, Table 4). This was mainly due to the feeding preference and movement habit of earthworms (Lavelle et al., 1998).

The hyperaccumulator *S. plumbizincicola* is a kind of shallow rooting herb, and its root depth is 0-15 cm, mainly occurring at 2-15 cm depth and particularly at 2-10 cm (Hu et al., 2019), while the key point is that both ecotype epigeic and anecic earthworms are feeding in the topsoil (Bouche et al., 1977; Keith and Robinson, 2012) and promote the interaction of plant roots with soil organisms. Additionally, the earthworms *A. cortices* are extraction cast in the topsoil, and the earthworms *A. aspergillum* and *M. guillelm* are preferably digging and extraction cast in the gallery (Zhang et al., 2022; Tibihenda et al., 2022; Ma et al., 2021), which promoted nutrient mineralization and root development (Table 1 and Fig. 1).

Metal concentrations in plant tissues reached high levels and showed the hyperaccumulator plant characteristics, consistent with Wang et al., (2020). The Cd and Zn concentration of aboveground and root tissues showed dilution effects, especially in root tissues (Fig. 1c and Fig. 2), which mainly caused by promoting growth effects of earthworm on the roots. For *S. plumbizincicola*, which has thin root systems and shows difficulty to separate the roots from soil, the critical factors of remove metals from soil is increasing metals transfer to the harvestable aboveground tissues. The underline mechanisms of earthworm activity promoting metals transfer from roots to aboveground tissues of *S. plumbizincicola* may be attributed to the increase of active matter chelating with metals (e.g., mucus (Huan et al., 2023), rich amino acids and proteins in cast (Lavelle et al., 1997), dissolved organic carbon), root development increasing soil metal bioavailability and roots uptake capacity by influencing endophytic and rhizosphere bacteria (Ma et al., 2015), upregulation of overexpression of function genes or increasing the activity of transport proteins (Li et al., 2024; Zhang et al., 2023), and stimulation by microbes to help the plants detoxify the metals (Kaur et al., 2019). One of them can be verified by the similarity trend of Cd and Zn transfer factors with the root biomass (Fig. 1c and Fig. 4). In terms of the ecological

categories of earthworm, *S. plumbizincicola* with the participation of epigeic *A. cortices* had higher metal transfer capacity than *S. plumbizincicola* with the participation of anecic *A. aspergillum* and anecic *M. guillelm*. This may be due to the intensive disturbance by epigeic *A. cortices* to the plant roots and rhizospheric environment, which further enhanced the interaction among earthworms, roots, and microorganisms. However, endogeic *A. robustus* did not shows obviously influence on the metal transfer in plants, mainly resulting from light movement and weekly interaction with plant roots. In different contaminated soils, the performance of *S. plumbizincicola* in moderate contaminated soils with high alkali-hydrolysable nitrogen was very well (Fig. 3 and Table 3). It exists positive relationship with soil nitrogen content (Fig. 5 a). van Groenigen et al., (2014) employed meta-analysis method and also concluded that earthworms increased the aboveground biomass in agroecosystems mainly through increased nitrogen availability.

4.2 The dominate factors for improving the remediation efficiency in soil-*S. plumbizincicola*-wild earthworm system

Overall, compared to the soil properties indexes and earthworm species, the factor of harvestable aboveground tissues biomass products drives the phytoremediation efficiency under earthworm activity, followed by transfer capacity of metals from roots to aboveground tissues and availability nutrient (Fig. 5 h and i). It could be founded that the first two key factors are directly related to plants. The underline mechanisms may be attributed to earthworm activities, which made indirectly effects on phytoremediation by creating healthy soil environment for the roots of *S. plumbizincicola*, promoting the root development and increasing the bioavailability of nutrient and metals (Blouin et al., 2013; Wang et al., 2018). Moreover, earthworm activity increased the leaf chlorophyll, so the high aboveground biomass was may benefit from the high leaf chlorophyll and photosynthesis efficiency by increase the expression of SpHMA1 to promote metals transfer to

outside from chloroplast (Zhao et al., 2019). Additionally, the endophytic and rhizosphere microbial communities (abundance, diversity and structure) may be the dominant pathway to increase the nutrient intake and accelerated translocation of metals entering the roots into the aboveground (Ma et al., 2015; Jusselme et al., 2015).

4.3 The trade-off of different earthworm species on soil nutrient mineralization and heavy resistance in phytoremediation system

Due to the feeding preference and movement habits, for the different ecological groups earthworms exist different ecological strategies and functions in soil environment (Lavelle et al., 1998; Eisenhauer and Eisenhauer, 2020). Additionally, earthworms may exist trade-off of satisfying its own survival needs and resistance to external environment stress. Ge et al., (2024) founded that metal stress and nutrient levels can change earthworm activities, such as cast production and burrow length. In this phytoremediation system, earthworm *A. cortices* shows strong nutrient mineralization, especially nitrogen element (Table 3). It also improved the remediation efficiency by increasing the metals availability and transfer from roots to aboveground part; this species may increase the soil remediation efficiency by improve metals bioavailability and the soil environment of *S. plumbizincicola* living in, such as improve the soil structure and nutrient availability. Due to the intensive interaction between earthworms and roots, soil after *A. cortices* activity can resupply the metals to roots in time. Earthworm *A. robustus* shows lightly nutrient mineralization, like increasing dissolved organic carbon, but its effects on *S. plumbizincicola* extraction metals is not obvious in general as compared with P treatment (Fig. 5 a, b, c and d). This species showed strong ability of metals activation (Table 4), but failed to increase the metals uptake amount and transfer ability in *S. plumbizincicola*, possibly resulting from insufficient development of roots and available metals resupply to roots unsuccessfully.

Laossi et al., (2009) found that anecic *Lumbricus terrestris* can increase plant biomass, while endogeic *Aporrectodea caliginosa* failure. Earthworm *A. aspergillum* promoted the roots development by mineralization of more phosphorus, and showed strong ability to activate soil metals (Fig. 5 a and c); the earthworm *A. aspergillum* increased *S. plumbizincicola* absorption of more soil metals by developing root system and bring more bioavailability metals to rhizosphere area, while earthworm *M. guillelm* shows great nutrient mineralization by increasing soil dissolved organic carbon (Fig. 5 b and d). The gut processes of *M. guillelm* promoted the soil organic matter mineralization and improved the soil environment, and then promoting *S. plumbizincicola* growth and secreting more root exudates rather than activating soil metals. Those processes are beneficial to plant root absorbing more metals from soil. The DGT-metals concentration in different hotspots soils could provide the direct evidence of the effects of anecic *A. aspergillum* and *M. guillelm* on soil metals bioavailability and resupply ability from soil to roots (Fig. 6 and Fig. 7). The DGT-Cd and DGT-Zn concentration in hotspot soils indicated the different strategies to enhance the phytoremediation effects, this phenomenon could be found in moderate contaminated soils (Fig. 6 a and b).

5. Conclusion

This study focuses on the physiological response and metals concentration in different hyperaccumulator *S. plumbizincicola* tissues and metals availability in soils to investigate whether wild earthworm can achieve the bioaugmentation aims in influencing phytoextraction efficiency under different levels soil contaminated by Cd and Zn. Generally, wild earthworm can improve soil nutrient mineralization and metals availability, promote *S. plumbizincicola* growth and increase the transfer capacity from roots to harvestable aboveground tissues, which achieved the bioaugmentation effects on phytoextraction. The specific-species effects of wild earthworm on *S.*

plumbizincicola were founded that the overall performance on phytoremediation ranked as *A. cortices* > *A. aspergillum* > *M. guillelmi*. However, earthworm species *A. robustus* lightly affected those processes. The bioaugmentation effects of earthworms dominated by increasing the biomass of *S. plumbizincicola*, followed by the metals transfer capacity in plants and available phosphorus. In addition, the trade-off of earthworm on nutrient mineralization and metal resistant capacity occur in different ecological categories. The feeding preference and movement habits of epigeic and anecic decided to the notably effects on the remediation improvement. Moreover, this performance of different species but same ecological groups shows different bioaugmentation strategies in this system. This study provides a clear understanding of the combined effects and underline mechanisms of hyperaccumulator *Sedum plumbizincicola* and wild earthworms on the soil remediation polluted by multiple-metals in abandoned mining area.

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Chapter III: Effect of individual and combined different wild earthworm species on the phytoextraction potential of *Sedum plumbizincicola*

Résumés: La contamination des sols par les métaux, en particulier dans les zones minières, constitue un défi environnemental important. À l'heure actuelle, la plupart des études se sont concentrées sur les effets d'une seule espèce de ver de terre sur le transfert ou le devenir des métaux dans l'écosystème, mais cela ne correspond pas au système naturel. Par exemple, l'enquête de terrain menée par Singh et al. en 2019 a révélé que les vers de terre endogènes et anéciques dominent les communautés de vers de terre sur le site étudié. Très peu d'études ont examiné les effets interactifs de différentes catégories de vers de terre sur la croissance des plantes hyperaccumulatrices (Laossi et al., 2009a, b). Les effets des activités des espèces de vers de terre individuelles et combinées sur l'activation des métaux restent également peu clairs. D'après les résultats du dernier chapitre, les vers de terre appartenant aux catégories écologiques épigée et anécique ont montré des effets de bioaugmentation plus importants que les vers de terre endogés. Par conséquent, les vers de terre *A. cortices* et *A. aspergillum* ont été sélectionnés, à la fois individuellement et en combinaison, dans le système de remédiation de *Sedum plumbizincicola* dans différents sols miniers contaminés par le Cd et le Zn dans des conditions de laboratoire contrôlées pendant 90 jours. Nous avons étudié les états physiologiques et les concentrations de métaux dans différents tissus de *S. plumbizincicola*, la concentration de métaux dans la solution du sol au ⁶⁰e jour et au ⁹⁰e jour, la spéciation des métaux dans le sol, ainsi que la biomasse et la structure de la communauté microbienne, afin d'explorer les effets de plusieurs espèces de vers de terre sur l'efficacité de la phytoremédiation et de révéler les mécanismes sous-jacents permettant

d'optimiser les processus de biorestauration. Les résultats montrent que la combinaison d'espèces de vers de terre avec *S. plumbizincicola* facilite la survie d'*A. cortices* et d'*A. aspergillum*. La biomasse des tissus aériens et des racines a montré une augmentation stable chez *S. plumbizincicola* inoculé avec *A. cortices*, *A. aspergillum*, et la combinaison des deux, atteignant 0,17-3,68 fois et 1,36-4,64 fois plus élevés que ceux de *S. plumbizincicola seul*. Un effet de dilution marqué a été observé dans différents tissus à un niveau de contamination élevé. En outre, la biomasse des différents tissus et le facteur de transfert dans *S. plumbizincicola* + *A. cortices* + *A. aspergillum* étaient similaires ou légèrement inférieurs à ceux de *S. plumbizincicola* + *A. cortices* et *S. plumbizincicola* + *A. aspergillum*. Néanmoins, l'activité des vers de terre a encore amélioré l'efficacité de la phytoremédiation. Les quantités de Cd et de Zn absorbées ont augmenté jusqu'à 435%, 540%, 315%, et 167%, 207%, 130% chez *S. plumbizincicola* + *A. cortices* + *A. aspergillum*, *S. plumbizincicola* + *A. cortices*, et *S. plumbizincicola* + *A. aspergillum*, respectivement. Les quantités les plus élevées d'absorption de Cd et de Zn ont atteint $124 \pm 10,3 \mu\text{g}$ et $5,09 \pm 0,34 \text{ mg}$ par 1,5 kg de sol dans *S. plumbizincicola* + *A. aspergillum* et *S. plumbizincicola* + *A. cortices*, respectivement. En outre, la mobilité et la biodisponibilité des métaux du sol ont augmenté sous l'activité des vers de terre avec le temps de culture, facilitant le transport des métaux du sol vers la solution du sol et finalement vers les racines des plantes. L'activité des vers de terre a influencé de manière significative la biomasse et la structure microbienne du sol, en particulier les bactéries, dans les sols en vrac et dans la rhizosphère. L'analyse statistique multivariée a indiqué que les bactéries et les champignons étaient fortement influencés par l'activité des vers de terre et jouaient un rôle vital dans le transfert des métaux dans le système sol contaminé - *S. plumbizincicola*- vers de terre sauvages. Cette étude pourrait donner un aperçu des effets et du potentiel des multi-organismes combinés pour l'assainissement des sols dans l'environnement naturel.

Effect of individual and combined different wild earthworm species on the phytoextraction potential of *Sedum plumbizincicola*

Highlights

- The bioaugmentation effects of combined earthworm species were similar to, or slightly lower than, those of individual species;
- Earthworm activity promoted Cd and Zn uptake by *S. plumbizincicola* by increasing metal mobility and bioavailability, facilitating metals transports from soil to soil solution and ultimately to plant roots;
- The bacteria and fungi were strongly influenced by earthworm activity and played a vital role in metal transform.

Abstract: Soil contamination with metals, particularly in mining areas, poses a significant environmental challenge. Based on the findings from the last chapter, earthworms belong to epigeic and anecic ecological categories showed greater bioaugmentation effects than endogeic earthworms. Hence, earthworms *A. cortices* and *A. aspergillum* were selected, both individually and in combination, within the *Sedum plumbizincicola* remediation system in different Cd and Zn contaminated mining soils under controlled laboratory conditions for 90 days. We investigated the physiological states and metal concentrations in different tissues of *S. plumbizincicola*, metal concentration in soil solution at 60th day and 90th day, soil metal speciation, and microbial community biomass and structure to assess the bioaugmentation effects and reveal the underline mechanisms. The results show that combining earthworm species with *S. plumbizincicola* facilitate the survival of *A. cortices* and *A. aspergillum*. The biomass of aboveground tissues and roots showed a stable increase in *S. plumbizincicola* inoculated with *A. cortices*, *A. aspergillum*, and combination of both, reaching 0.17-3.68 folds and 1.36-4.64 folds higher than those in *S. plumbizincicola* alone. A marked dilute effect was observed in different tissues in high contaminated level. Moreover, the biomass of different tissues and transfer factor in *S. plumbizincicola*+ *A. cortices* + *A. aspergillum* were similar to or lightly lower than those in *S. plumbizincicola* + *A. cortices* and *S. plumbizincicola* + *A. aspergillum*. Nevertheless, earthworm activity still improved the phytoremediation efficiency. Cd and Zn uptake amounts increased by up to 435%, 540%, 315%, and 167%, 207%, 130% in *S. plumbizincicola* + *A. cortices* + *A. aspergillum*, *S. plumbizincicola* + *A. cortices*, and *S. plumbizincicola* + *A. aspergillum*, respectively. The highest Cd and Zn uptake amounts reached $124 \pm 10.3 \mu\text{g}$ and $5.09 \pm 0.34 \text{ mg}$ per 1.5 kg soil in *S. plumbizincicola* + *A. aspergillum* and *S. plumbizincicola* + *A. cortices*, respectively. Furthermore, soil metal mobility and bioavailability increased under earthworm

activity with cultivation time, facilitating metal transport from soil to soil solution and ultimately to plant roots. Earthworm activity significantly influenced soil microbial biomass and structure in bulk and rhizosphere soils, particular bacteria. Multivariate statistical analysis indicated that the bacteria and fungi were strongly influenced by earthworm activity and played a vital role in metal transfer within the contaminated soil-*S. plumbizincicola*-wild earthworm system. This study could provide a sight in effects and potential of combined multi-organisms for soil remediation in natural environment.

Keywords: wild earthworm species, *Sedum plumbizincicola*, bioaugmentation effect, microbial community structure

1. Introduction

Soil metal contamination resulting from human activities has become a worldwide issue. For instance, mining activities have led to high metals concentration in surrounding environment mediums (Wang et al., 2024a), and increased the risk to human beings or other organisms through food chains (Doabi et al., 2018). Generally, the soil quality in mining areas is low, for the lack of nutrients, poor soil structure and scarce soil microorganisms (Kumi and Adu-Poku and Attiogbe, 2023). It is urgent to remediate the contaminated soils to protect the surrounding agricultural soil in order to achieve sustainable development.

Phytoextraction by hyperaccumulator plants is an environmentally friendly, economic and in situ remediation approach (Lavanya and Viswanath and Sivapullaiah, 2024). It has become popular in remediation field by making a minimum disturbance on soil biological and ecological health. However, the limitation of phytoextraction is time consuming because of low growth rates and low plant biomasses (Fadzil et al., 2023). The low soil quality in mine areas further increases the difficulty and results in low remediation efficiency. Earthworm as “soil ecosystem engineers”

can reconstruct soil structure, improve soil quality, expand the soil beneficial microflora (Brenard et al., 2012) and increase the metal availability by digging, feeding, and excretion activities (Zhang et al., 2022). *E. fetida*, compost earthworm, was introduced to phytoextraction system and improved the remediation efficient through promoting plant growth (Wang et al., 2024b), while wild native earthworms show higher bioremediation potential of metal contaminated soils than compost earthworm *E. fetida*, as for improving soil quality, metal accumulation and activation (Zhang et al., 2022). Most importantly, *E. fetida* is unable to survive long-term in natural ecosystems. Generally, the different earthworm species can be identified and classified as epigeic, endogeic and anecic by morphology, distribution in soil and feeding habits, etc. (Keitha and Robinson, 2012). Feeding preference of different earthworm ecotypes makes different impacts on biogeochemical cycle (Gong et al., 2023). Zhang et al., (2021) showed that the metal activation ability of epigeic and epi-endogeic species is higher than that of endogeic. At present, most of studies have focused on the effects of single earthworm species on metal transfer or fate in ecosystem, but this is not consistent with the natural system. For example, Singh et al., 2019 field investigation found that endogenic and anecic earthworm dominate the earthworm communities in the investigated site. Very few studies have investigated the interactive effects of different earthworm categories on hyperaccumulator plant growth (Laossi et al., 2009a; b). The effects of earthworm activities of individual and combined earthworm species on metal activation also remain unclear.

The metal accumulation by hyperaccumulator plants depend on soil metals mobility and bioavailability. The concentration of metals in soil solution has been considered to reflect the metals available concentration for plants in the soil. In addition, the BCR extraction method also be employed to estimate metals availability. While the transformation and dynamics of metals in

soil environment result from the combined action of multiple biotic and abiotic factors. In *S. plumbizincicola*-earthworm-contaminated soil system, earthworm activities make great influence on soil properties, mineralization of soil organic matter and microbial activity by feeding, burrowing and casting (Blouin et al., 2013), which return to impact on soil metals. Those impact factors also exist complex interaction relationship, especially in “hotspot” areas, such as rhizosphere and drilosphere. However, the microbial community in bulk and rhizosphere area after phytoextraction by *S. plumbizincicola* and earthworms are still poorly characterized. For instance, bacteria, fungi, and actinomycetes may exist differently to plant metal accumulation due to their different abundance and compositions in soil (Mahohi and Raiesi, 2021). Therefore, further research should focus on the relationship and interaction of different earthworm species and organisms to reveal the overall effect and underline metal uptake mechanisms.

Overall, we established a mine contaminated soil-multiple earthworm species-hyperaccumulate plant system to explore the effects of multiple earthworm species on phytoremediation efficiency and reveal the underline mechanisms to optimize bioremediation processes. The aims of the current study were to investigate the effect of individual and combined earthworm species (*Amythnas cortices* and *Amythnas aspergillum*) belonging to different ecological categories on remediation efficiency of hyperaccumulator plant *S. plumbizincicola* in different Cd and Zn contaminated soils. Therefore, the research objects were i) to analyze and compare the effects of individual and combined earthworm species on phytoremediation efficiency of *S. plumbizincicola* under different Cd and Zn pollution stress; ii) to reveal the impact of combined wild earthworm with *S. plumbizincicola* on soil microbial community in rhizosphere and bulk soils; iii) to elucidate the effects of Cd and Zn pollution stress on this process and the underline

mechanisms. This study could provide a sight in effects and potential of combined multi-organisms for soil remediation.

2. Materials and Methods

2.1 Experiment materials

Three Cd contaminated level soils were collected from Dabaoshan mining area (24° 30' 01" -24° 35' 26" N, 113° 41' 53" -113° 46' 40" E), Guangzhou city, Guangdong province, China. Mining activity started 50 years ago and thoroughly stopped around 15 years ago. The soil type is clay loam soil. The three contaminated soil were Low Cd, Medium Cd and High Cd contaminated soil, namely L, M, and H, respectively. Rice straw passing 2 mm sieve was employed as exogenous organic fertilizer and food for plant and earthworm. The basic properties of soil and rice straw are shown in Table 1.

S. plumbizincicola was employed to perform metals phytoextraction from soil. In addition, two species of wild earthworm (native species of south China), *A. cortices* and *A. aspergillum* were collected at tea garden from Guangdong province, China.

Table 1 Soil and rice straw properties before phytoremediation

	pH	Clay %	CEC (cmol/kg)	SOC (g/kg)	TN (g/kg)	C:N	Cd (mg/kg)	Zn (mg/kg)	Cu (mg/kg)	Pb (mg/kg)
Soil I (L)	6.26±0.07	22.0±1.57	9.37±0.14	13.0±0.10	1.25±0.04	10.4±0.35	1.53±0.25	224±19.5	195±4.67	99.0±5.68
Soil II (M)	6.37±0.10	18.8±0.73	15.1±1.10	16.3±0.34	1.54±0.04	10.6±0.31	5.20±0.53	431±20.5	38.9±2.72	76.6±25.6
Soil III (H)	6.61±0.09	21.8±0.94	12.2±4.25	16.9±0.45	1.62±0.05	10.5±0.41	9.56±0.38	536±38.2	48.0±6.91	128±4.64
Rice straw				242±3.03	9.37±0.14	25.8±0.68	0.01±0.00	6.72±1.55	1.24±0.90	3.25±0.26
Threshold ^a				5.5<pH≤6.5			0.3	200	50	90
				6.5<pH≤7.5			0.3	250	100	120

Note: results are expressed as the mean value ± SD (n=3). The uppercase letter L, M and H represent Low soil, Medium Cd content soil and High Cd content soil, respectively; OM represents organic matter; TN represents total nitrogen; The ratio of C:N represents

organic carbon content/total nitrogen content; CEC represents cation exchange capacity. Threshold ^a represents the risk control standard for soil metal contamination of agricultural land (GB 15618-2018).

2.2 Experiment design

There were three contaminated level for soils and six treatments for each soil, with five replicated per treatment, 120 pots in total. There were eight treatments: (1) control soil (CK); (2) inoculation with earthworm *A. cortices* (E1); (3) inoculation with earthworm *A. aspergillum* (E2); (4) inoculation with earthworm *A. cortices* and *A. aspergillum* (E1E2); (5) planting *S. plumbizincicola* seedlings (P); (6) planting *S. plumbizincicola* seedlings and inoculation with earthworm *A. cortices* (PE1); (7) planting *S. plumbizincicola* seedlings and inoculation with earthworm *A. aspergillum* (PE2); (8) planting *S. plumbizincicola* seedlings and inoculation with earthworm *A. cortices* and *A. aspergillum* (PE1E2).

One and half kilograms air-dried soil was placed in each plastic pot (dimensions of caliber × bottom diameter × height of 176 × 143 × 185 mm) with a microporous bottom. The soil bulk density was 1.14 g/cm³. All treatments were equilibrating and aged at 70% of maximum soil water holding capacity (WHC) for four weeks after mixing soil with 5% rice straw. Then, four similar size *S. plumbizincicola* seedlings (~8 cm height) were planted into each pot. After one month for seedling roots stabilization, 15 ± 2 g of earthworms of different species were inoculated in PE treatments, approximately 0.55 ± 0.11 g·individual⁻¹ for *A. cortices*, 4.69 ± 1.09 g·individual⁻¹ for *A. aspergillum*. In combined earthworm species treatments, each species was added at half amount of the total biomass. To prevent earthworms from escaping, the top and bottom of each pot were covered with a fine nylon mesh with a 1 mm pore diameter. Soil moisture in each pot was maintained at ~70% of soil maximum water holding capacity (MWHC) and the temperature was maintained at 25 °C.

The soil solution was collected by RhizoMOM devices at 60th day and 90th day. The plants were harvested and the soil samples were collected at the 90th day (Lu et al., 2014). Earthworms were hand-picked, and then counted and weighted. The rhizosphere soil and bulk soil in the treatments planting with *S. plumbizincicola* was collected. Additionally, the soil without *S. plumbizincicola* was also collected. One part of the fresh soil was storage in -40 °C. Another part of the soil was thoroughly air-dried, ground, and sieved for the analysis of soil physical and chemical properties. The live seedlings were counted. The high and fresh weight of plants were measured and weighted. The Cd and Zn concentration of plant tissues and soils were also determined.

2.3 Soil chemical and biological properties

Soil pH was measured using a glass electrode in a 1:2.5 (w/v) soil/water suspension. Soil clay, soil organic matter (SOC), total nitrogen (TN) and alkali-hydrolysable nitrogen (AN) content were analyzed by the pipette (Lu, 1999), dichromate oxidation (Sparks, 1996), Kjeldahl (Brookes et al., 1985) and alkali N-proliferation (Cornfield, 1960) methods, respectively. dissolved organic matter (DOC) content was extracted with deionized water (1:5) and determined (Dai et al., 2004). Available phosphorus.

2.4 Total metal content in rice straw, plant tissues and soils, and speciation of metals

Total Cd, Pb, Cu, Zn in the soils were extracted by microwave digestion with aqua regia (HNO₃:HCl (v/v)=1:3). The three-stage BCR (Community Bureau of Reference) sequential extraction procedure was used to analyze the combined fraction of Cd and Zn. The detailed BCR extraction procedures were referenced to the methods reported by Pueyo et al. (2008) and Wang et al. (2018). Total Cd and Zn in rice straw and plant tissues were extracted by microwave digestion with HNO₃. GBW07455 (GSS-26) was employed as certified reference material for soil analyses,

and the measured concentrations were within 85%-105% of the reported certified concentration for Cd and Zn. GSBZ5001-88 ESS was employed as certified reference material for rice straw and plant tissue analyses, and the measured concentration were 95%-110% of the reported certified concentration for Cd and Zn. Quality control measures included three repetitions for each soil and three blanks for each batch to ensure the quality of the analysis.

2.5 PLFAs determination and grouping

PLFAs fingerprints were employed as biomarkers to estimate soil microbial biomass and community composition. The PLFAs technique involved the extraction of lipids from the soil and separation of phospholipids from the lipids. The obtained phospholipids were then methylated to form fatty acid methyl esters, which were quantified by gas chromatography. To evaluate the extraction efficiency, an internal standard fatty acid (19:0) was used, and another fatty acid (10:0) was used as an instrumental standard for the gas chromatography analysis, which was added prior to detection and quantification (Au-Quideau et al., 2016). In this study, the PLFAs were divided into three groups according to Frostegård and Bååth, 1996: (1) bacteria, including common bacteria (14:00, 15:00, 17:00 and 18:00), gram-positive (G^+) bacteria (i14:0, i15:0, a15:0, i16:0, i17:0, a17:0, i15:1 w6c, i17:1 w9c), and gram-negative (G^-) bacteria (16:1 w7c, cy17:0, 17:1 w8c, 18:1 w5c, 18:1 w7c, 18:1 w9c, cy19:0 w8c). (2) fungi (18:2 w6c); and (3) actinomycetes (ACT, 16:0 10-methyl, 17:0 10-methyl, 18:0 10-methyl, 18:1w7c 10-methyl). The ratio of fungal biomass to total bacterial biomass was used to estimate the ratio of fungi to bacteria in the rhizosphere and bulk soils (Moore-Kucera and Dick, 2008). The total content of PLFAs (total PLFAs) was calculated as the sum of all identified lipids and expressed as nmol/g soil (Frostegard et al., 1996). All microbial analysis was conducted in triplicate.

2.6 Data analysis

Data were analyzed and plotted with SPSS statistical software (Version 24.0), Origin 9.0, R (Version 3.4.3). Duncan's test AND variance (ANOVA) analysis were performed. Data were presented as the mean $\pm$ SD. Transfer factor (TF) = metal concentration in aboveground tissue ($\text{mg}\cdot\text{kg}^{-1}$) / metal concentration in root tissue ($\text{mg}\cdot\text{kg}^{-1}$). Principal components analysis (PCA) was conducted to analyze the relationships of plant physiological items, metals concentration in plant tissues, as well as soil properties, BCR-metals concentration and PLFAs. In addition, the PCA was also be employed to analyze the relationships of microbial community biomass and structure in rhizosphere and bulk soils. The co-inertia analysis was performed to explore the influences of earthworms and *Sedum plumbizincicola* on soil properties, BCR-metals concentration, and soil microbial community biomass and structural. The PCA and co-inertia analysis were performed in R version 3.4.3 using the “ade4” packages.

3. Results

3.1 The physiological characteristics and Cd and Zn uptake of *S. plumbizincicola* under earthworm activities

3.1.1 The physiological characteristics

The physiological characterizes of *S. plumbizincicola* are shown in Fig. 1. The earthworm activities significantly increased the leaf chlorophyll, stem thickness, plant height, and biomass ($P < 0.05$). The dry weight of the whole plant and the aboveground tissues biomass in PE treatments were significantly increased with metal contaminated levels ($P < 0.05$) (Fig. 1b and c). The dry weight of aboveground tissues and roots were significantly increased in PE treatment ($P < 0.05$), which were 0.17-3.68 folds and 1.36-4.64 folds higher than those in corresponding P treatments,

respectively. In addition, the biomass of the aboveground tissues and roots biomass in PE1E2 treatments were similar to or lightly lower than those in PE1 and PE2 treatments (Fig. 1b and c).

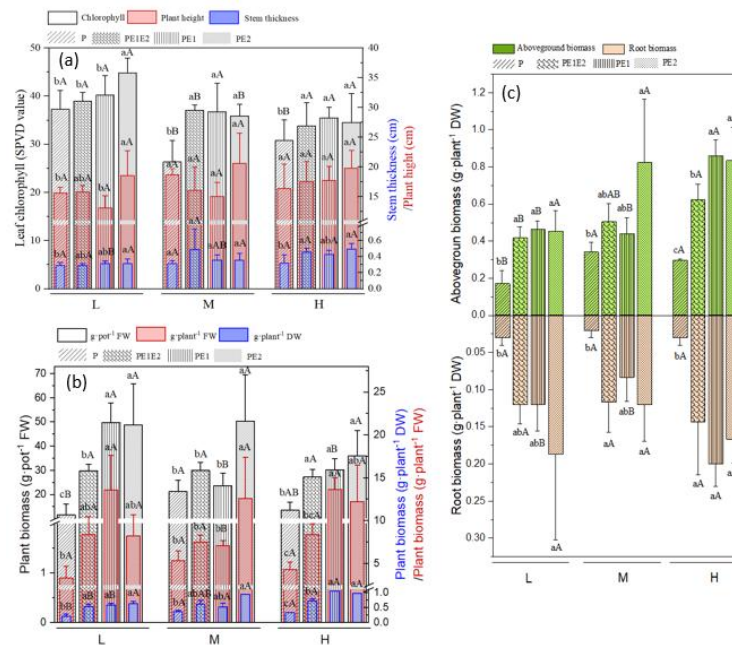


Fig. 1 The physical characteristics of *Sedum plumbizincicola* in different treatments. Results are expressed as the mean value $\pm$ SD ($n=3$). The uppercase letters L, M, and H represents the soil L, soil M, and soil H. P, PE1E2, PE1 and PE2 represents the treatments with *Sedum plumbizincicola*, *Sedum plumbizincicola* + *A. cortices* + *A. aspergillum*, *Sedum plumbizincicola* + *A. cortices*, *Sedum plumbizincicola* + *A. aspergillum*. Different lowercase letters indicate significant differences ($P < 0.05$) between different treatments. Different uppercase letters indicate significant differences ($P < 0.05$) between different soils.

3.1.2 Cd and Zn concentrations and accumulation in *Sedum plumbizincicola*

The metals concentration in different tissues of *Sedum plumbizincicola* and transfer factors are shown in Fig. 2. Except for the Cd and Zn concentration in aboveground tissues significantly increasing in PE treatment in L soil ($P < 0.05$), it was significantly higher in P treatment than those in PE treatments for each soil ($P < 0.05$). In M and H soils, except for Cd in roots in M-PE1E2, the Cd and Zn concentration in aboveground tissues and roots in PE1E2 treatment were higher than that in PE1 or PE2 treatments. Except for roots in H-PE1, the Cd and Zn concentration in

aboveground tissues and roots in PE treatments were increased with soil contaminated levels. However, the two metals concentration in aboveground tissues in P treatment were not increased with soil contaminated levels. The Cd and Zn transfer factors were significantly increased in PE1E2, PE1 and PE2 treatments in three soils ($P < 0.05$) (Fig. 2e and f). Furthermore, the transfer factor in PE1E2 treatment were similar to or in between those in the PE1 and PE2 treatments.

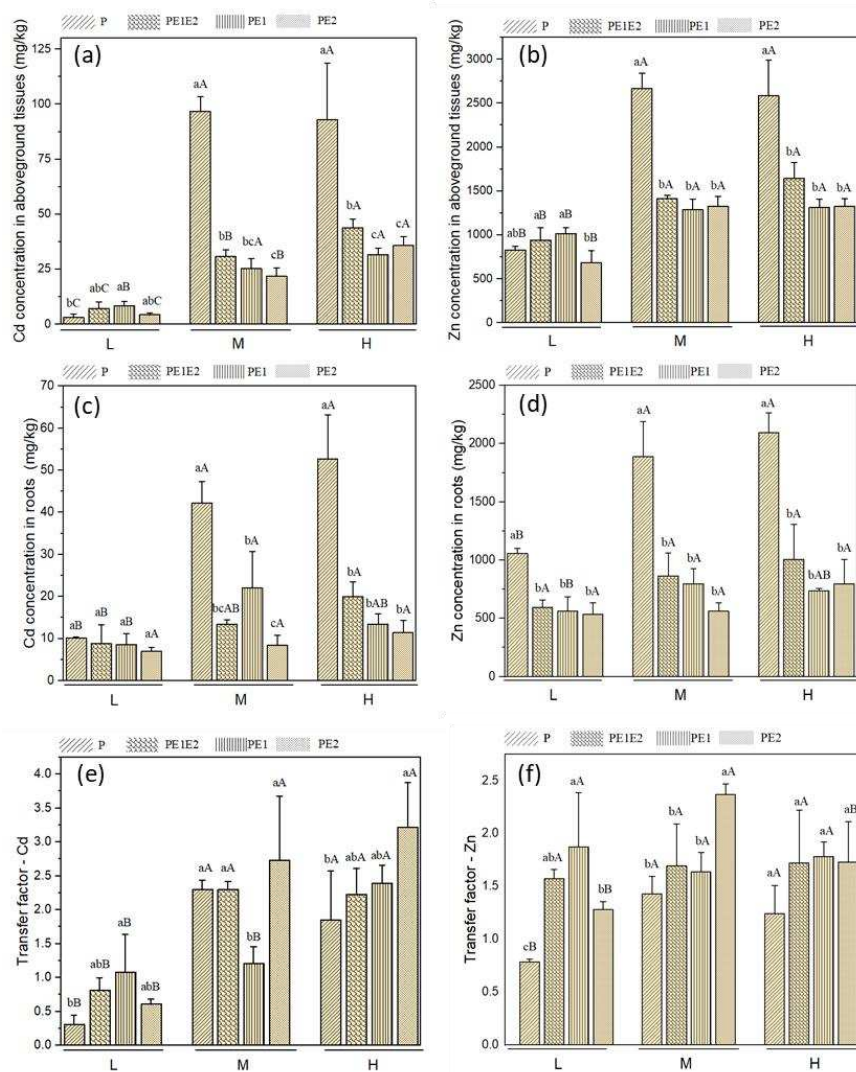


Fig. 2 Cd and Zn concentration in different tissues of *Sedum plumbizincicola* and transfer factor (TF). Results are expressed as the mean value $\pm$ SD (n=3). The uppercase letters L, M, and H represents the soil L, soil M, and soil H. PE1, PE2, PE3, and PE4 represents the treatments with *Sedum plumbizincicola* + *A. cortices*, *Sedum plumbizincicola* + *A. aspergillum*, *Sedum*

plumbizincicola + *A. robustus*, *Sedum plumbizincicola* + *M. guillelm*. Different lowercase letters indicate significantly differences ($P < 0.05$) between different treatments. Different uppercase letters indicate significantly differences ($P < 0.05$) between different soils.

The Cd and Zn uptake amount of *Sedum plumbizincicola* are shown in Fig. 3. The Cd and Zn uptake amount were significantly increased in PE1E2, PE1 and PE2 treatments in L and H soils ($P < 0.05$), while Cd uptake amount was significantly decreased in PE treatments in M soil ($P < 0.05$). Specifically, the Cd uptake amount was increased in PE1E2 (16.6 ± 10.4 $\mu\text{g/pot}$), PE1(19.8 ± 4.69 $\mu\text{g/pot}$), and PE2 (12.8 ± 3.57 $\mu\text{g/pot}$) treatments by 435%, 540%, and 315% in L soil (Fig. 3a). The highest Cd uptake amount reached at in 124 ± 10.3 $\mu\text{g/pot}$ in H-PE2. The Zn uptake amount was increased in PE1E2 (1.87 ± 0.46 mg/pot), PE1(2.15 ± 0.28 mg/pot), and PE2 (1.61 ± 0.32 mg/pot) treatments by 167%, 207%, and 130% in L soil and were increased in PE1E2 (4.68 ± 0.52 mg/pot), PE1 (5.09 ± 0.34 mg/pot), and PE2 (4.93 ± 0.80 mg/pot) treatments by 40.5%, 52.9%, and 48.0% in H soil (Fig. 3b). The highest Zn uptake amounts reached at 5.09 ± 0.34 mg per 1.5 kg soil in H-PE1.

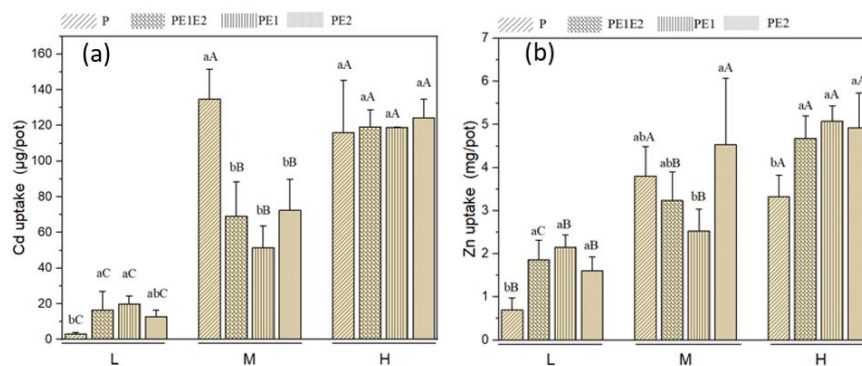


Fig. 3 The Cd and Zn uptake by *Sedum plumbizincicola* in different treatments. Results are expressed as the mean value $\pm$ SD ($n=3$). The uppercase letters L, M, and H represents the soil L, soil M, and soil H. PE1, PE2, PE3, and PE4 represents the treatments with *Sedum plumbizincicola* + *A. cortices*, *Sedum plumbizincicola* + *A. aspergillum*, *Sedum plumbizincicola* + *A. robustus*, *Sedum*

plumbizincicola + *M. guillelm*. Different lowercase letters indicate significant differences ($P < 0.05$) between different treatments. Different uppercase letters indicate significant differences ($P < 0.05$) between different soils.

3.2 Variations of soil properties after phytoextraction

The soil chemical properties after phytoremediation are shown in Table 2. The soil pH shows a similar trend for the three contaminated soils. The pH ranked as $CK \geq E2 > E1 > E1E2$, and $PE2 > PE1E2 > P > PE1$. In L and M soils, SOC was decreased by 12.5%, 5.99% and 12.5% in L-E1E2, M-E1E2 and M-E1 respectively, while it increased by 8.46% and 8.23% in E1 and E2 treatments in H soil. In PE treatments, it was significantly decreased by 10.8%, 14.6% and 11.7% in PE1E2, PE1 and PE2 in L soil ($P < 0.05$), conversely, it was significantly increased by 11.4%, 13.0% and 12.0% in PE1E2, PE1 and PE2 in M soils ($P < 0.05$). There was no significant change in H soils. Comparing with CK, the PE1E2 and PE2 significantly increased TN in L soil ($P < 0.05$), while TN significantly decreased in M and H soils ($P < 0.05$), and ranked as $PE1E2 > PE1 = PE2$. Moreover, this effect was enhanced with soil contaminated levels. However, comparing with P treatment, the PE treatments significantly increased TN ($P < 0.05$), and ranked as $PE2 > PE1 > PE1E2$ while this effect was diminished with soil contaminated levels. The ratio of C:N showed similar trend with SOC. Moreover, it significantly decreased with soil contaminated levels ($P < 0.05$).

The DOC showed opposite trends to SOC. In L and M soils, it was significantly increased by 86.9%, 69.3%, 37.6%, 50.9% and 34.9% in L-E1E2, L-E1, L-E2, M-E1E2 and M-E1, respectively ($P < 0.05$) while in H soil, it was significantly decreased by 32.2% and 23.9% in E1E2 and E1. In the three soils, DOC content ranked as $P > PE1 > PE2 > PE1E2$. In addition, it shows DOC in with P treatments lower than that in without P treatment. AN was increased in E2 and PE2

compared with CK and P, respectively; while it was significantly decreased in E1E2, E1, PE1E2 and PE1 treatments ($P < 0.05$). The AP content ranked as $M \geq H > L$ in the three soils, and ranked as $E1E2 > E1 > CK \geq E2$; in addition, compared with the corresponding CK, AP was decreased in the E2 by 0.67%, 7.76% and 25.0% in L, M and H soils, while in the with P treatments, it showed different trend. Specifically, it ranked as $P > PE2 \geq PE1 > PE1E2$ in L soil, $PE2 > PE1 > P \geq PE1E2$ in M soil, and $PE1 > PE1E2 \geq PE2 > P$ in H soil.

Table 2 Soil chemical properties after 90 days

Soil	Treatments	pH	SOC (g·kg ⁻¹)	TN (g·kg ⁻¹)	C:N	DOC (mg·kg ⁻¹)	AN (mg·kg ⁻¹)	AP (mg·kg ⁻¹)
C	CK	6.65±0.26 abA	27.5±0.74 bcA	1.41±0.02 cdB	19.5±0.24 bA	59.0±6.21 cdC	142±2.37 bcdA	5.93±1.00 bB
	E1	6.56±0.06 bcA	27.8±0.61 bcA	1.42± 0.04bcdB	19.6±0.80 bA	99.9±0.00 aB	135±0.25 deA	9.25±1.94 abC
	E2	6.56±0.12 bcB	28.4±1.34 aA	1.49±0.02 abA	19.1±1.10 bA	81.1±6.24 bB	144±3.51 bcA	5.89±1.20 bC
	E1E2	6.35±0.38 bcA	24.1±0.94 bA	1.51±0.05 aB	15.9±0.18 dA	110±15.7 aA	131±3.13 eB	15.7±1.06 aB
	P	6.70±0.21 abA	30.0±0.27 aA	1.28±0.07 eC	23.5±1.26 aA	113±9.38 aA	149±3.29 abA	8.93±1.46 abB
	PE1	6.24±0.06 cB	25.7±0.57 dAB	1.44±0.02 bcB	17.8±0.16 cA	67.6±0.00 bcA	138±3.41 cdeA	7.19±0.85 abB
	PE2	6.95±0.06 aA	26.5±0.35 cdA	1.48±0.02 abC	17.9±0.35 cA	73.7±6.14 bcA	154±8.86 aA	7.37±0.45 abC
	PE1E2	6.66±0.03 abA	26.8±0.95 cdA	1.37±0.03 dC	19.6±0.53 bA	46.6±6.21 dA	143±4.52bcdA	5.07±1.60 bB
L	CK	6.79±0.03 bcA	29.0±0.96 aA	1.67±0.04 aA	17.3± 0.22abcB	81.7±7.17 bB	141±6.99abcA	23.6± 2.77bcdA
	E1	6.65±0.05 deA	25.4±1.43 cdB	1.52±0.03 cA	16.7±1.00 bcB	110±3.60 aA	133±3.00 cA	28.5±2.95 abA
	E2	6.84±0.08 bA	28.6±0.63 abA	1.52±0.01 cA	18.8±0.30 aA	83.2±14.4 bB	141±2.63abcA	21.7± 0.48cdeA
	E1E2	6.35±0.05 fA	27.3± 2.82abcA	1.54±0.01 bcB	17.8±1.94 abA	123±7.24 aA	136± 1.78bcAB	30.6±1.79 aA
	P	6.55±0.09 eA	23.6±0.18 dC	1.59±0.04 bB	14.9±0.26 dB	69.6±9.38 bcC	148±4.01 aA	18.7±3.81 deA
	PE1	6.35±0.02 fB	26.7± 1.00abcA	1.69±0.04 aA	15.8±0.71 cdB	71.7±7.09 bA	136±3.70 bcA	22.1± 1.19cdeA
	PE2	6.96±0.09 aA	26.5± 1.16abcA	1.69±0.01 aA	15.6±0.55 cdB	54.8±9.48 cdA	144±1.18 abA	26.5± 5.56abcA
	PE1E2	6.69±0.02 cdA	26.3±0.75 bcA	1.64±0.04 aB	16.0±0.85 cdB	44.5±7.17 dA	142±8.40abcA	16.8±0.74 eA
H	CK	6.85±0.04 abA	25.8±0.45 bB	1.69±0.11 abA	15.2±0.25 bcC	120±7.50 aA	140±2.31 aA	20.5±1.31 bcA
	E1	6.59±0.14 cdA	27.9±0.71 aA	1.53±0.02 dA	18.3±0.57 aAB	91.5±7.21 bC	134±1.36 abA	21.0±2.78 bB
	E2	6.95±0.03 aA	27.9±1.32 aA	1.50±0.03 dA	18.6±1.07 aA	110±9.53 aA	138±6.01 abA	15.3±1.13 dB
	E1E2	6.50±0.09 dA	25.9±1.21 bA	1.61±0.03 cA	16.1±1.00 bA	81.6±10.9 bcB	138±4.29 abA	30.9±2.98 aA
	P	6.60±0.17 cdA	25.4±0.48 bB	1.69±0.03 abA	15.0±0.05 bcB	90.1±3.55 bB	142±2.32 aA	16.1±0.50 cdA
	PE1	6.56±0.14 cdA	24.7±0.95 bB	1.75±0.07 aA	14.1±0.54 cC	69.6±9.38 cdA	142±2.85 aA	21.0±2.68 bA
	PE2	6.71±0.05 bcB	26.4±1.11 abA	1.64±0.04 bcB	16.1±0.40 bB	54.8±9.48 deA	144±9.52 aA	17.3± 1.90bcdB
	PE1E2	6.72±0.06 bcA	26.0±0.40 bA	1.73±0.04 aA	15.0±0.53 bcB	52.8±0.00 eA	129±8.93 bA	19.0± 4.41bcdA

Note: Results are expressed as the mean value ± SD (n=3). The uppercase letter L, M and H represent Low soil, Medium Cd content soil and High Cd content soil, respectively; CK, E1, E2, E1E2, PE1E2, PE1 and PE2 represents the treatments with control, *A. cortices*, *A. aspergillum*, *A. cortices*+ *A. aspergillum*, *Sedum plumbizincicola*, *Sedum plumbizincicola* + *A. cortices*+ *A.*

aspergillum, *Sedum plumbizincicola* + *A. cortices*, *Sedum plumbizincicola* + *A. aspergillum*. Different lowercase letters indicate significantly differences ($P < 0.05$) between different treatments. Different uppercase letters indicate significantly differences ($P < 0.05$) between different soils.

3.3 Cd and Zn concentration in soil and soil solution

3.3.1 The different fractions concentration of Cd and Zn in soil

The different fractions determined by BCR of Cd and Zn are shown in Fig. 4. Earthworms did not make great influence on different fraction of Cd, except for oxidizable Cd in PE1E2 treatment in the three soils, reducible and oxidizable Cd in the inoculation with earthworm treatments in H soil. The acid-soluble Zn and reducible Zn was significantly increased in only inoculation earthworm treatments in the three soils, and the concentration ranked as $E1E2 > E2 > E1$ ($P > 0.05$). Moreover, PE1 further significantly increased acid-soluble Zn in L and M soils, whereas PE2 and PE1E2 did not. In H soil, compared to P (251 ± 21.1 mg/kg), it significantly decreased in PE1 (148 ± 12.9 mg/kg), PE2 (185 ± 9.95 mg/kg) and PE1E2 (218 ± 35.3 mg/kg). The reducible Zn was significantly increased in PE treatments in three soils ($P > 0.05$). PE1E2 significantly increased the oxidizable Zn and Cd; it worth to note that all the treatments with earthworm inoculation significantly increased oxidizable Zn in H soil ($P > 0.05$).

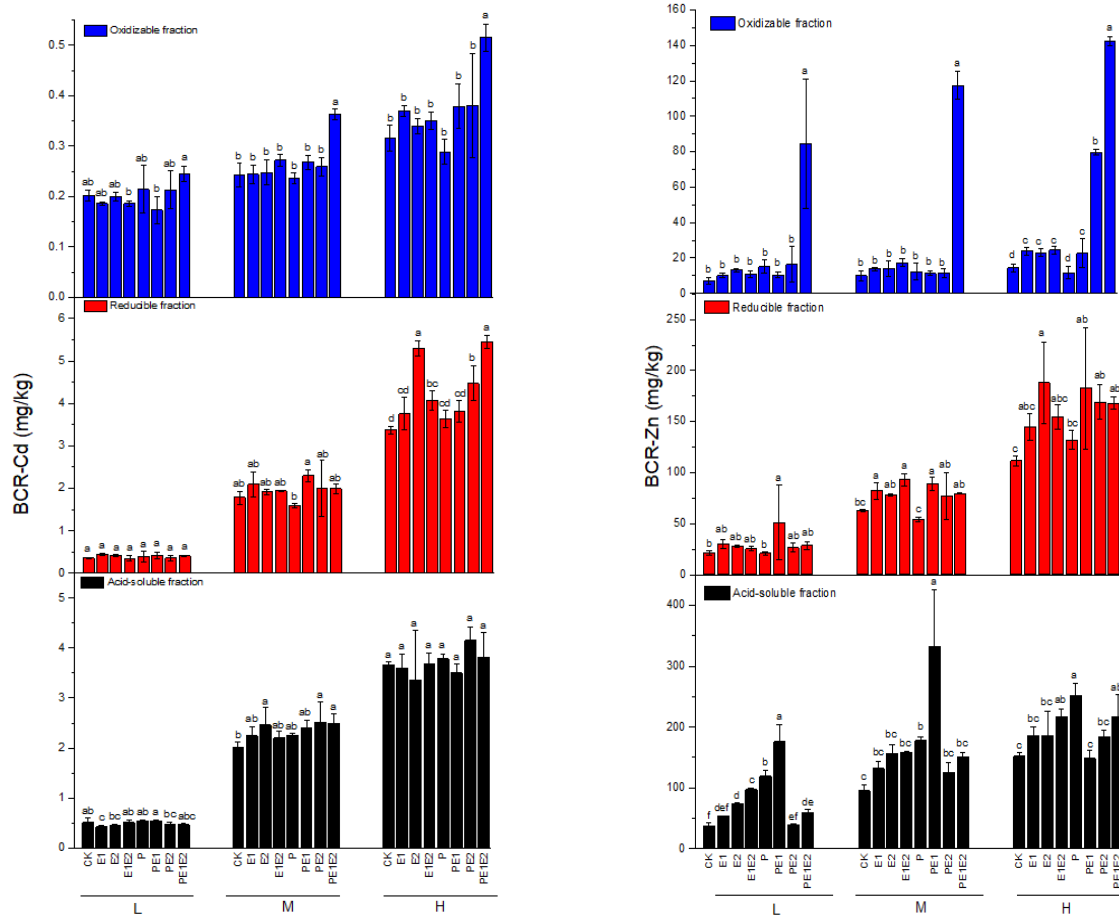


Fig. 4 Percentages of Cd and Zn speciation released by BCR sequential extraction. Results are expressed as the mean value $\pm$ SD (n=3). The uppercase letters L, M, and H represents the soil L, soil M, and soil H. PE1, PE2, PE3, and PE4 represents the treatments with *Sedum plumbizincicola* + *A. cortices*, *Sedum plumbizincicola* + *A. aspergillum*, *Sedum plumbizincicola* + *A. robustus*, *Sedum plumbizincicola* + *M. guillelm*. Different lowercase letters indicate significantly differences ($P < 0.05$) between different treatments. Different uppercase letters indicate significantly differences ($P < 0.05$) between different soils.

3.3.2 Metals concentration in soil solution changing with cultivation time

The Cd and Zn concentration in soil solution at day 60 and day 90 are shown in Fig. 5. In L soil, the Cd concentration in soil solution in the treatments with earthworm inoculation was higher at 90 day than at 60 day, while it shows conversely in M and H soils. The Cd concentration was increased in the only inoculation earthworm treatments in L soil at 60 days, and shows conversely in M and H soils. It was increased in PE treatments, not including in H soil. Except for only inoculation earthworm treatments in H soil, the Cd concentration was increased in all

inoculation earthworm treatments at 90 days. The Zn concentration in soil solution in the treatments with earthworm inoculation was higher at 90 days than at 60 days in three contaminated soils. At 60 days, only inoculation earthworm treatments and PE1E2 increased Zn concentration in soil solution. In the three contaminated soils, the Zn concentration in soil solution in the treatments with earthworm inoculation was increased at 90 days comparing to that in the corresponding CK and P treatments.

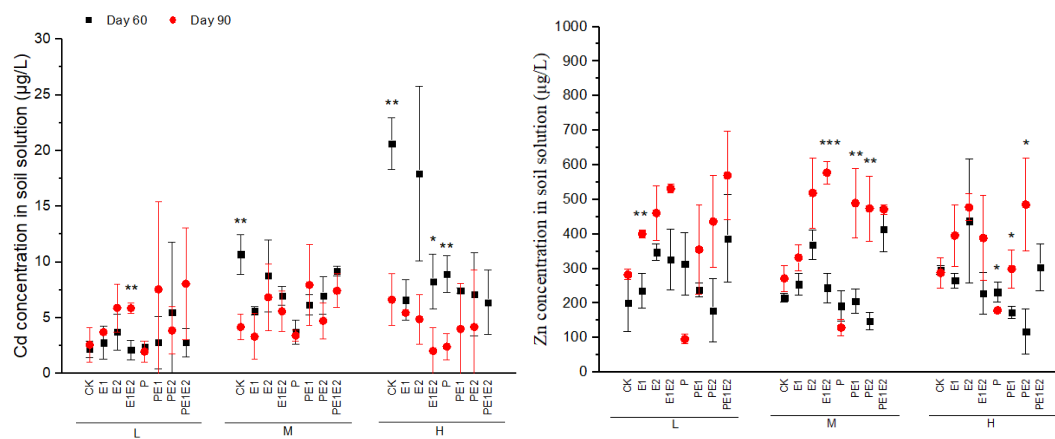


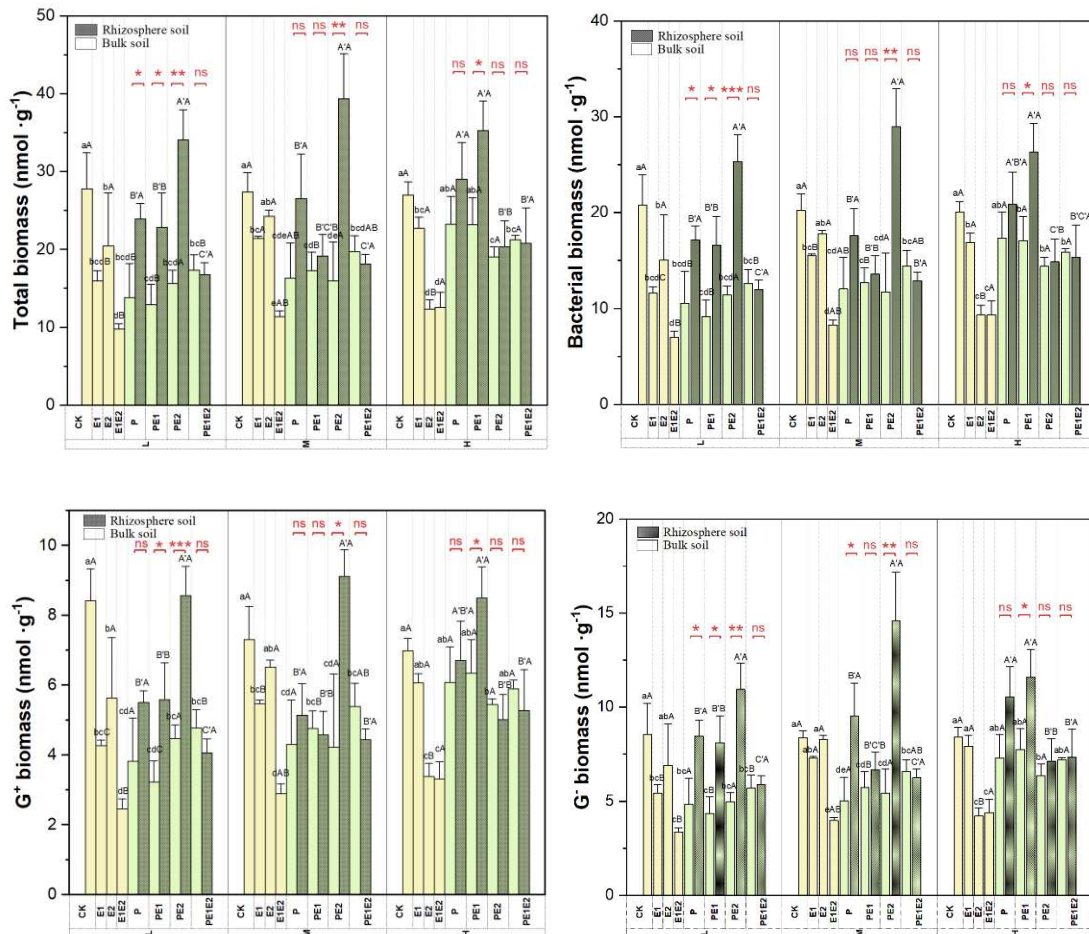
Fig. 5 Cd and Zn concentration in soil solution at day 60 and day 90. Results are expressed as the mean value $\pm$ SD (n=3). The uppercase letters L, M, and H represents the soil L, soil M, and soil H. PE1, PE2, PE3, and PE4 represents the treatments with *Sedum plumbizincicola* + *A. cortices*, *Sedum plumbizincicola* + *A. aspergillum*, *Sedum plumbizincicola* + *A. robustus*, *Sedum plumbizincicola* + *M. guillelm*. T test.

3.4 Impacts of earthworms on soil microbial biomass and community structure in the Cd and Zn contaminated soil

The total PLFAs and the biomasses of soil microbial communities are shown in Fig. 6. In general, the trend of total PLFAs, bacterial, G^+ , G^- and ACT biomass was consistent. Fungi biomass show different trend with total PLFAs. Specifically, the total PLFAs was significantly decreased in only inoculation with earthworm treatments (E) comparing to CK, in particularly in E1E2 treatment and decreasing by 64.8%, 58.4%, and 53.5% in L, M, and H soils respectively; in

addition, the total PLFAs of E2 treatment was also significantly decreased by 54.3% in H soils; it ranked as $CK > E2 > E1 > E1E2$ in L and M soils, and $CK > E1 > E2 > E1E2$ in H soil. Interestingly, the variation of total PLFAs in bulk soil was smaller in PE treatments than in E treatments. Conversely, the variation was larger in rhizosphere soil than in bulk soil, and the trend in rhizosphere soil was consistent with the corresponding total PLFAs in bulk soil of without *S. plumbizincicola*. Additionally, in rhizosphere soil, the total PLFAs in the PE1 and PE2 treatments shown thoroughly different in H soil with L and M soils. Except for there no significant difference between bulk soil and rhizosphere soil of PE1E2 treatment in the three soils, the total PLFAs shows higher in the rhizosphere than in the bulk soil, and showing significant difference in P, PE1, and PE2 in L soil, in PE2 in M soil, and in PE1 in H soil. As for bacterial biomass, it shows a similar trend to total PLFAs. It is worth to noticed that the bacterial biomass reached significantly different in P, PE1, and PE2 in L soil, in PE2 in M soil, and in PE1 in H soil, and the highest occurring in PE2 (25.3 ± 2.78 n mol/g), PE2 (29.0 ± 3.91 n mol/g), and PE1 (26.3 ± 2.99 n mol/g) treatments in L, M, and H soils. In terms of G^+ biomass, it shows similar trend with total PLFAs, and reached significantly different in PE1, and PE2 in L soil, in PE2 in M soil, and in PE1 in H soil, and the highest occurring in PE2 (8.58 ± 0.82 n mol/g), PE2 (9.12 ± 0.76 n mol/g), and PE1 (8.51 ± 0.88 n mol/g) treatments in L, M, and H soils. Moreover, in terms of G^- biomass, it shows similar trend with total PLFAs, and reached significantly different in P, PE1, and PE2 in L soil, in P and PE2 in M soil, and in PE1 in H soil, and the highest occurring in PE2 (11.0 ± 1.39 n mol/g), PE2 (14.6 ± 2.60 n mol/g), and PE1 (11.6 ± 1.48 n mol/g) treatments in L, M, and H soils. The ACT biomass and Fungi biomass show a similar trend to total PLAFs and bacterial biomass in without *Sedum plumbizincicola* treatments. Furthermore, compare to CK, inoculation with earthworm and *Sedum plumbizincicola* decreased ACT biomass, and further decreased in PE treatments comparing with

P treatment. Except for E2 and PE2, the ACT biomass was increased with soil contaminated levels. Interestingly, the fungi biomass in rhizosphere soil showed a different trend in H soil with in L and M soil, and it ranked as $P \geq PE2 > PE1 \geq PE1E2$ in L soil, $P > PE2 \geq PE1 \geq PE1E2$ in M soil, and $P > PE1 > PE2 > PE1E2$ in H soil.



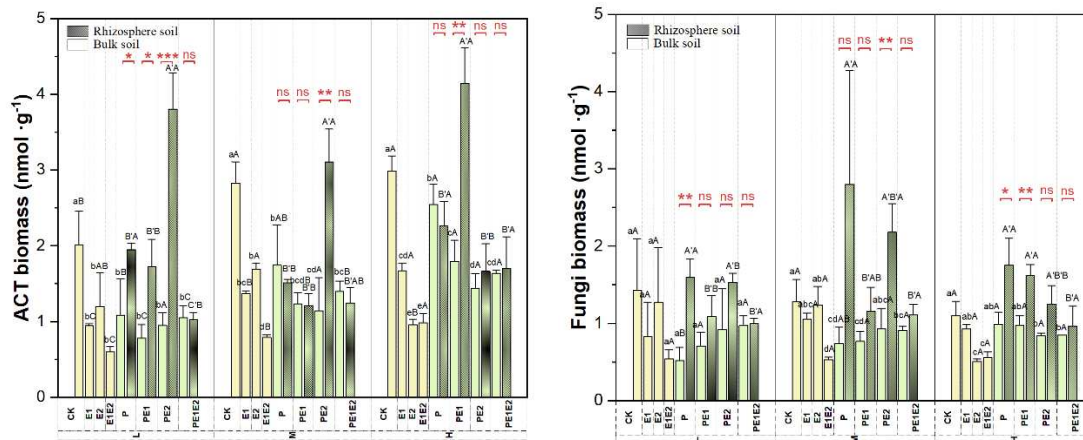


Fig. 6 Effects of earthworm and *Sedum plumbizincicola* inoculation on the total PLFAs biomass and the biomasses of soil microbial communities at different Cd and Zn contamination levels. Note: Different lowercase letters indicate significantly differences in bulk soil ($P < 0.05$) between different treatments. Different uppercase letters indicate significantly differences ($P < 0.05$) between different soils. Different uppercase letters with “ ’ ” indicate significantly differences ($P < 0.05$) in rhizosphere soil between different treatments.

The $G^+:G^-$ and $F:B$ ratio in different treatments are shown in Fig. 7. The $G^+:G^-$ ratio significantly decreased in only earthworm inoculation treatments comparing to CK ($P < 0.05$), while it significantly increased in rhizosphere soil in PE treatments comparing to P treatment ($P < 0.05$). The difference of $G^+:G^-$ between bulk soil and rhizosphere soil are increasingly significant with contaminated levels, and the difference of it between bulk and rhizosphere soils ranked as P ($P < 0.001$) $>$ PE1 ($P < 0.01$) $\geq$ PE2 ($P < 0.01$) $>$ PE1E2 ($P < 0.05$) in H soil. As for earthworm species, the $G^+:G^-$ ratio shows $E2 > E1$ and $PE2 > PE1$. The $F:B$ ratio increased in all inoculation earthworm treatment comparing to CK, especially in L and M soils. In contrast comparing to $F:B$ ratio in the rhizosphere soil of P treatment, it decreased in PE treatments in the rhizosphere soils, and significantly decreasing in M and H soils ($P < 0.05$).

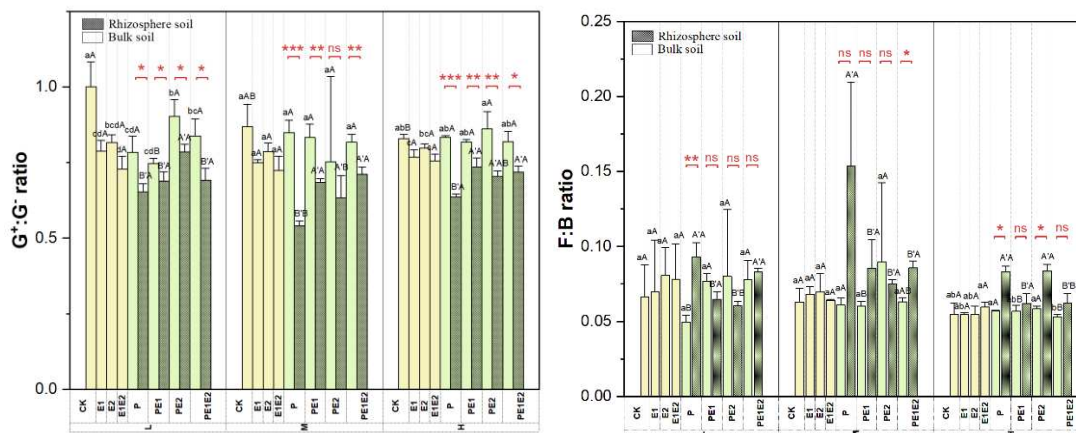


Fig. 7 Gram-positive to Gram-negative bacteria ratio ($G^+:G^-$) and fungi to bacteria ratio (F:B) in the treatments with earthworm and *Sedum plumbizincicola* inoculation at different Cd and Zn concentration levels. Note: Different lowercase letters indicate significantly differences in bulk soil ($P < 0.05$) between different treatments. Different uppercase letters indicate significantly differences ($P < 0.05$) between different soils. Different uppercase letters with “ ’ ” indicate significantly differences ($P < 0.05$) in rhizosphere soil between different treatments.

3.5 Relationships between plant Cd concentrations and soil parameters

Principal Component Analysis (PCA) was carried out to identify the overall trends in plant physiological, plant metals uptake amount, metals transfer factor, soil chemical properties and microbial community of bulk soil in response to *S. plumbizincicola* and earthworm activities in different Cd and Zn contaminated soils (Fig. 8). Axis 1 (39.9% variance explained) was primarily associated with TN, Uptake Cd, Oxidizable Cd, Oxidizable Zn, C:N, G^+ , G^- , B, Total PLFAs, Uptake Zn, TF-Cd, Reducible Zn, Acid-solution Cd, Reducible Cd. Axis 2 (14.5% variance explained) was primarily associated with Acid-solution Zn, ACT, DOC, Leaf chlorophyll, F:B, Plant biomass FW, Root biomass DW, TF-Zn, Aboveground biomass DW, F, Plant height and Stem thickness. Axis 3 (10.5% variance explained) was primarily associated with AP, pH, SOC, AN and $G^+:G^-$ (Fig. 8a and b). Soil with inoculation earthworms were significantly differentiated from the control soils (Fig. 8c and d) ($P=0.001$).

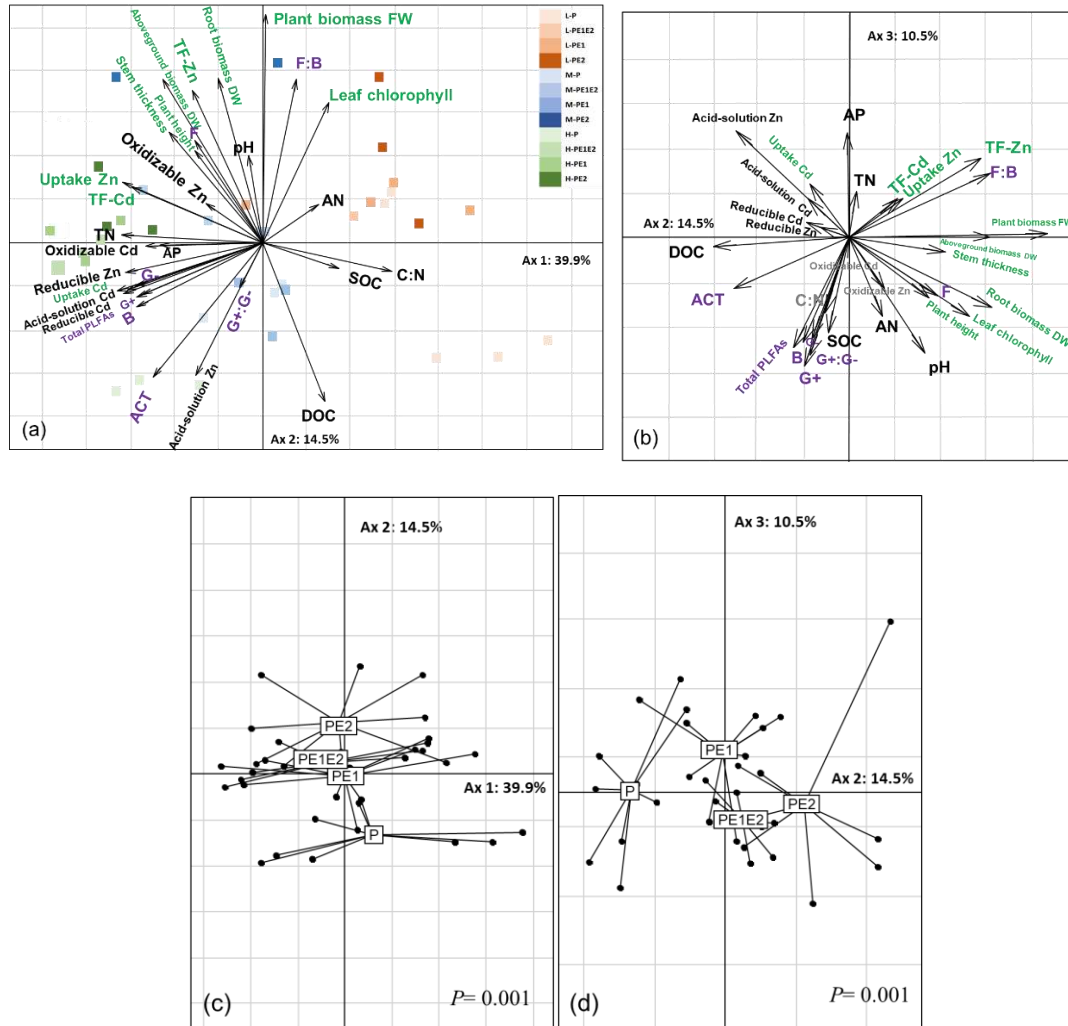


Fig. 8 Principal component analysis (PCA) of the effect of *Sedum plumbizincicola* and earthworms on plant physiological, plant metals uptake amount, metals transfer factor, soil chemical properties and microbial community of bulk soil in low, medium, high Cd and Zn contaminated level soils in fractal F1, F2 and F3 planes. (a) Correlation circles of soil microbial communities with the $G^+ : G^-$ and F:B ratios in fractal F1 and F2 planes; (b) Correlation circles of soil microbial communities with the $G^+ : G^-$ and F:B ratios in fractal F2 and F3 planes; (c) Projection of experimental points according to treatments with or without different species earthworms in fractal F1 and F2 planes; (d) Projection of experimental points according to treatments with or without different species earthworms in fractal F2 and F3 planes.

The effect of earthworm activities on soil chemical properties and microbial community are shown in Fig. 9. The co-inertia analysis showed that the first axis explained 66.7% of the total variation, and the second axis explained 27.4% of the total variation (Fig. 9). TN and Acid-soluble

Cd were significantly positively correlated with ACT, G^- , B, Total PLFAs, G^+ (Fig. 9a, b), which clearly distinguished the treatments between high and low Cd and Zn contamination levels (Fig. 9d). Compared with the low contamination levels, those with high contamination levels exhibited higher TN, Acid-soluble Cd, ACT, G^- , B, Total PLFAs and G^+ . In addition, Soil pH and AN were positively correlated with F and $G^+:G^-$. The treatments with earthworm and without *S. plumbizincicola* (E1, E2 and E1E2) were separated from control (CK), with the former associated with higher projected values of DOC, Acid-solution Zn but a lower projected value of soil pH, AN, F and $G^+:G^-$; Additionally, those effects ranked as $E1E2 > E1 > E2$. (Fig. 9c). In L and M soils, the treatments with *S. plumbizincicola* and earthworm inoculation (PE2 and PE1E2) were separated from those only planting *S. plumbizincicola* (P), with the former associated with higher projected values of pH, AN, TN, Oxidizable Zn, Total PLFAs, B, G^+ , G^- , F and $G^+:G^-$ (Fig. 9c). PE1 was lightly separated from P, with the former associated with higher projected values of Acid-solution Zn and Reducible Zn.

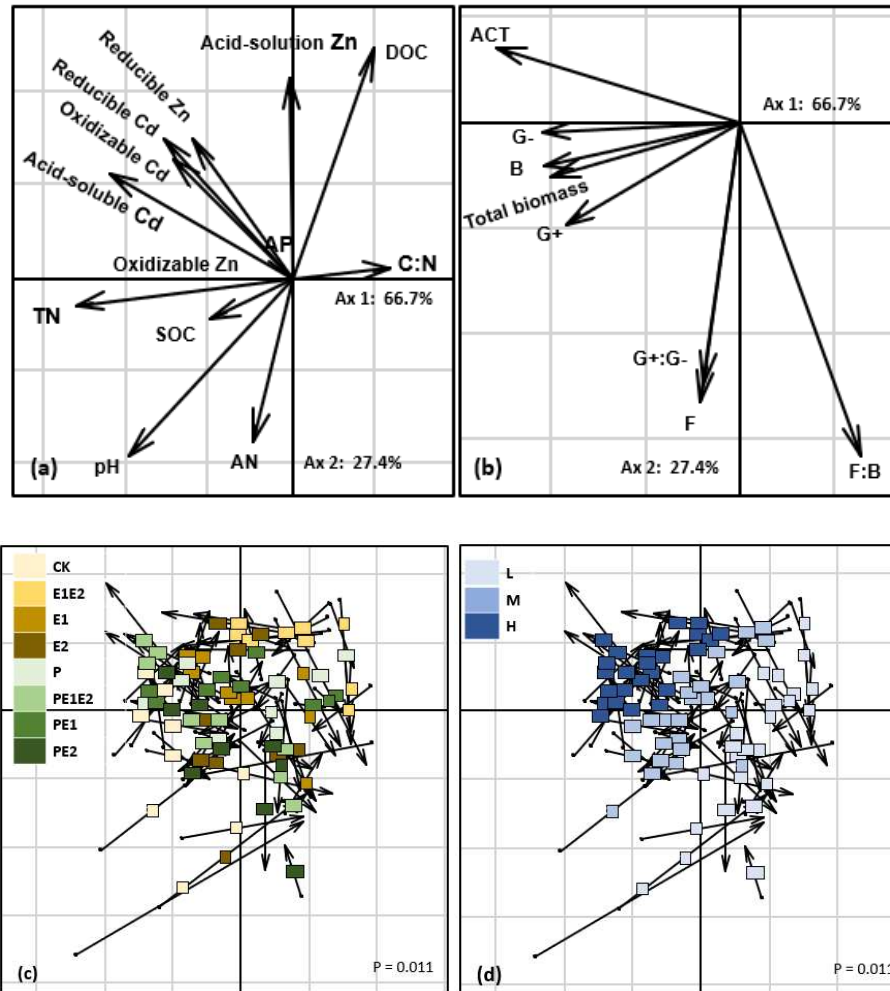


Fig. 9 Co-inertia analysis between soil chemical properties and soil microbial community characteristics of bulk soil in the different treatments. (a) Co-inertia projection of soil chemical properties of bulk soil; (b) Co-inertia projection of soil microbial community characteristics of bulk soil; (c) Score plot of all the treatments; (d) Score plot of different Cd and Zn contaminated levels. Note: the squares and arrows represent the projected coordinates of soil chemical properties and soil microbial community characteristics of each pot. CK: control; E1E2, inoculated *A. cortices* and *A. aspergillum*; E1, inoculation *A. cortices*; E2, inoculation *A. aspergillum*; P, planting *Sedum plumbizincicola*; PE1E2, planting *Sedum plumbizincicola* and inoculation *A. cortices* and *A. aspergillum*; PE1, planting *Sedum plumbizincicola* and inoculation *A. cortices*; PE2, planting *Sedum plumbizincicola* and inoculation *A. aspergillum*. Cd and Zn contamination levels was shown as L, M and H, representing low, medium and high contaminated levels respectively.

The PCA was employed to identify the overall trends in microbial community of bulk and rhizosphere soils in response to *S. plumbizincicola* and earthworm activities in different Cd and Zn contaminated soils (Fig. 10). Axis 1 (66.2% variance explained) was primarily associated with

G⁺, ACT, B, Total PLFAs, G⁻ and F; Axis2 (24.6% variance explained) was primarily associated with G⁺:G⁻ and F:B. Soil with inoculation earthworms were significantly differentiated from the control soils (Fig. 10b, d-e) ($P < 0.05$).

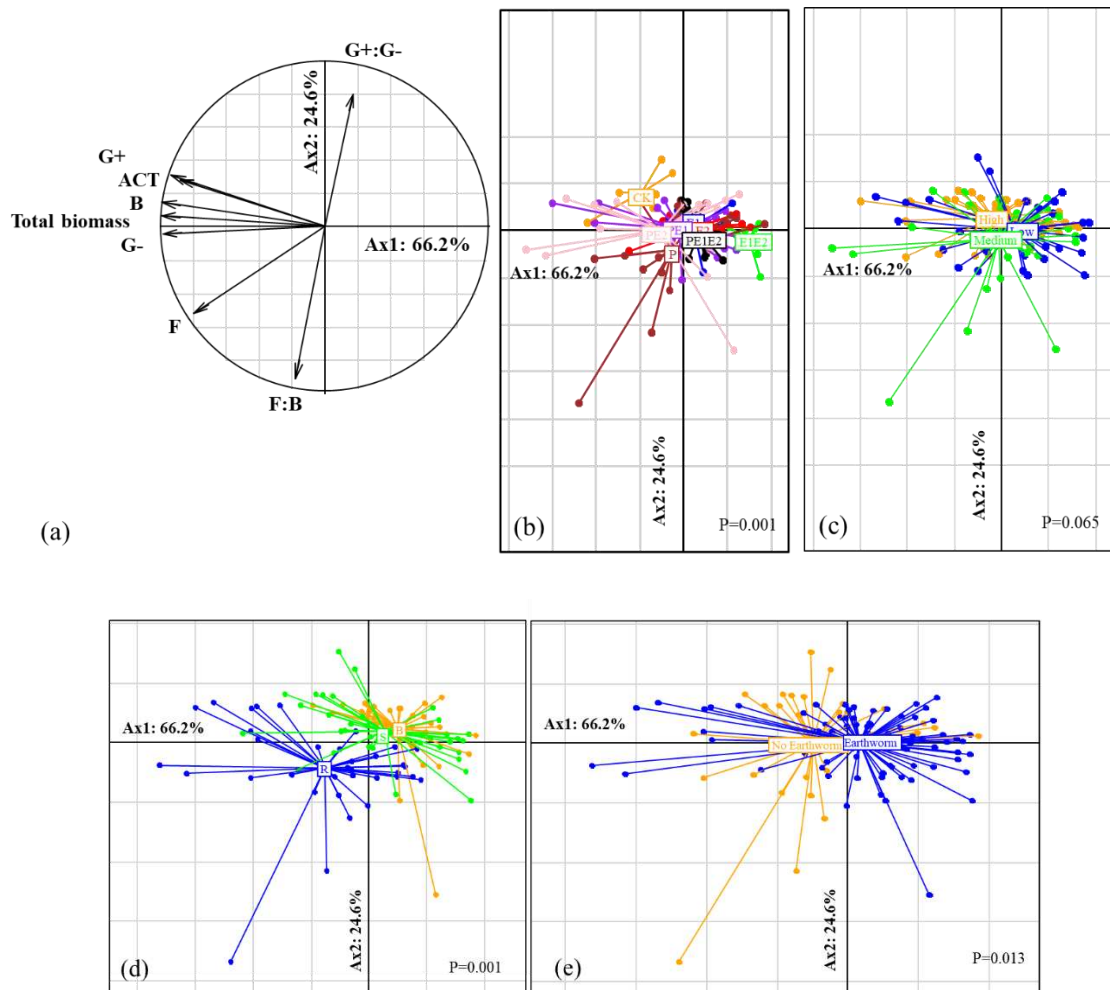


Fig. 10 Principal component analysis (PCA) of the effect of *Sedum plumbizincicola* and earthworms on soil microbial community in low, medium, high Cd and Zn contaminated level soils in fractal F1 and F2 planes. (a) Correlation circles of soil microbial communities with the G⁺:G⁻ and F:B ratios in fractal F1 and F2 planes; (b) Projection of experimental points according to treatments with or without *Sedum plumbizincicola* and earthworms; (c) Projection of experimental points according to different Cd and Zn contaminated levels; (d) Projection of experimental points according to different hotspots soil; (e) Projection of experimental points according to with or without *Sedum plumbizincicola*; (f) Projection of experimental points according to with or without

earthworms. Note: R, rhizosphere soil; B, bulk soil of the treatments with *Sedum plumbizincicola*; S, soil of the treatments without *Sedum plumbizincicola*.

4. Discussion

4.1 Effects of earthworm species, individual and combined different species on the phytoremediation efficiency

The keys of broken the phytoextraction bottleneck are individually or simultaneously increasing the biomass and the metals concentration of hyperaccumulator (Suman et al., 2018). In the combined earthworm and *S. plumbizincicola* system, earthworm activity not only enhanced the plant physiological trait but also increase soil Cd and Zn bioavailability (Fig. 1, Fig. 4 and Fig. 5). Noteworthy, the biomass of *S. plumbizincicola* and the Cd and Zn concentration in different tissues were not further increased with contaminated levels in only cultural *S. plumbizincicola*, while it increased continuously under earthworm activities (Fig. 2 and Fig. 3); it suggested that earthworm activity alleviated the metals stress to *S. plumbizincicola* in high metals contaminated environment and also increased the phytoremediation efficiency. Similarly, Elmer, 2019 reveled that earthworm activity can decrease the diseases caused by metal pollution through regulating soil microbial community. Furthermore, it may be due to that the photosynthesis efficiency was guaranteed with high leaf chlorophyll under earthworm activities (Fig. 1), and may also benefit from low Cd toxicity to chloroplast by the high expression of SpHMA1 to increase the Cd transporter protein in chloroplast periplasm to promote metals transfer to outside from chloroplast (Zhao et al., 2019), further increasing the biomass. The plant biomass was found as the main factor of increasing metals uptake amount. This finding is consistent with Wang et al., 2020 and Chapter II. Specifically, the plant biomass was significantly increased in the treatments with simultaneously inoculation with earthworm and *S. plumbizincicola* in L and H soils and the soil of M-PE2 treatment, resulting in high Cd and Zn uptake amount of *S. plumbizincicola* (Fig. 1b and

c; Fig. 3), while the biomass was slightly increased in M soil and the metals uptake amount lower than in only *S. plumbizincicola* treatment because of slight dilution effect (Fig. 3). The metals concentration in different tissues were reversed with the biomass (Fig. 2 and Fig. 1c). The evident of dilution effect was observed in high metals concentration soil, like in M and H soils (Fig. 2). This phenomenon occurred possibly to due to high growth rate of *S. plumbizincicola*, but the metal absorption needs of roots was increasing and cannot be satisfied in time. This suggested that the high biomass make a greater positive influence on increasing metal uptake amount than the metal concentration in plant tissues in this system, and also provide the direct evidence that the bioaugmentation effects of earthworms mainly realized by promoting *S. plumbizincicola* growth. Moreover, the promoting growth effects was enhanced with contaminated levels (Fig. 1b and c); it may be attributed to earthworm activity enhances plant tolerance to metal by increasing antioxidative enzyme activities, gene expression and reactive oxygen species inhibition (Kaur et al., 2019); Additionally, wild earthworm may accelerate the microbial community succession in rhizosphere soil and mitigate of pollution stress by enhancing the activity of microorganisms that symbiotically interact with plant roots or promote plant growth. Wang et al., 2024a found that *Sphingomonas* sp. Hbc-6, plant growth-promoting bacteria, can promote plant growth and drought resistance by manipulating the rhizosphere microbiome. Moreover, the metals transfer from roots to harvestable tissues in time are secondary dominant factor and essential to improve the phytoremediation efficiency. The metals uptake amount in only cultivation *S. plumbizincicola* treatment in medium contaminated soil was higher than in the treatment inoculation with earthworm; it suggested that earthworm activities may damage the root system, and it is necessary to explore the appropriate earthworm inoculation densities in the further research. Nevertheless, earthworm facilitated the roots development and expanded the range of restoration.

The different earthworm species make different ecological effects by feeding preference, digging habits, and gut transits (Arrazola-Vasquez et al., 2022). The augmentation effects of epigeic *A. cortices* were greater than anecic *A. aspergillum*, while the effects of combined those two species were somewhere in between (Fig. 8). The bioaugmentation effects of combined *A. cortices* and *A. aspergillum* was not further increased compared with those two species individual, but may be due to the competition for resources (Edwards and Bohlen, 1996; Lavelle and Spain, 2001; Marinissen and van den Bosch, 1992). Nevertheless, this strong relationship between two species makes positive effects on earthworm survival, such as high survival rate and biomass (Fig. S1).

4.2 Microbial property variations in hotspots induced by earthworms in Cd and Zn contaminated soil

The earthworms and roots activities have a great influence on soil microbial community biomass and structure, especially in hotspots areas (Fig. 9 and Fig. 10), consistent with Jusselme et al., 2012. The root exudates can restructure the microbial community in rhizosphere area, and earthworm activities further influence on it (Fig. 6 and Fig. 7). This finding aligns with previous research indicating that the behavioral and feeding traits of earthworms could change soil microbial community composition (Brown, 1995; Ren et al., 2021).

In only inoculation earthworm treatments, earthworm activities decreased the microbial biomass, especially in *A. aspergillum* treatment and in *A. cortices* + *A. aspergillum* treatment (Fig. 6), it was consistence with Tibihenda et al., 2023, which may be due to earthworm feeding (Edwards and Arancon, 2022). The microbes ingested by earthworms may be killed by the microbicidal substances in the gastrointestinal fluid or may not be able to survive the anaerobic conditions in the earthworm gut (Nechitaylo et al., 2010; Gómez-Brandón et al., 2011; Takur et

al., 2022; Ahmed and Al-Mutairi, 2022). In addition, the variation of total microbial biomass, bacterial, G^+ and G^- biomass in bulk soil among different treatments were decreased in *S. plumbizincicola* + earthworm treatments compared to only cultivation *S. plumbizincicola* treatment; it may be caused by earthworm activity influencing on soil microbial community (Medina-Sauza et al., 2023) and the high biodiversity increasing the interconnectivity and integration of microbial communities in the rhizosphere and bulk soils, the stability of soil microbial community (Jacquiod et al., 2020; Wagg et al., 2014). Noteworthy, the trend of total microbial biomass and all the bacterial components in the rhizosphere was consistence with in the soil only inoculation with earthworm treatment. This suggests that earthworm activity made greater impact on soil microbial community in rhizosphere. It also be founded in Delgado-Baquerizo et al., 2016, and shows that soil bacteria dominated influence by soil properties and environment; moreover, earthworm, as “soil ecosystem engineer”, have great influence on soil properties and environment (Tibihenda et al., 2023; Zhang et al., 2023). In rhizosphere soil, the different performance of the different microbial components in *S. plumbizincicola* + *A. cortices* treatment and *S. plumbizincicola* + *A. aspergillum* treatment between in H soils and in L and M soil, may be caused by the metal stress inducing the change of earthworm metabolites and root exudates, and in turn promoting or inhibiting some special microbial communities (Ahmed and Al-Mutairi, 2022) such as Proteobacteria, Actinobacteriota, Bacteroidota, Firmicutes and Ascomycota with high metal resistance ability (Liu et al., 2020; Sibanda et al., 2019; Wu et al., 2022; Egidi et al., 2019). In terms of earthworm species, the $G^+ : G^-$ ratio ranked *A. aspergillum* > *A. cortices* and *S. plumbizincicola* + *A. aspergillum* > *S. plumbizincicola* + *A. cortices*, it may be due to *A. cortices* being more favorable G^- . Similarly, Tibihenda et al., 2023 found that *E. fetida* and *A. aspergillum* were favorable for G^- and fungal communities, respectively. Interestingly,

earthworm activities increased the ratio of the $G^+:G^-$ in rhizosphere soil, and showed combined earthworm species > individually earthworm species (Fig. 7); it indicated that combined earthworm species increase the nutrient cycling and pollution resistance ability of microbial community in rhizosphere soil. The F:B ratio decreased in rhizosphere soil when earthworm participated, which suggested that earthworm disturbance did not facilitate for fungal growth.

4.3 Influence of earthworm and soil microorganisms on Cd and Zn uptake by *S. plumbizincicola* with earthworm inoculation

Soil solution plays an important role in the transfer of metals to plant roots. Inoculation individual earthworm species promoted Cd and Zn releasing from soil solid phase to soil solution with cultivation time especially Zn, opposite of the soil aging process (Fig. 5). Furthermore, the combined different earthworm species further increase metal releases to soil solution. The high acid-soluble fraction content generally accompanied by high metal concentration in soil solution. At end of the experiment, we found that soil Cd and Zn trend to high mobility, especially Zn (Fig. 4). The reservoir of trace elements in soil solution depends on the balance between element mobilization and fixation, which is further influenced by plant and microorganisms. Earthworms improve the soil environments and root development through affecting soil physical-chemical properties and mitigating the metal toxic effects on plant roots (Table 2, Fig. 8 and Fig. S2), to enhance metal uptake by *S. plumbizincicola*. In terms of nutrient supplying to plant roots, earthworm activity increase soil available nitrogen, meeting the nitrogen demand for straw decomposition; the gut process also accelerates straw breakdown and promotes organic matter mineralization (Table 2). Moreover, it could be found that earthworm activities had greater promoting effects on roots than on aboveground tissues (Fig. 1c). Furthermore, the high root biomass increased the metals uptake amount by roots, and earthworm activities facilitated metals

transfer from roots to aboveground tissues (Fig. 2e and f). This indicated that earthworm activity alleviated the metals stress to the roots of *S. plumbizincicola*. The high root biomass may be attributed to earthworm mucus stimulate root development, which further increase the nutrient uptake by enlarging root-soil contact area. In addition, the microbial community also played a vital role in those processes (Fig. 9). The metals accumulating microbes might serve as a potential metal reservoir for plant metals uptake (Pham et al., 2022; Rajivgandhi et al., 2022), in particularly in rhizosphere area. The increase in total microbial biomass in hotspots area due to earthworm activities suggests a likely more diverse soil microbial community with a more diverse genetic pool for more diverse soil functions to influence metals uptake by plants (Tardy et al., 2014; Li et al., 2018). The strain SC2b and RC6b were isolated from the rhizosphere of *S. plumbizincicola*, and found that those promotion microorganisms increase the plant biomass by secretion of probiotic substances, activation of nutrients and metals to improve the phytoremediation efficiency (Ma et al., 2016; Ma et al., 2015; Ma et al., 2015b). The NSE1 fungi strain was also isolated from rhizosphere and be found the cadmium tolerant function by adsorbing cadmium on cell wall, as well as promoting function for *S. plumbizincicola* by secretion of probiotic substances, activation of phosphorous (Liu et al., 2016). Earthworm make direct impact on soil microbial community in casts through gut digestion and indirect impact on soil microbial community in burrow wall by changing the soil properties, which leads to the discrepancy of soil microbial community between hotspots area and bulk soil. During those processes, earthworm can activate and expand the soil beneficial microflora (Edwards, 2004), which can promote soil organic matter decomposition and metal bioavailability, and accelerate soil biogeochemical cycling. Moreover, it may also increase the gene SpMTL and SpMT expression to enhance metal tolerance and promote metal long-

distance transfer in *S. plumbizincicola* (Peng et al., 2017; Pent et al., 2020), achieving high metal accumulation amounts in the harvestable aboveground tissues.

5. Conclusion

This study focuses on the phytoremediation efficiency of *S. plumbizincicola* inoculation with individual and combined different species of wild earthworm to explore the effects of multiple earthworm species on phytoremediation efficiency and reveal the underline mechanisms to optimize bioremediation processes. Generally, combining earthworm species with *S. plumbizincicola* facilitate the survival of *A. cortices* and *A. aspergillum*. The biomass of *S. plumbizincicola* show stable increase under earthworm activities. Even with the diluted effects, earthworm activity still improved the phytoremediation efficiency. Earthworm affect Cd and Zn uptake by *S. plumbizincicola* through improving soil environment, metal mobility and bioavailability and strengthen the interaction with soil microbial community in bulk and rhizosphere soils. This study provides a sight in effects and potential of combined multi-organisms for soil remediation in natural environment.

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Supplementary materials

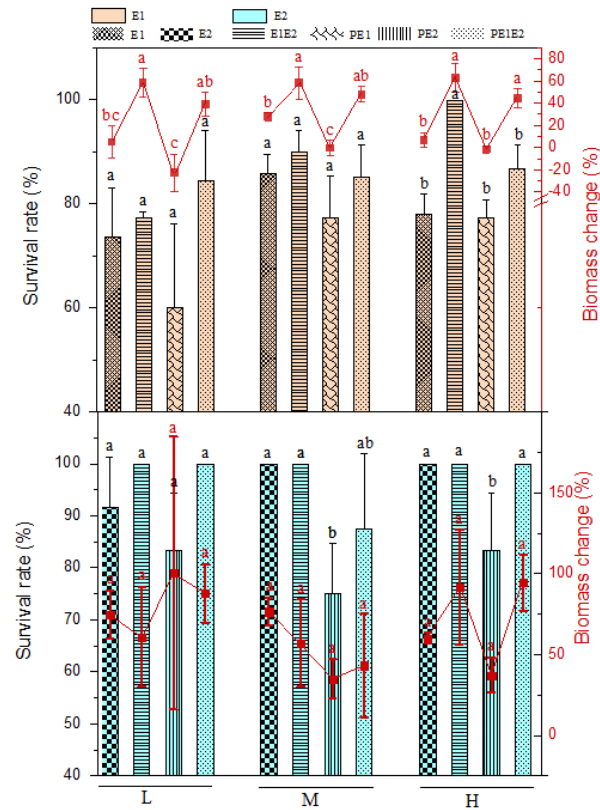


Fig. S1 Survival rate and biomass increase of four earthworm species at the 90th day. Results are expressed as the mean value $\pm$ SD (n=4). The uppercase letters L, M, and H represents the soil L, soil M, and soil H. E1, E2, E1E2, PE1E2, PE1 and PE2 represents the treatments with *A. cortices*, *A. aspergillum*, *A. cortices*+ *A. aspergillum*, *S. plumbizincicola* + *A. cortices*+ *A. aspergillum*, *S. plumbizincicola* + *A. cortices*, *S. plumbizincicola* + *A. aspergillum*. Different lowercase letters indicate significantly differences ($P < 0.05$) between different treatments. Different uppercase letters indicate significantly differences ($P < 0.05$) between different soils.

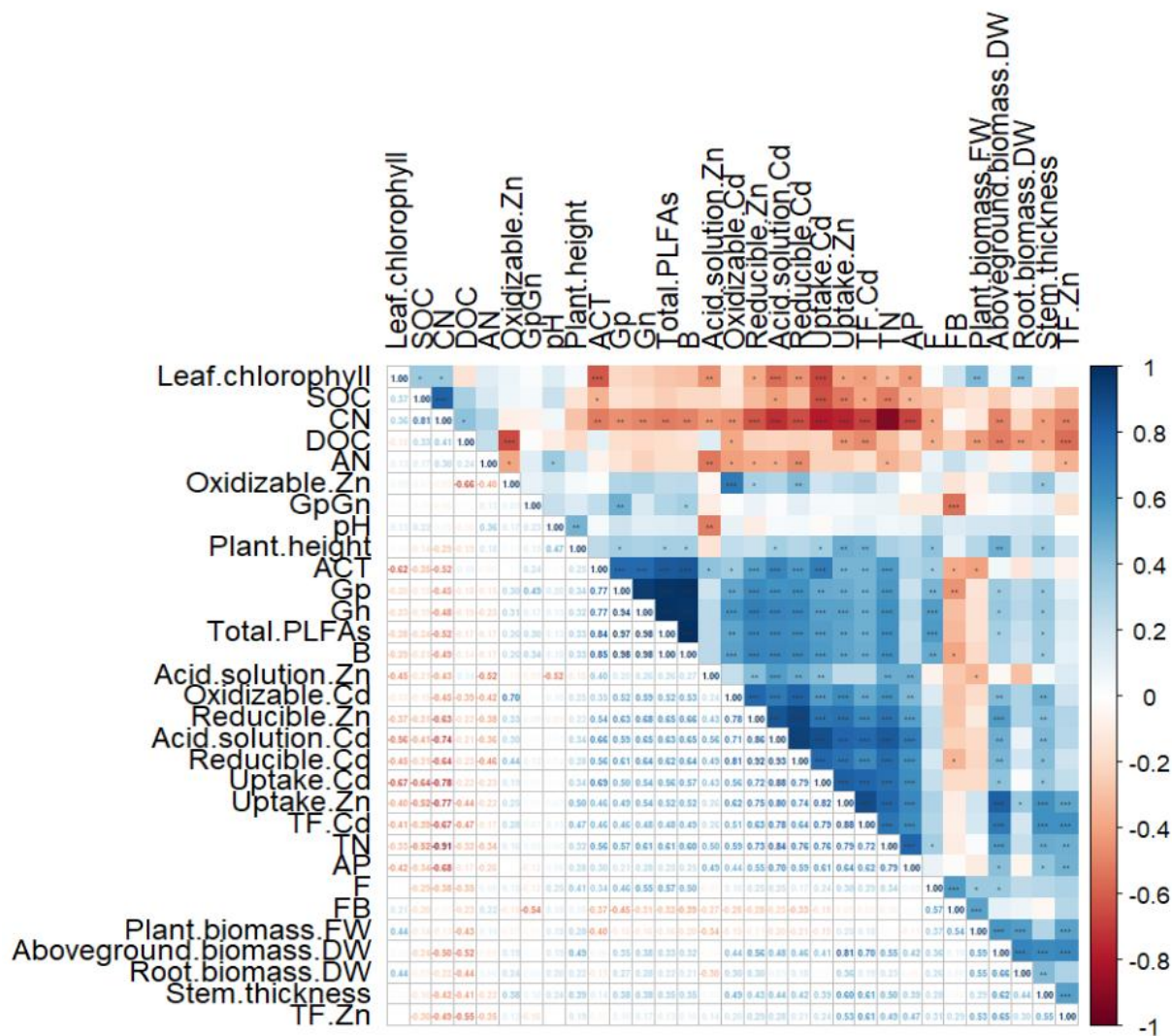


Fig. S2 Pearson's correlation coefficients between plant physiological, plant metals uptake amount, metals transfer factor, soil chemical properties and microbial community of bulk soil in low, medium, high Cd and Zn contaminated level soils. Note: *, **, *** indicate the significant levels at $p < 0.05$, 0.01 and 0.001, respectively.

Chapter IV: Responses of soil microbial communities to the involvement of individual and combined different wild earthworm species in the uptake of Cd and Zn by *Sedum plumbizincicola*

Résumés: Les activités des vers de terre ont une influence significative sur la composition taxonomique et la diversité des communautés microbiennes de bactéries et de champignons (Medina-Sauza et al., 2019). Dans les environnements de sol soumis à un stress métallique, les vers de terre pourraient participer à la réponse à la croissance des plantes, en façonnant le microbiote du sol d'une manière favorable pour les plantes. Les processus de transit intestinal des vers de terre peuvent faciliter ou inhiber certains taxons microbiens, et les effets hérités de la distribution des vers de terre affectent également la communauté microbienne du sol (Yang et al., 2024). Il est intéressant de connaître les effets de l'activité des vers de terre sur la communauté microbienne dans les sols en vrac et la rhizosphère, et l'interaction (in)directe avec la croissance des plantes et l'absorption des métaux. Pour comprendre l'impact de l'activité des vers de terre sur la composition et la structure de la communauté microbienne du sol et trouver les prédictors microbiens dominants de la quantité d'absorption de Cd et de Zn de *S. plumbizincicola* à différents degrés de contamination, nous avons approfondi la communauté microbienne dans les sols en vrac et dans la rhizosphère. Les résultats ont montré que les activités des vers de terre augmentaient de manière significative l'abondance des phyla dominants et la diversité microbienne α et β . En présence de vers de terre, davantage de taxons biomarqueurs de bactéries et de champignons ont augmenté dans la rhizosphère, en particulier à des niveaux de contamination moyens et élevés, les fonctions contribuant principalement à la décomposition de la matière organique du sol, au cycle du carbone et de l'azote, à la survie dans un environnement soumis à un stress métallique, et

affectant indirectement le transfert de métaux, etc. Les modèles d'équation structurelle ont révélé que les activités des différentes espèces de vers de terre avaient un effet positif et (in)direct sur la quantité de Cd et de Zn absorbée par *Sedum plumbizincicola* en influençant les propriétés du sol et la diversité microbienne dans la rhizosphère et les sols en vrac. Le microbiote clé de l'amélioration de l'absorption du Cd et du Zn par *S. plumbizincicola* a changé avec les niveaux de contamination du sol. En détail, les genres dominants *Fusarium*, *Desulfitobacterium*, *Pseudeurotium*, *Cellulomonas*, *Georgenia*, *Edaphobacter*, *Anaerosporobater*, *Sorangium* ont été remplacés par les genres *Defluviicoccus*, *Methyloceanibacter*, *Microtholunatus*, *Skermanella*, *Aridibacter*, *Myxococcus*, *Intrasporangium*, *Rubrobacter*, *Defluviicoccus*, *Stenotrophobacter*, *Puia*, et la famille Vicinamibacteraceae et Caldilineaceae, dans les sols fortement contaminés par les métaux. En conclusion, les vers de terre participent à la réponse à l'absorption de Cd et de Zn par *S. plumbizincicola*, en façonnant le microbiote fonctionnel du sol, en particulier ceux liés au cycle des nutriments. Nous fournissons une base théorique pour comprendre les effets combinés et le potentiel de plusieurs organismes dans la remédiation des sols.

Responses of soil microbial communities to the involvement of individual and combined different wild earthworm species in the uptake of Cd and Zn by *Sedum plumbizincicola*

Highlights

- The characteristics of soil microbial communities in bulk and rhizosphere soils tend to become more diverse, stable, and exhibit favorable microbial phylogeny after earthworm activity.
- The rhizosphere shows an increase in biomarker taxa in earthworm with *S. plumbizincicola* system, with functions primarily contributing to biogeochemistry cycles and metal resistance.
- The synthesis effects of earthworm on soil microbiome development showed remarkable higher in combined two species than that in individual species.
- The key microbiota enhancing Cd and Zn uptake by *S. plumbizincicola* changed with soil contaminated levels.

Abstract

Earthworm activities significantly influenced on the taxonomic composition and diversity of bacteria and fungi microbial communities. To understand the impact of earthworm activity on the composition and structure of soil microbial community and to explore the dominant microbial predictors for Cd and Zn uptake by *S. plumbizincicola* under different contaminated levels, we further conducted an in-depth analysis of the microbial community in bulk and rhizosphere soils. The results showed that earthworm activities significantly increased the abundance of the dominate phyla and the microbial α and β diversity. In the presence of earthworms, more biomarker taxa of bacteria and fungi increased in the rhizosphere, especially in medium and high contamination levels, the functions mainly contributing to the soil organic matter decomposition, carbon and nitrogen cycling, survival in metal-stress environmental, and indirectly affecting metals transfer, etc. Structural equation models reveled that the activities of different species of earthworm had a positive and (in)direct effect on Cd and Zn uptake amount of *S. plumbizincicola* through influencing the soil properties and the microbial diversity in rhizosphere and bulk soils. The key microbiota of enhance Cd and Zn uptake by *S. plumbizincicola* changed with soil contaminated levels. In details, the dominant genus *Fusarium*, *Desulfitobacterium*, *Pseudeurotium*, *Cellulomonas*, *Georgenia*, *Edaphobacter*, *Anaerosporobater*, *Sorangium* were replaced by genus *Defluviicoccus*, *Methyloceanibacter*, *Microtholunatus*, *Skermanella*, *Aridibacter*, *Myxococcus*, *Intrasporangium*, *Rubrobacter*, *Defluviicoccus*, *Stenotrophobacter*, *Puia*, and family Vicinamibacteraceae and Caldilineaceae, in high metals contaminated soils. In conclusion, earthworm take part in the response to Cd and Zn uptake by *S. plumbizincicola*, by shaping soil functional microbiota, especially those related to nutrient cycling. We provide a theoretical

foundation for understanding the combined effects and potential of multiple organisms in soil remediation.

Keywords: wild earthworm species, *Sedum plumbizincicola*, bioaugmentation effect, rhizosphere soil, keystone microbial community

1. Introduction

Earthworms are well known for their positive effect on plant growth (van Groenigen et al., 2014, Blouin et al., 2019). Earthworms produce mucus and modifies soil physics and chemistry (Blouin et al., 2013; Vidal et al., 2023) in a way analogous to plants, which produce root exudates and modify the physico-chemical properties of the rhizosphere (Hinsinger et al., 2005). These earthworm activities affect microbial communities, with consequences on microbial functions involved in metals cycling and hormone production which reverberate on plant growth (Medina-Sauza et al., 2019). The results obtained are promising. Nevertheless, understanding the behavior of the microbial communities in the process of bioaugmentation and their role in improving metal phytoremediation is very important to fully validate the application of this biological technology.

In metal-stress soil environments, earthworm could take part in the response to the plant growth, by shaping soil microbiota in a favorable way for the plants. The earthworm gut transit processes can facilitate or inhibit some microbial taxa, and the legacy effects of earthworm cast also affect the soil microbial community (Yang et al., 2024). The soil microbiota, shaped by both earthworms and plant roots, is the critical mediator of biogeochemical cycles in soil-earthworm-plant system (Yang and van Elsas, 2018). It is worth to know that the effects of earthworm activity on microbial community in bulk and rhizosphere soils, and the (in)direct interaction to plant growth and metals uptake.

Hence, we tested the hypothesis that earthworm can modify the soil microbiome and the abundance of metals resistance and nutrient cycles microorganisms, which could help the plant in metals-stress and unfertile conditions. The objectives of this study were (1) to reveal the impacts of earthworm activities of individual and combined different species on the microbial community composition, structure and biomarkers function in bulk soil; (2) to reveal the impacts of earthworm activities on the microbial community in rhizosphere soil and the communication of microbial community between bulk and rhizosphere soils under earthworm disturbing; (3) to clarify the key factors and identified the keystone taxa for driving the Cd and Zn uptake of *S. plumbizincicola*. This research could provide a theoretical foundation for understanding the combined effects and potential of multiple organisms in soil remediation.

2. Materials and methods

2.1 Experiment design, soil sampling and measurement of soil physicochemical properties

The experiment design and materials and methods were same as Chapter III. Moreover, the rhizosphere and bulk soils were also from Chapter III. More details can be found in section 2.1 to 2.3 of Chapter III.

2.2 Genomic DNA extraction, amplicon sequencing, bioinformatic analyses

Soil genomic DNA was extracted using the sample was extracted by CTAB/SDS method, and then the purity and concentration of DNA were detected by agarose gel electrophoresis. The primers used were 16S V4 region primers (515F/ 806R) and ITS1 region primers (ITS5-1737F/ ITS2-2043R) to amplify the bacterial 16S rRNA gene and fungal ITS region, respectively. According to the manufacturer's instructions, amplicons were amplified as follows: initial denaturation at 98°C for 1 min, followed by denaturation at 98°C for 10 s (30 cycles), annealing and extension at 50°C and 72°C for 30 s, and centrifugation at 72°C for 5 min. PCR products were

detected by electrophoresis using agarose gel with a 2% concentration. For the target strip, the Qiagen Gel Recovery Kit (Qiagen, Germany) was used to recover the products. The library was constructed using the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA). The constructed library was quantified by Qubit and Q-PCR and sequenced on the Illumina Nova Seq platform.

Raw tags were filtered with reference to the QIIME Tags quality control process (V 1.9.1, http://qiime.org/scripts/split_libraries_fastq.html) (Caporaso et al., 2010). The tag sequence was compared with the species annotation database to detect the chimeric sequence (v2.22.1, <https://github.com/torognes/vsearch/>), and the chimeric sequence was ultimately removed to obtain effective Tags (Rognes et al., 2016). The effective tags were assembled into amplicon sequence variants (ASV) using Deblur v1.1.1 (Knight et al., 2018; Amir et al., 2017) by QIIME2 (Bolyen et al. 2019). Deblur compares Hamming distances (sequence-to-sequence) within a sample to an upper bound error curve, and combines this greedy algorithm to obtain the ASV. The species annotation analysis was performed using the Mothur (v1.48) method and SILVA138.1 (<http://www.arb-silva.de/>) SSUrRNA database (Set the threshold to 0.8–1) for bacteria (Quast et al., 2012), and the Mothur (v1.48) method and UNITE (ver9, 29.11.2022, <https://unite.ut.ee/>) for fungi (Nilsson et al. 2019). The Chao1, Shannon, Simpson, and ACE indices were calculated using QIIME software (Version 1.9.1).

2.3 Statistical analyses

The advanced stacked bar charts were plotted using the “ggplot2” package in R (version 4.2.0). The star plots were plotted by Origin 9.0. To determine the potential compositional differences in the bacterial and fungal community across treatments and microsite samples, principal coordinates analysis (PCoA) was performed using the “phyloseq” package in R based on

the Bray-Curtis dissimilarity (Qiu et al., 2023). Permutational multivariate analysis of variance (PERMANOVA) was conducted using the “phyloseq” package (Qiu et al., 2023) to test the difference in the bacterial and fungi community compositions between different treatments and microsite samples. Significant differences in the relative abundances of bacterial and fungal taxa between treatments and microsite samples were identified using the linear discriminant analysis (LDA) effect size (LEfSe). Random Forest analysis was performed with the “randomForest” package to identify the main microbial predictors of the Cd and Zn uptake amount in bulk soil and rhizosphere soil, respectively. Structural equation models (SEMs) were established to test the direct and indirect effects of earthworm activity, bacterial diversity, fungal diversity, and soil properties of bulk soil on the Cd and Zn uptake amount. The a priori models included all possible pathways among these factors. The significance of each path-coefficient was analyzed by calculating its critical ratio ($P < 0.05$). The SEM was performed using R. The heatmaps were plotted with the “Complex Heatmap 2.12.0”.

3. Results

3.1 Microbial community structure

Amplicon sequencing of bacterial 16S rRNA gene and fungal ITS region yielded a total of 9,470,244 and 11,608,623 high-quality tags, respectively, after quality filtering. The Good's coverage for the observed ASVs of bacterial and fungal communities was $96.99\% \pm 1.13\%$ and $99.78\% \pm 1.00\%$, respectively, which indicated that the sequencing depth was sufficient to reliably describe the microbial community diversity (Fig. S1-S3).

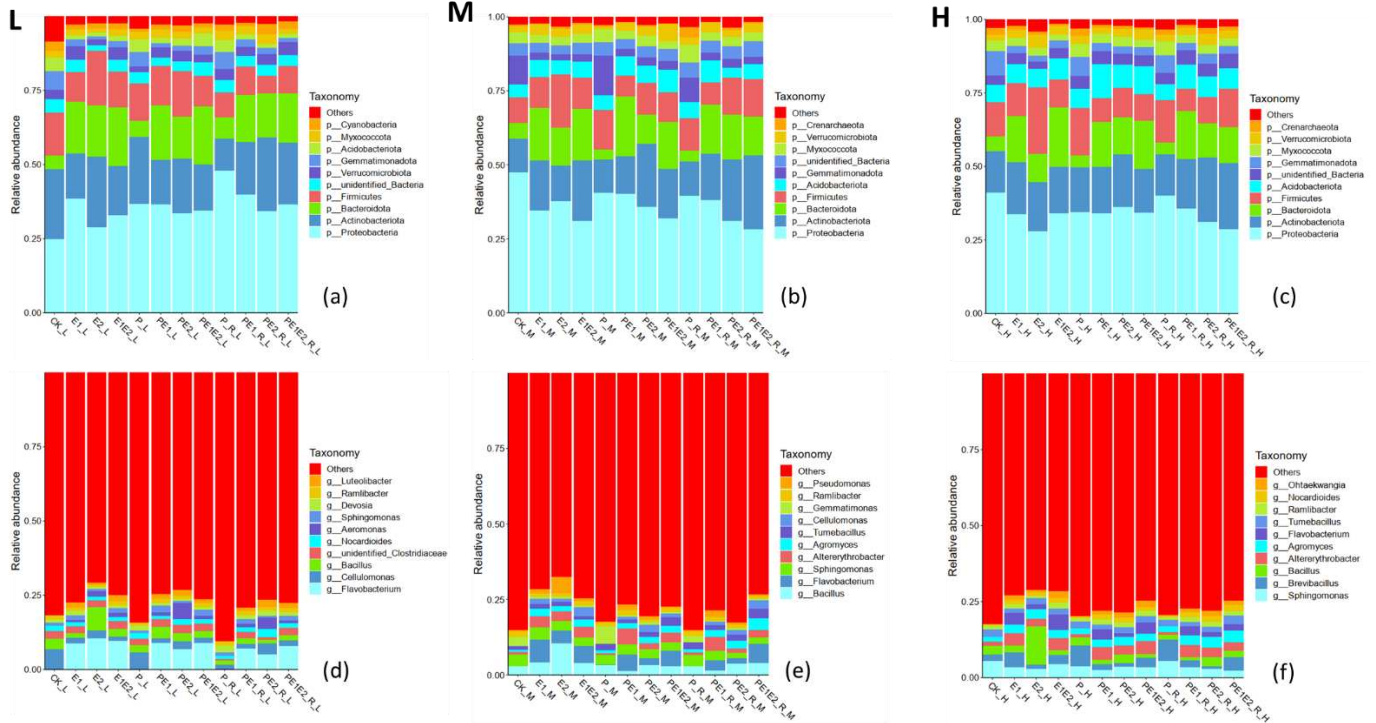
Variance in microbial composition in bulk and rhizosphere soils was studied at the phylum and genus levels (Fig. 1). In phylum level, the bacterial community in rhizosphere and bulk soil was predominated by p_Proteobacteria (24.9%-48.0%), p_Actinobacteriota (11.2%-25.0%),

p_Bacteroidota (3.47%-20.2%), p_Firmicutes (7.48%-18.3%) and p_Acidobacteriota (1.31%-11.5%), p_Gemmatimonadota (1.53%-13.4), accounting for 78.3%-90.5% of the whole community (Fig. 1a, b and c). The relative abundance of p_Proteobacteria and p_Gemmatimonadota shown in CK>E, P>PE, and P_R>PE_R in L, M and H soils, while p_Bacteroidota and p_Actinobacteriota conversely. In genus level, the dominant microbial in bulk and rhizosphere soils included *g_Flavobacterium*, *g_Cellulomonas*, *g_Bacillus*, *g_Nocardioides*, *g_Sphingomonas*, *g_Altererythrobacter*, *g_Agromyces*, *g_Brevibacillus* (Fig. 1d, e and f). Interestingly, *g_Flavobacterium*, *g_Nocardioides* and *g_Altererythrobacter* be founded high relative abundance in E and PE samples, not in CK and P samples. The abundance of *g_Bacillus* was the highest in E2 treatment in three contaminated soils.

In terms for fungi, the microbial composition was predominated by p_Ascomycota (77.4%-98.0%), p_Zygomycota (0.007%-53.9%) and p_Basidiomycota (0.076%-9.63%), accounting for 85.3%-99.4% of the whole community (Fig. 1g, h and i). In genus level, the dominant microbial in bulk and rhizosphere soils included *g_unclassified_Lasiosphaeriaceae*, *g_Papulaspora*, *g_Westerdykella*, *g_Mucor*, *g_Zopfiella*, *g_Chaetomium* (Fig. 1j, k and l). It is worth noting that the relative abundance of p_Ascomycota significantly decreased in E1, E2, PE1, PE1E2 and PE1_R samples with soil contaminated levels in particularly in E1 and E2 samples, while p_Zygomycota and *g_Mucor* in E1 and E2 samples conversely; In addition, the dramatic changes of p_Zygomycota not be found in other PE treatments. Additionally, the abundance of p_Basidiomycota and *g_Bolbitius* (belonging to p_Basidiomycota) increased in PE1 and PE1_R samples in three contaminated soils. Interestingly, *g_Papulaspora* significantly lower in CK, P and P_R samples than other inoculation earthworm samples in three contaminated soils, especially in M and H soils. The abundance of *g_unclassified Lasiosphaeriaceae* and *g_Mucor* was

significantly increased with soil contaminated levels and became dominant genus, while *g_Zopfiella* and *g_Fusarium* shows conversely.

Bacteria



Fungi

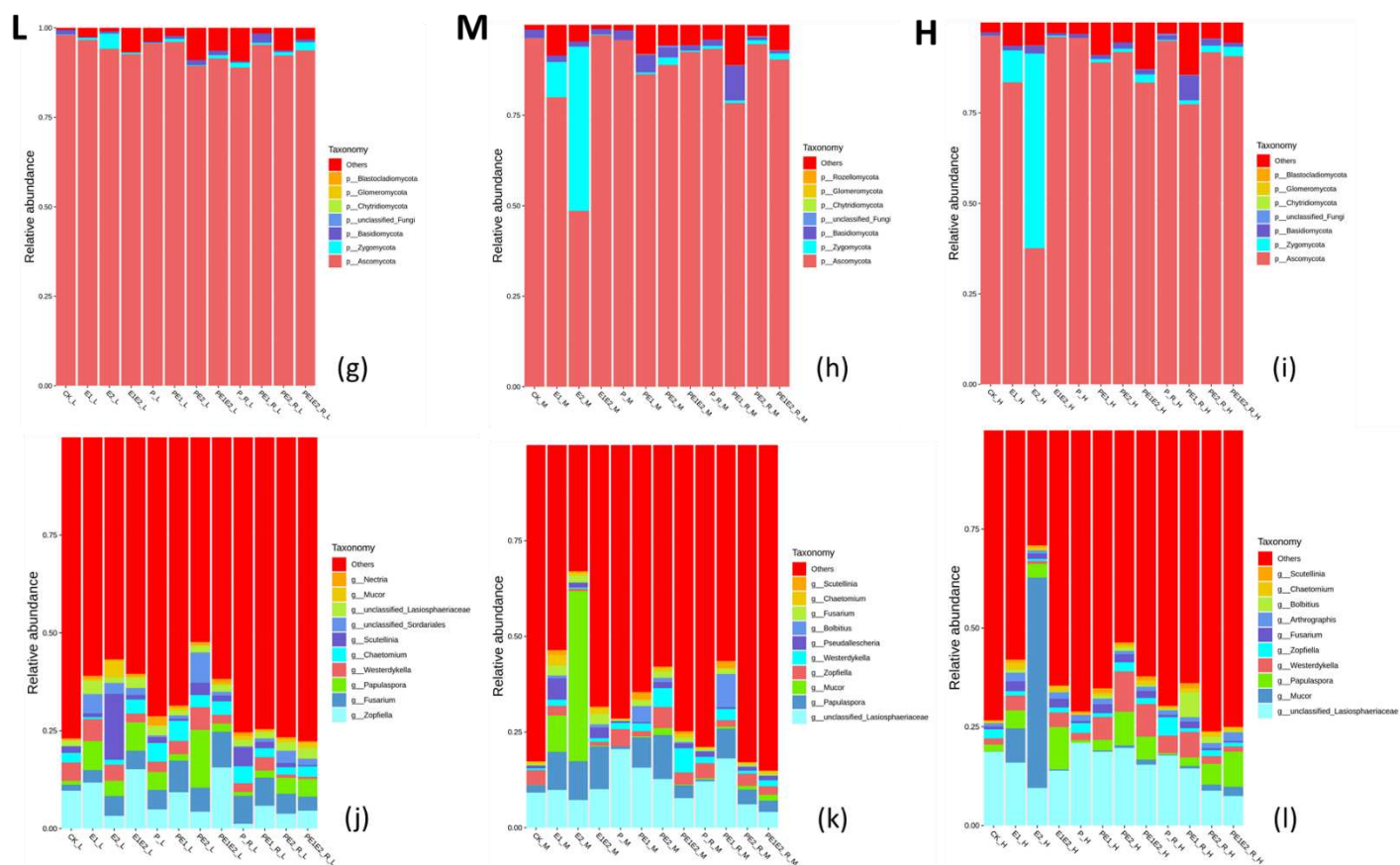


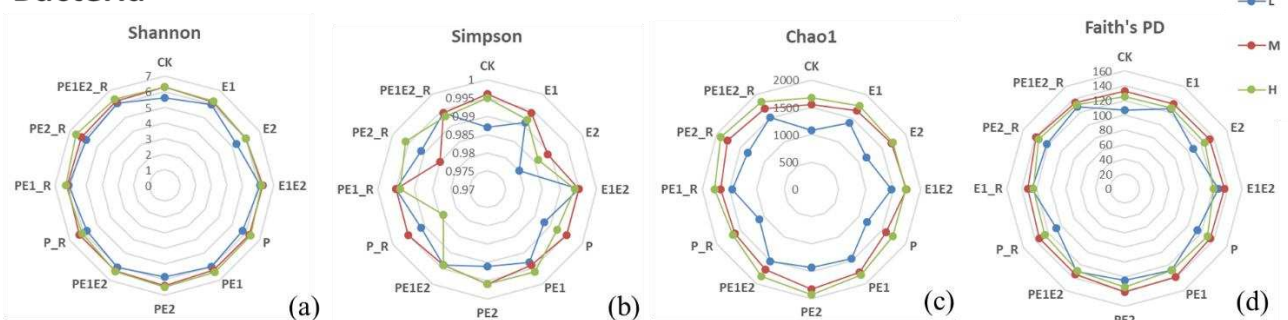
Fig. 1 Taxonomic composition of bacteria and fungi microbial communities at phylum level and genus level based on 16 S RNA sequences in different treatments. The uppercase letters L, M and H represents low contaminated soil, medium contaminated soil and high contaminated soil, respectively. CK, E1, E2, E1E2, P, PE1, PE2 and PE1E2 represents the treatments with control, *A. cortices*, *A. aspergillum*, *A. cortices*+ *A. aspergillum*, *Sedum plumbizincicola*, *Sedum plumbizincicola* + *A. cortices*, *Sedum plumbizincicola* + *A. aspergillum*, *Sedum plumbizincicola* + *A. cortices*+ *A. aspergillum*, respectively.

3.2 Microbial richness and diversity

The α -diversity indexes and richness were calculated at the ASV level to characterize of microbial diversity, such as Shannon, Simpson and Chao1 index (Fig. 2). The evolutionary diversity was represented by Faith's PD index. The Shannon and Simpson indexes of bacteria and fungi were applied to indicate the community diversity. In totally, the Shannon (Fig. 2 a, e), Simpson (Fig. 2 b, f), Chao1 (Fig. 2 c, g) and Faith's PD (Fig. 2 d, h) index of bacteria and fungi

were slightly increased with soil contaminated levels, and it generally was higher in M and H soils than in L soil except for Simpson of bacteria. Moreover, those four indexes of bacteria and fungi were higher variation in all treatments of inoculation without earthworms and E2, and higher abundance in E, PE and PE_R samples than in CK, P and P_R samples respectively, expect for Simpson of bacteria. Specifically, those four indexes ranked as $E1E2 > E1 > E2 > CK$, and $PE1E2 > PE1 > PE2 > P$.

Bacteria



Fungi

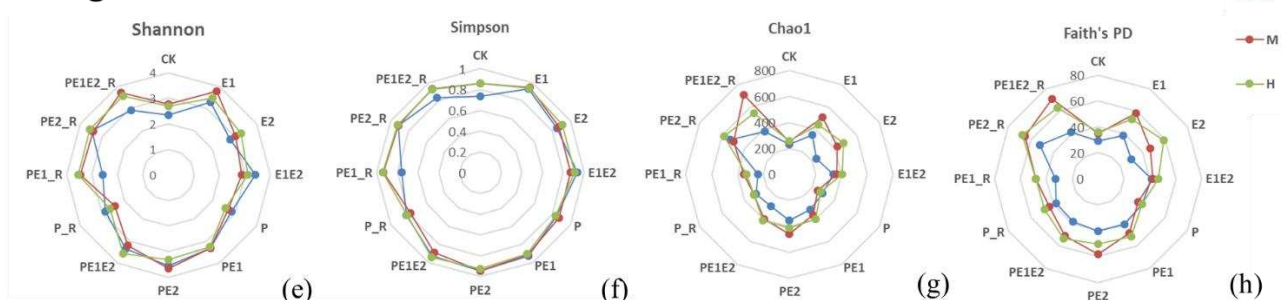


Fig. 2 Star plots of α -diversity indexes of bacteria and fungi community in bulk and rhizosphere soils. The uppercase letters L, M and H represents low contaminated soil, medium contaminated soil and high contaminated soil, respectively. CK, E1, E2, E1E2, P, PE1, PE2 and PE1E2 represents the treatments with control, *A. cortices*, *A. aspergillum*, *A. cortices*+ *A. aspergillum*, *Sedum plumbizincicola*, *Sedum plumbizincicola* + *A. cortices*, *Sedum plumbizincicola* + *A. aspergillum*, *Sedum plumbizincicola* + *A. cortices*+ *A. aspergillum*, respectively.

The PCoA analysis of bacteria and fungi based on Bray-Curtis was employed to analyze the β -diversity of microbial community (Fig. 3). The treatments with earthworms in bacteria and fungi were significantly separated with P and CK treatments ($P < 0.001$). Moreover, as for bacteria,

the points were clearly gathered into three groups with soil contaminated levels, without inoculation earthworms (CK, P, P_R), inoculation with E2 (E2, PE2, PE2_R), inoculation with E1 or E1E2 (E1, PE1, PE1_R; E1E2, PE1E2, PE1E2_R). The distance of the points between rhizosphere soil and bulk soil of P and PE treatments and soil of E treatments was large ($P < 0.001$). While the microbial community in the rhizosphere soil of P and PE treatments was not distant from the bulk soil of those two treatments. However, as for fungi, the points of E, PE and PE_R were clearly separated together, especially inoculation with E2 and E1E2 points.

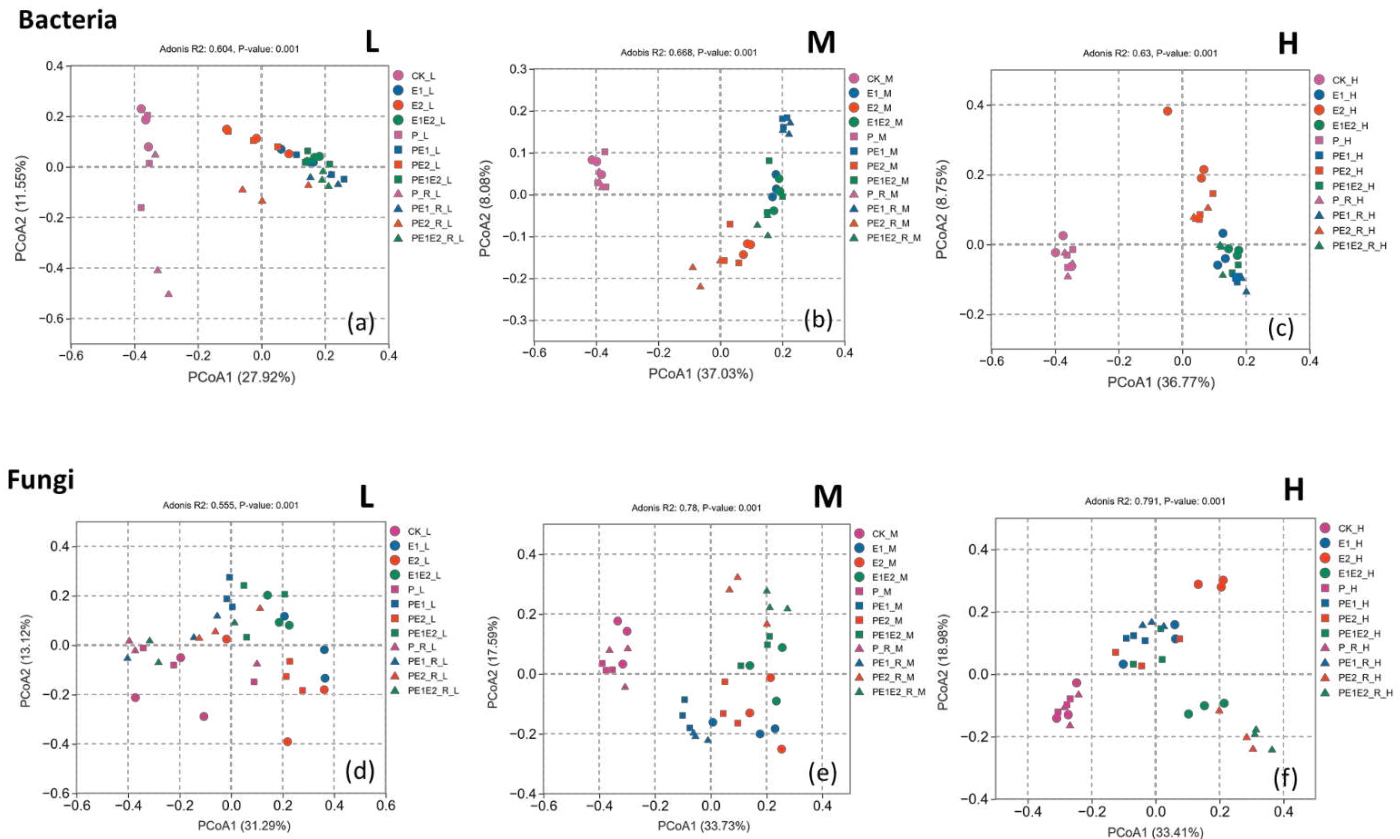
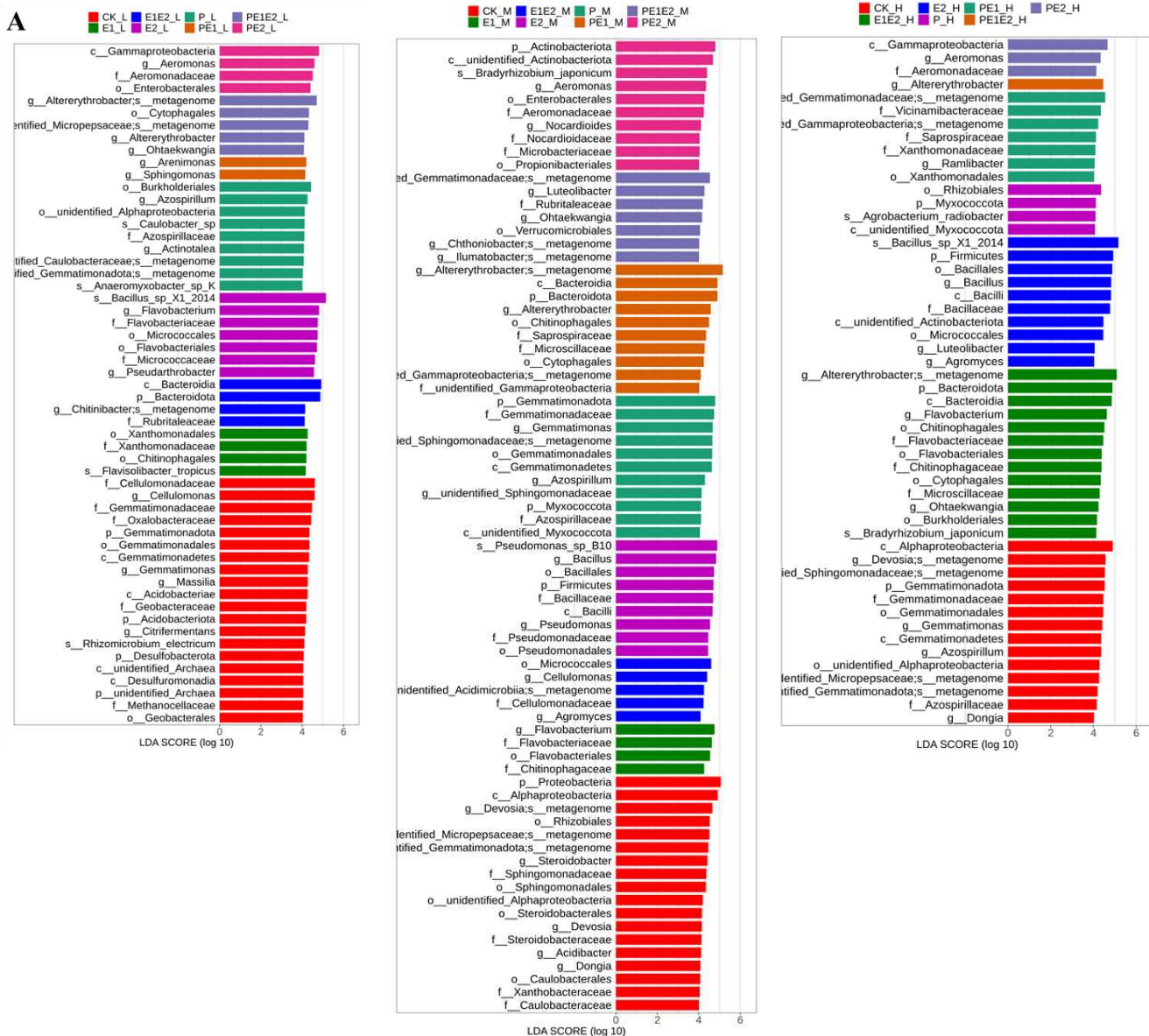


Fig. 3 Principle coordinate analysis (PCoA) plot with Bray-Curtis dissimilarity among soil microbial communities of bulk soil and rhizosphere soil in different treatments. The uppercase letters L, M and H represents low contaminated soil, medium contaminated soil and high contaminated soil, respectively. CK, E1, E2, E1E2, P, PE1, PE2 and PE1E2 represents the treatments with control, *A. cortices*, *A. aspergillum*, *A. cortices*+ *A. aspergillum*, *Sedum plumbizincicola*, *Sedum plumbizincicola* + *A. cortices*, *Sedum plumbizincicola* + *A. aspergillum*, *Sedum plumbizincicola* + *A. cortices*+ *A. aspergillum*, respectively.

3.3 Earthworm activity shaping the soil microbial communities in earthworm-Sedum plumbizincicola system

The linear discriminant analysis (LDA) was conducted to identify the major microbial taxa that were significantly impacted by earthworm across rhizosphere and bulk soil (Fig. 4 and Fig. 5). The Linear Discriminant analysis effect size (LEfSe) tool was employed to search for biomarkers from the phylum to the genus level. LDA scores of 4 or more were confirmed by LEfSe. There were greater number of bacteria species enriched at significant level ($LDA > 4$) in L (55), M (74) and H (52) contaminated soils in bulk soil (Fig. 4A), and in L (31), M (52) and H (24) contaminated soils in rhizosphere soil (Fig. 4B). There were greater number of fungi species enriched at significant level ($LDA > 4$) in L (14), M (40) and H (38) contaminated levels in bulk soil (Fig. 5A), and in L (7), M (26) and H (23) contaminated levels in rhizosphere soil (Fig. 5B). As for different treatments, the number of the taxonomic biomarker of bacteria and fungi shows different in CK (bacteria-49, fungi-7), E1(8, 8), E2(26, 12), E1E2(22, 10), P (23, 12), PE1(19, 10), PE2(14, 6), PE1E2(12, 13) bulk soils and in P_R (32, 8), PE1_R (33, 10), PE2_R (11, 5), PE1E2_R (22, 23) rhizosphere soils. Interestingly, more microbes with growth promoting and symbiotic rhizobacteria (f_Burkholderiaceae, g_Azospirillum, g_Luteibacter, g_Sphingomonas, g_Devosia, s_Bradyrhizobium_japonicum, etc.) and fungi (g_Penicillium, g_Arthrographis, o_Hypocreales, f_Nectriaceae, o_Sebacinales, and g_Papulaspora, etc.), and participating or promoting soil carbon and nitrogen cycling of bacteria (g_Flavobacterium, o_Chitinophagales, g_Cellulomonas, g_Chitinibacter, etc.) and fungi (g_Mucor, g_Sordariomycetes, g_Penicillium, g_Zopfiella, g_Westerdykella, etc.) were enriched in E and PE treatments.



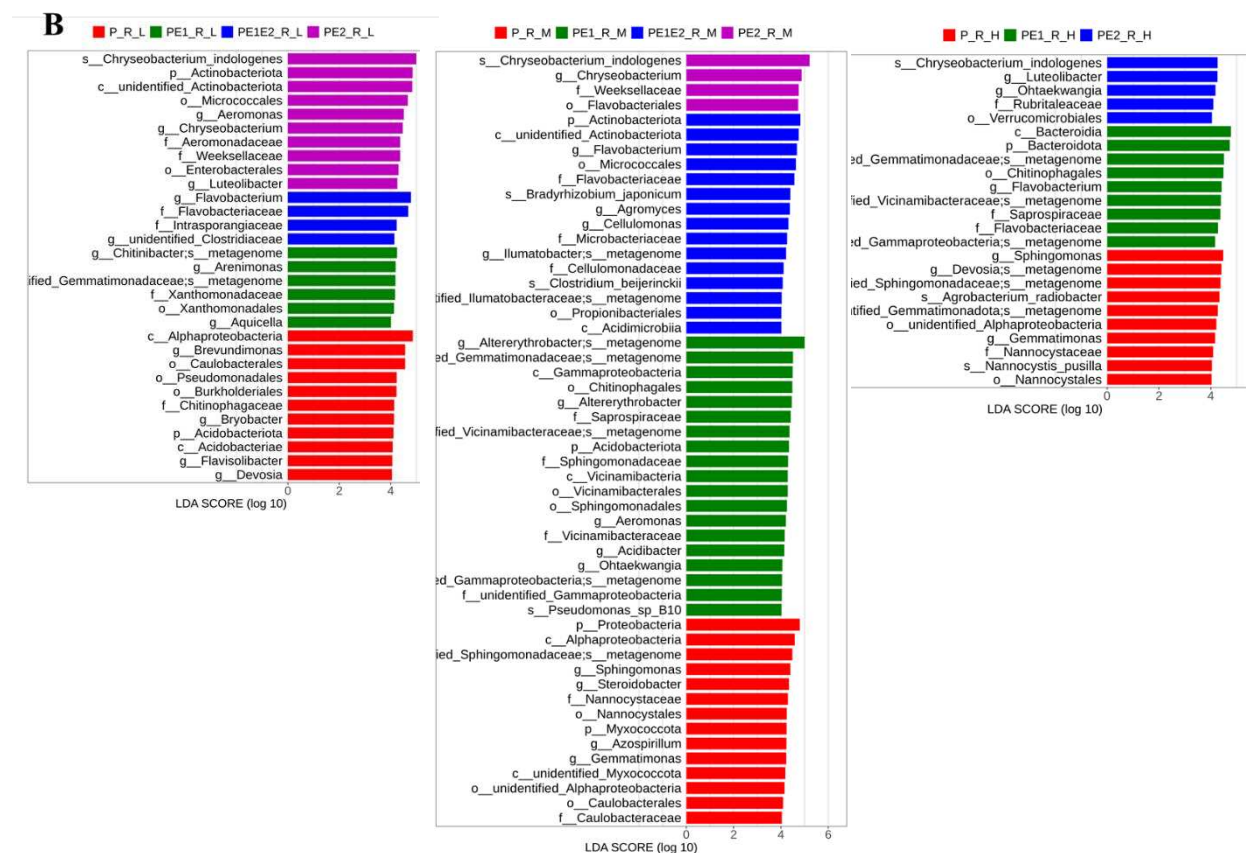


Fig. 4 Liner discriminant analysis (LDA) indicating the differences of bacterial taxa in bulk soil among treatments (A). LDA indicating the differences of bacterial taxa in rhizosphere soil among treatments (B).

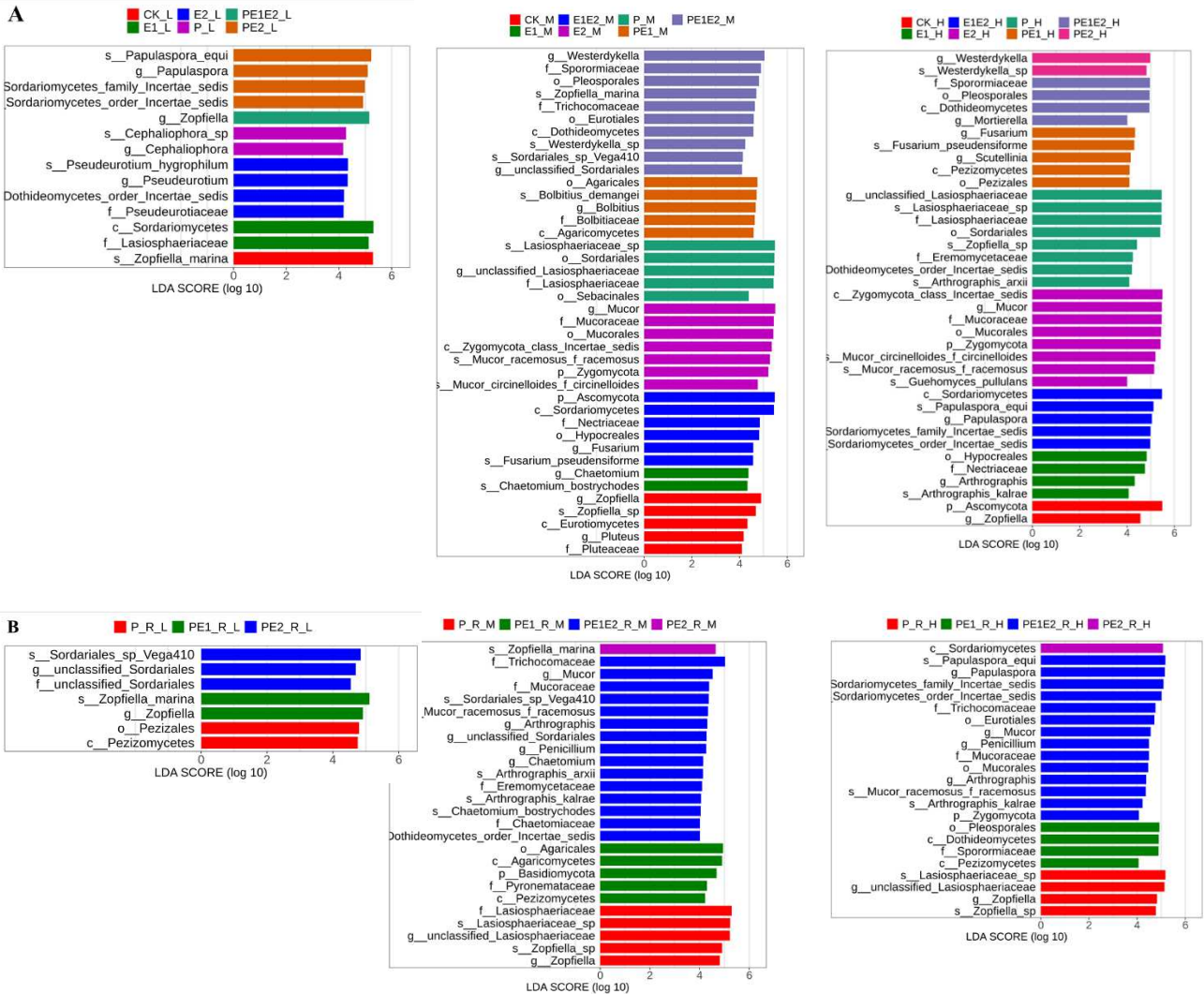


Fig. 5 Liner discriminant analysis (LDA) indicating the differences of fungal taxa in bulk soil among treatments (A). LDA indicating the differences of fungi taxa in rhizosphere soil among treatments (B).

3.4 Relationships between environmental factors and microbial communities with Cd and Zn uptake amount

We constructed structural equation models (SEMs) to explore the direct and indirect relationships among microbial communities in rhizosphere and bulk soils, soil properties, Cd and Zn uptake amount by *S. plumbizincicola* (Fig. 6). Earthworm activity (individual or combined different species) treatment had a positive and indirect effect on Cd and Zn uptake amount through i) positively influencing soil properties or ii) positively influencing the fungal diversity and iii)

negatively influencing the bacterial diversity in in bulk and rhizosphere soils (Fig. 6A). The bacterial and fungal diversity in rhizosphere soils was founded to have the standardized negative effects on Cd uptake amount and Zn uptake amount of *Sedum plumbizincicola* (Fig. 6B). The earthworm species treatment and fungal diversity in bulk soils had positive effects on Zn uptake amount. The soil properties and bacteria diversity in bulk soils were found positive effects Cd uptake amount and Zn uptake amount, and the soil properties had the largest standardized positive effects.

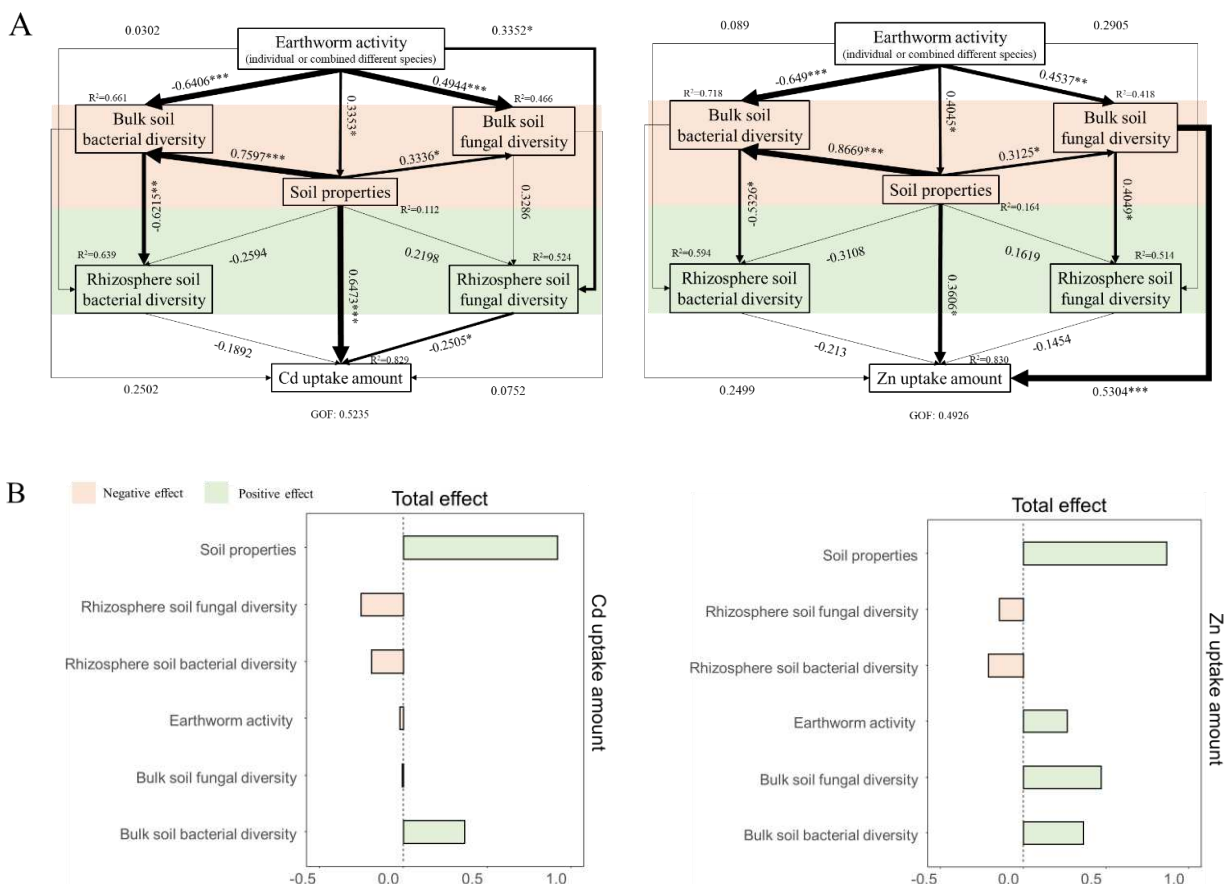


Fig. 6 Structural equation models (SEM) to evaluate the direct and indirect effects of soil properties, microbial communities in rhizosphere and bulk soils on the Cd uptake amount and Zn uptake amount (A). The total standardized effects of multiple factors on the Cd uptake amount and Zn uptake amount (B).

3.5 Soil microbial communities as predictors of Cd and Zn uptake amount of *S. plumbizincicola*

The Cd and Zn uptake amount of *Sedum plumbizincicola* per kilogram soil were selected as indexes to represent the remediation efficiency. Compare with the treatment with only *Sedum plumbizincicola*, inoculation earthworm treatments significantly increased the Cd and Zn uptake amount of *Sedum plumbizincicola* ($p < 0.05$, Chapter III). Random forest regression modelling was conducted to identify the bacterial and fungal taxa in bulk and rhizosphere soils that can predict Cd and Zn uptake amount across different inoculation earthworm species treatments (Fig. 7). The top 15 microorganisms in bulk soils and rhizosphere soils that best predicted the Cd uptake amount and Zn uptake amount of *Sedum plumbizincicola* were shown, and its belonging to a total of 7 bacterial (p_Acidobacteriota, p_Actinobacteriota, p_Proteobacteria, p_Myxococcota, p_Firmicutes, p_unidentified_Bacteria, p_Bacteroidota) and 2 fungal (p_Ascomycota, p_Basidiomycota) phyla were incorporated as the main biological predictors in our models (Fig. 7a, b, c and d). The bacteria *g_Stenotrophobacter* and *g_Aridibacter* (belonging to p_Acidobacteriota), *g_Rubrobacter* and *g_Microthunium* (belonging to p_Actinobacteriota), *g_Defluviicoccus*, *g_Methyloceanibacter* and *g_Skermanella* (belonging to p_Proteobacteria), *g_Desulfotobacterium* (p_Firmicutes), and *g_Haliangium* and *g_Sandaracinus* (belonging to p_Myxococcota), *g_Dehalococcoidia* (p_unidentified bacteria), as well as fungi *g_Arthrographis*, f_Lasiosphaeriaceae, *g_Pseudeurotium*, *g_Fusarium* (belonging to p_Ascomycota) and *g_Piriformospora* (p_Basidiomycota) were the dominant predictors of Cd and Zn uptake amount. In addition, the two unidentified genus of p_Actinobacteriota and p_Acidobacteriota was also the dominant predictors.

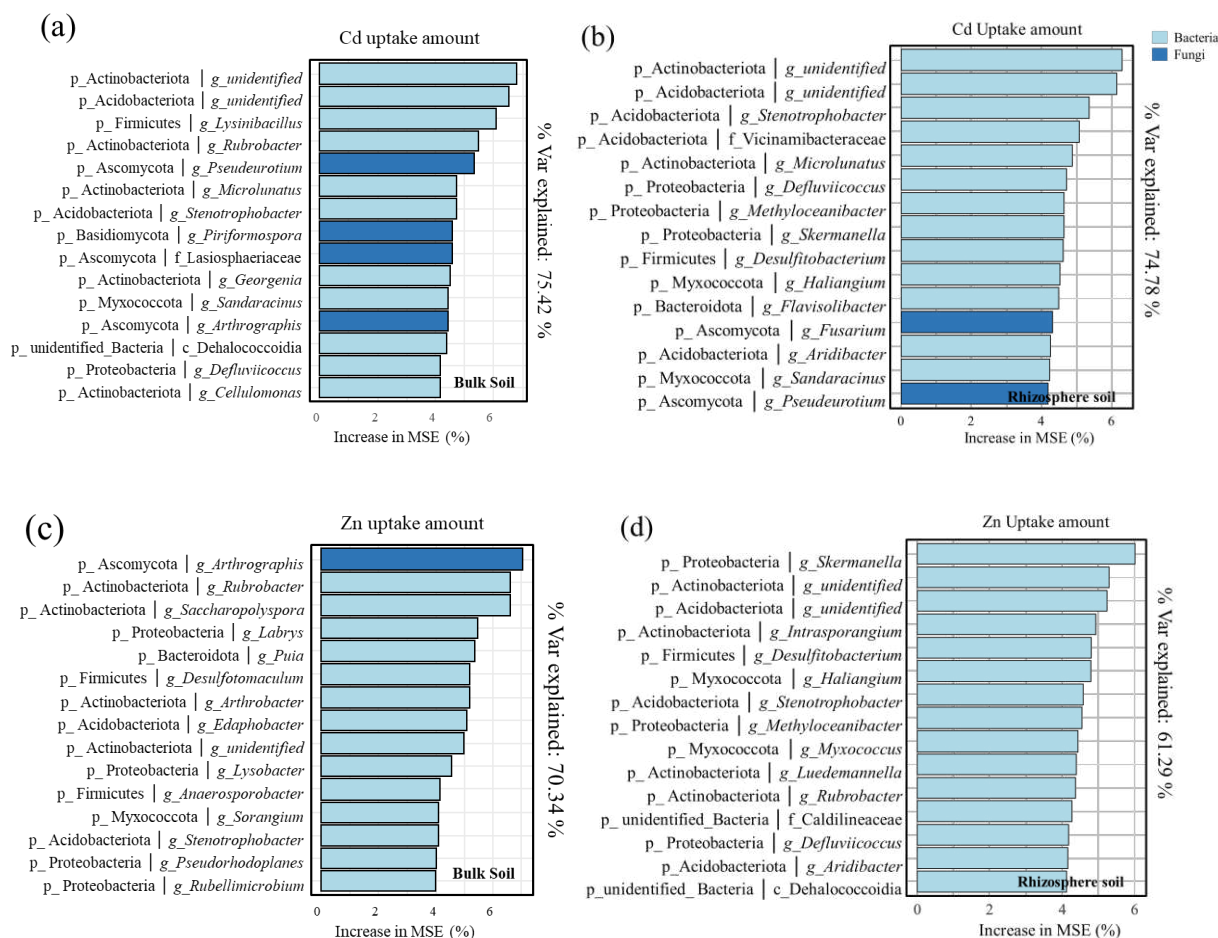


Fig. 7 Random Forest regression model shows the top 15 most important taxa of bacteria and fungi at different level in rhizosphere and bulk soils as key drivers of Cd uptake amount and Zn uptake amount of *Sedum plumbizincicola*. The bars indicate that the increased in mean squared error (Increase in MSE (%)) scores computed for the contribution of microbiome among four *Sedum plumbizincicola* and *Sedum plumbizincicola*+Earthworms treatments (at the genus level) to Cd uptake amount and Zn uptake amount of *Sedum plumbizincicola*.

The clustering heatmap was employed to reveal the characteristics of specific bacteria and fungi species abundance in bulk and rhizosphere soils in L, M and H contaminated soils (Fig. 8). The abundance of the majority specific bacteria and fungi increased with soil contaminated levels, and shows the highest abundance in H soil, such as *f_Lasiosphaeriaceae*, *g_Sandaracinus*, *g_Stenotrophobacter*, *g_Defluviococcus*, *g_Microlunatus*, *c_Dehalococcoidia*, *g_Rubrobacter*, *g_Arthrographis*, *g_Aridibacter*, *g_Skermanella*, *g_Methyloceanibacter*, *g_Haliangium*,

g_Lysinibacillus, *f_Vicinamibacteraceae*, *g_Lysobacter*, *g_Pseudorhodoplanes*, *g_Labrys*, *g_Arthrobacter*, *g_Puia*, *g_Rubellimicrobium*, *f_Caldilineaceae*, *g_Luedemannella*, *g_Intrasporangium*, *g_Piriformospora*, *g_Myxococcus* et al. Additionally, the abundance of some specific microbial decreased with soil contaminated levels, such as *g_Cellulomonas*, *g_Pseudeurotium*, *g_Georgenia*, *g_Desulfotomaculum*, *g_Pseudeurotium*, *g_Desulfitobacterium*, *g_Edaphobacter*, *g_Sorangium*.

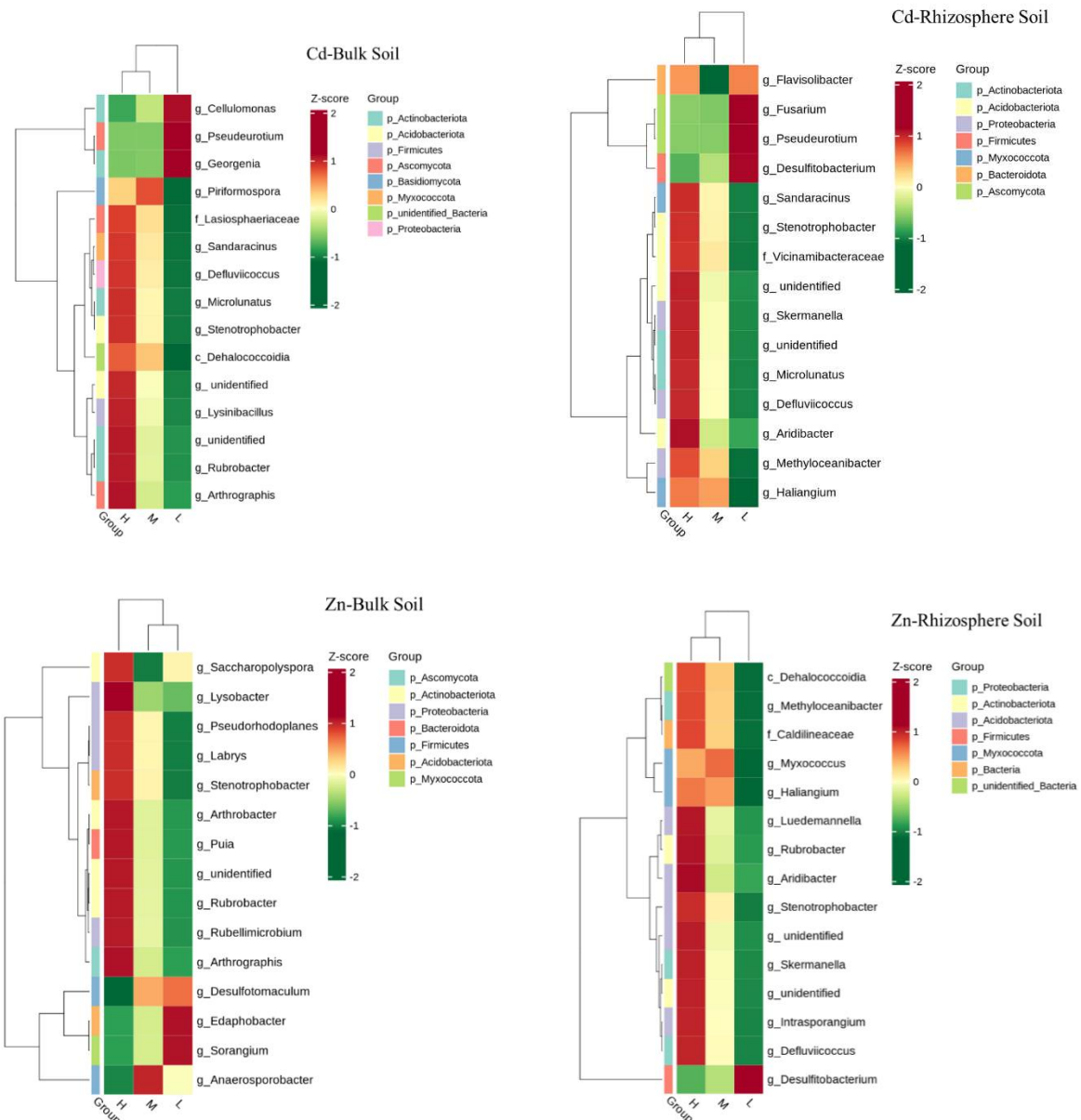


Fig. 8 Specific-species abundance clustering heatmap of bulk and rhizosphere soil bacteria and fungi under different contaminated levels. Horizontal is the soil contaminated levels, vertical is the species classification information, and the clustering tree is the species clustering tree and the contaminated levels clustering tree. The values corresponding to the heatmap are the relative quantitative data standardized by Z-Score. The colors indicate the abundance and read indicated relatively high abundance and green indicated relatively low abundance. The uppercase letters L, M and H represents low contaminated soil, medium contaminated soil and high contaminated soil, respectively. CK, E1, E2, E1E2, P, PE1, PE2 and PE1E2 represents the treatments with control, *A. cortices*, *A. aspergillum*, *A. cortices*+ *A. aspergillum*, *Sedum plumbizincicola*, *Sedum plumbizincicola* + *A. cortices*, *Sedum plumbizincicola* + *A. aspergillum*, *Sedum plumbizincicola* + *A. cortices*+ *A. aspergillum*, respectively.

The clustering heatmap was employed to reveal the characteristics of specific bacteria and fungi species abundance in bulk and rhizosphere soils in different treatments (Fig. 9). In bulk soil (Fig. 9 a and c), the bacteria of *g_Sandaracinus*, *f_Lasiosphaeriaceae*, *c_Dehalococcoidia*, *g_Piriformospora*, *g_Defluviicoccus*, *g_Cellulomonas* in CK and P was higher than in E and PE, and shows the highest abundance in P. However, the other species did not show same trend in P and PE with in CK and E. The highest abundance of *g_Stenotrophobacter* exist in PE2, and low abundance in P, CK and E1E2; The abundance of *g_Arthrographis* and *g_Microlunatus* shows high abundance in E1 and E2 and low abundance in CK and E2, while this trend did not exist in P and PE. The highest abundance of *g_Pesudeurotium* be founded in E2. The abundance of *g_Defluviicoccus* shows high abundance in CK and P and the lowest abundance in PE1, and CK > E, P > PE. The abundance of *g_Rubrobacter* ranked as CK > E2 > E1E2 > E1, PE2 > P > PE1E2 > PE1. In rhizosphere soil (Fig.9 b and d), the abundance of *g_Defluviicoccus*, *g_Stenotrophobacter*, *g_Sandaracinus*, *g_Flavisolibacter*, *g_Haliangium*, *g_Pseudeurotium*, *g_Dehalococcoidia*, *g_Stenotrophobacter*, *g_Haliangium*, *g_Defluviicoccus*, *g_Caldilineaceae* in P_R were higher than in PE_R, while the abundance of *g_Methyloceanibacter*, *g_Skermanella*, *g_Aridibacter*, *g_Intrasporangium*, *g_Rubrobacter* conversely. The abundance of *g_Fusarium*, *f_Vicinamibacteraceae*, *g_Skermanella* and the lowest abundance of *g_Haliangium* and

g_Microlunatus, *g_Luedemannella* exist in PE1_R. The abundance of *g_Rubrobacter*, *g_Aridibacter*, *g_Luedemannella* and the lowest abundance of *g_Myxococcus*, *g_Desulfotobacterium* exist in PE2_R. The abundance of *g_Desulfotobacterium*, *g_Microlunatus*, *g_Myxococcus* and the lowest abundance of *g_Defluviococcus* and *g_Fusarium* exist in PE1E2_R.

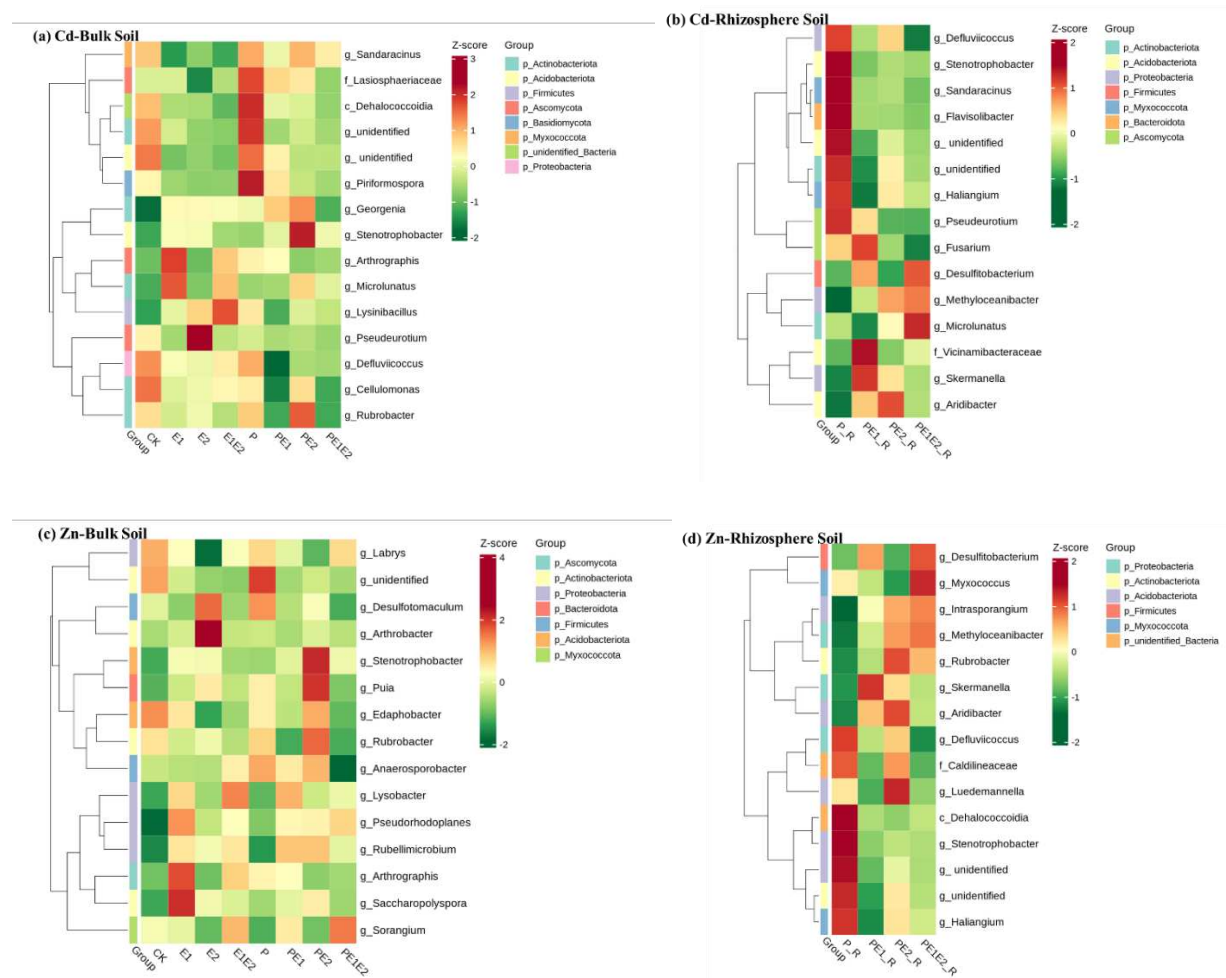


Fig. 9 Species abundance clustering heatmap of bulk and rhizosphere soil bacteria and fungi under different treatments. Horizontal is the different treatments, vertical is the species classification information, and the clustering tree is the species clustering tree and the treatments clustering tree. The values corresponding to the heatmap are the relative quantitative data standardized by Z-Score. The colors indicate the abundance and read indicated relatively high abundance and green indicated relatively low abundance. The uppercase letters L, M and H represents low contaminated soil, medium contaminated soil and high contaminated soil, respectively. CK, E1, E2, E1E2, P, PE1, PE2 and PE1E2 represents the treatments with control, *A. cortices*, *A. aspergillum*, *A. cortices*+ *A.*

aspergillum, *Sedum plumbizincicola*, *Sedum plumbizincicola* + *A. cortices*, *Sedum plumbizincicola* + *A. aspergillum*, *Sedum plumbizincicola* + *A. cortices* + *A. aspergillum*, respectively.

4. Discussion

4.1 Effects of earthworm activities of individual and combined different species on bulk soil microbial community composition and diversity, functions characterization of biomarker taxa

The earthworm activity can reconstruct the soil structure, improve soil organic matter decomposition, and increase available nutrient for microorganisms by digging, feeding, gut digestion, excretions and secretion mucus behaviors, which can directly or indirectly facilitate or inhibit some microbiota growth (van Groenigen et al., 20019; Jia et al., 2024). The earthworm gut is a microbiological filter (Medina-Sauza et al., 2023) and recently research shows that earthworm activities exist legacy effects on soil microbiome by cast, and that effects can last beyond a vegetation period (Yang et al., 2024). During cast aging, the microbiome is strongly influenced by soil environments. In soil-earthworm-*Sedum plumbizincicola* remediation system, the main phyla of bacteria and fungi was Proteobacteria, Actinobacteriota, Bacteroidota, Firmicutes and Ascomycota, it may be shaped by the soil environment, like high metals concentration (Fig.1 a-c, g-i). Chen et al., 2013 found that Proteobacteria was highly enriched in neutral and slightly acidic Pb/Zn tailings. The widespread distribution of Proteobacteria and Actinobacteriota can be attributed primarily to the complexation and adsorption capacities of microorganisms, aiding them in adapting to metal-rich environment (Fig. 1 a, b and c) (Liu et al., 2020; Sibanda et al., 2019; Wu et al., 2022). Moreover, Ascomycota are frequently noted to carry a high number of genes associated with stress tolerance and nutrient acquisition, such as nitrogen immobilization and phosphate transport (Egidi et al., 2019). Nevertheless, the legacy effects also can be found by changes of the relative abundance of dominant phyla and genus in bacteria and fungi by comparing

with or without earthworm treatments (Fig. 1). The phyla Bacteroidota (especially genus *Flavobacterium*) and Actinobacteriota, and genus *Papulaspora* were stimulated by earthworm activities, and the abundance increased dramatically after earthworm activities, which was consistence with some previous studies Yang et al., 2024, Yang et al., 2021 and Sun et al., 2022. The abundance of dominate genus *Bacillus* in earthworm gut and fungi genus *Mucor* (belonging to phyla Zygomycota) preferential feeding by earthworms was strongly increased in soils only inoculation with *A. cortices* and *A. aspergillum*, phyla Proteobacteria, Gemmatimonadota and Ascomycota conversely; it is may due the preference feeding habits of earthworm and the gut transit promoting or facilitating its growth, resulting in high abundance in cast and soil (Dai et al., 2015; Bonkowski and Griffiths and Ritz., 2000); moreover, it may also be active and thrive in burrow walls, which were strongly enriched with organic matter (Tiunov and Dobrovolskaya, 2002). The inhibition of phyla Proteobacteria, Gemmatimonadota, Ascomycota may be caused by inhibiting substance in casts (Nechitaylo et al., 2010; Gómez-Brandón et al., 2011; Takur et al., 2022). However, this performance was not founded in earthworm + *Sedum plumbizincicola* treatment, it may be attributed to the high biodiversity of organisms contributes to soil ecological balance (Jia et al., 2024); it is the synthesis consequences of the different organisms. Furthermore, the soil microbial community of bulk soil in different treatments trend to be more diversity, stable and favorable microbial phylogeny after earthworm activities (Fig. 2 and Fig. 3); it may be attributed to inoculation earthworm can improve the soil environment quality by metabolic activity, supporting microbial communities and enhancing the connectivity of micobial communities between different microhabitats (Jacquiod et al., 2020). In terms of earthworm species, the dominate microbiome in casts were similar among different ecological categories of earthworm (Dai et al., 2015), contributing to similar microbial composition in soils through legacy effects, it

is in line with our results. In addition, the diversity, stability and microbial phylogeny degree of bacteria and fungi were poorer performance in all inoculation without earthworm and with *A. aspergillum* treatment than in all inoculation of *A. cortices* and *A. cortices*+ *A. aspergillum* treatments; it may due to the fact that the earthworm *A. aspergillum* activities greatly influenced on the microbial community structure by strongly changing the micro-environment and organic matter cycling, while the same results in inoculation without earthworm treatments may due to the unstable and high variable of microbial community structure in highly heterogeneous soil environments (Guan et al., 2021). Meanwhile, the microbial community significantly different in *A. aspergillum* treatment with in *A. cortices* and *A. cortices* + *A. aspergillum* treatments (Fig. 3), indicated that the different biological effect of *A. cortices* and *A. cortices*+ *A. aspergillum* with *A. aspergillum* on soil microbial community. The number of bacteria and fungi biomarker was greatly changed among different contaminated soils (Fig. 4A and B, Fig. 5 A and B), indicated that the soil metal contaminated levels have influence on the internal processes and intensities of soil-earthworm-*Sedum plumbizincicola* remediation system. The evidence of growth promoting effect of earthworms on *Sedum plumbizincicola* was founded. In details, in terms of the functions of biomarker taxa, it could be founded that earthworm activity enrich the relevant microbial communities that can promote the soil organic matter decomposition and nutrient cycling. In addition, the growth promoting bacteria and symbiotic rhizobacteria and fungi were active by wild earthworms.

The bacteria and fungi community were significantly influenced by soil contaminated levels (Fig. 2 and Fig. 3). The microbiome can be selected by metal resistance ability under different metal contaminated levels. Due to their ability of metal resistance, the dominant genus of bacteria and fungi was occupied by different microbial taxa with soil contaminated levels (Fig. 1

d-f, j-l), like high relative abundance of genus *Sphingomonas* of bacteria and *unclassified Lasiosphaeriaceae*, *Mucor* and *Papulaspora* of fungi in high contaminated soil, and low relative abundance of genus *Flavobacterium* and *Cellulomonas* of bacteria and genus *Zopfiella* and *Fusarium* of fungi. In addition, the soil microbial diversity slightly increased with soil contaminated levels (Fig. 3), which may be due to metals-resistant microorganisms being stimulated and active in high metals concentration environments, and promoting the occupation of more ecological niches (Guan et al., 2021). The biomarker taxa of bacteria and fungi was increased with soil contaminated levels, indicating the earthworm effects was enhanced under metal stress.

4.2 Effects of earthworm activities of individual and combined different species on rhizosphere soil microbial community composition and diversity, functions characterization of biomarker taxa

Earthworm improve the plant growth in a soil-dependent way, notably via modifications of rhizosphere microbiota and its functions (Ruben and Samuel and Manuel, 2023; Medina-Sauza et al., 2019; Medina-Sauza et al., 2023), and some microbial taxa stimulated by the plant are also stimulated by earthworms (Jacquiod et al., 2020). In totally, the predominant phyla of bacteria and fungi in rhizosphere soil were consistence with that in bulk soil except for the abundance, and the legacy effects also can be found (Fig. 1 a-c and Fig. 2 j-i). While the dominate phyla of bacteria were not consistence with the study of Hou et al., 2021 who identified that the six dominant phyla of bacteria occupied by Actinobacteria, Chloroflexi, Acidobacteria, Alphaproteobacteria, Betaproteobacteria and Firmicutes in the rhizosphere as *Sedum plumbizincicola* growth proceeded under different Cd contaminated levels. Furthermore, in the presence of earthworms, the soil microbial community in rhizosphere soil trend to be higher diversity, stable and favorable microbial phylogeny than that in bulk soil after earthworm activities, especially Fungi (Fig. 2); it

is suggested that earthworm activity affect the microbial habitats, and can makes great effects on soil microbiome in rhizosphere soil, which can also be evidence by SEMs analysis (Fig. 6A). Moreover, the bacteria community compositions in rhizosphere soil became increasingly similar to bulk soil communities, while fungi not. In the presence of earthworms, rhizosphere rise more biomarker taxa of bacteria and fungi especially in mediate and high contaminated soils (Fig. 4 and Fig. 5), the functions mainly contributing to the soil organic matter decomposition, carbon and nitrogen cycling, survival in metal-stress environmental, and indirectly affecting metals transfer, etc.

4.3 The pathway and key microbiota of improving Cd and Zn uptake amounts by *S. plumbizincicola* under earthworm activity

The microbial communities in rhizosphere and bulk soils were closely related to the earthworm activity induced changes in Cd and Zn uptake amounts (Fig. 6). In details, the nutrient cycling and metals transfer processes were improved when cultural *Sedum plumbizincicola* into metals-contaminated soil; furthermore, those processes were further enhanced by earthworm activities through the intensity interactions of soil abiotic and biotic, the frequent microorganism communication between rhizosphere soil and bulk soil, and increasing the metals resistant ability of plant roots, which significantly impacted metals uptake amounts. Additionally, the SEM results showed that the bacterial diversity in different hotspots area plays the vital roles than fungi diversity on Cd and Zn uptake of *Sedum plumbizincicola* (Fig. 6B). In soil-*Sedum plumbizincicola*-earthworm system, the functions of mostly keystone microbiota for improving Cd and Zn uptake by *Sedum plumbizincicola* was focused on highly metal resistance, promoting plant growth or root symbiotic bacteria, and participating organic matter decomposition processes (Fig. 7). The highly metal-tolerance microbiota emergence to increase the resistance on metal-stress and

to help *Sedum plumbizincicola* uptake metals from soil with contaminated degrees, like genus *Methyloceanibacter* and *Rubrobacter* (Fig. 8 and Fig. 9). The key microbiota of enhance Cd and Zn uptake by *Sedum plumbizincicola* changed with soil contaminated levels (Fig. 7-Fig. 9), suggesting that metal stress can change the competition relationship within microbial populations, and can drive the replacement of some non-dominant microorganisms in clean soils by dominant microorganisms with metals resistance (Li et al., 2020). Therefore, the relative low metal tolerance genus *Cellulomonas* and *Anaerosporebacter* was reasonably as keystone microbiota in low contaminated soil. The genus *Pseudoeurotium* was identified as the common dominate genus in casts of earthworms *Eisenia fetida* and *Amyntas robustus* under low soil metals contaminated environments (Cd, 0.7-1.4 mg/kg; Zn, 246-450 mg/kg) (Dai et al., 2015). The relative high metal tolerance genus *Methyloceanibacter* and *Rubrobacter* was reasonably as keystone microbiota in high contaminated soil.

5. Conclusion

This study focuses on the impact of earthworm activity on the composition and structure of soil microbial community and the dominant microbial predictors for Cd and Zn uptake by *S. plumbizincicola* under different contaminated levels. Overall, earthworm activities significantly enhance the abundance of dominant microbial phyla and improve both microbial diversity in contaminated soils. The presence of earthworms promotes an increase in biomarker bacterial and fungal taxa, particularly in rhizospheres at medium and high contamination levels. These taxa contribute to essential soil functions such as organic matter decomposition, carbon and nitrogen cycling, and adaptation to metal-stress environments, which in turn influence the transfer and uptake of heavy metals. Structural equation models further reveal that earthworm activities have positive direct and indirect effects on the uptake of Cd and Zn by *S. plumbizincicola* by modifying

soil properties and microbial diversity in both rhizosphere and bulk soils. The composition of key microbiota that facilitate Cd and Zn uptake changes as contamination levels increase, shifting to microbiota that not only tolerate metal contamination but also promote nutrient cycling in highly contaminated soils. This study underscores the crucial role of earthworms in shaping functional soil microbiota that support nutrient cycling and metal uptake, providing a theoretical basis for leveraging multi-organism interactions for effective soil remediation strategies.

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Supplementary Figures

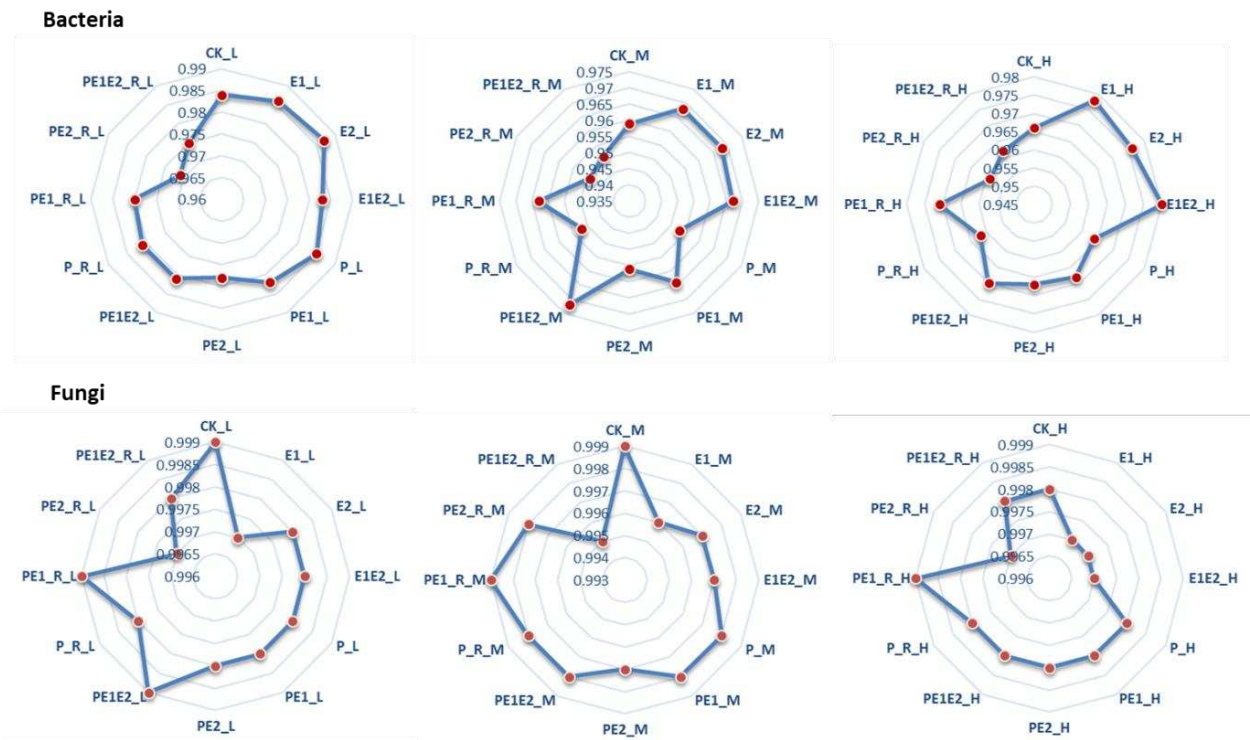


Fig. S1. Good's coverage of bacterial and fungal.

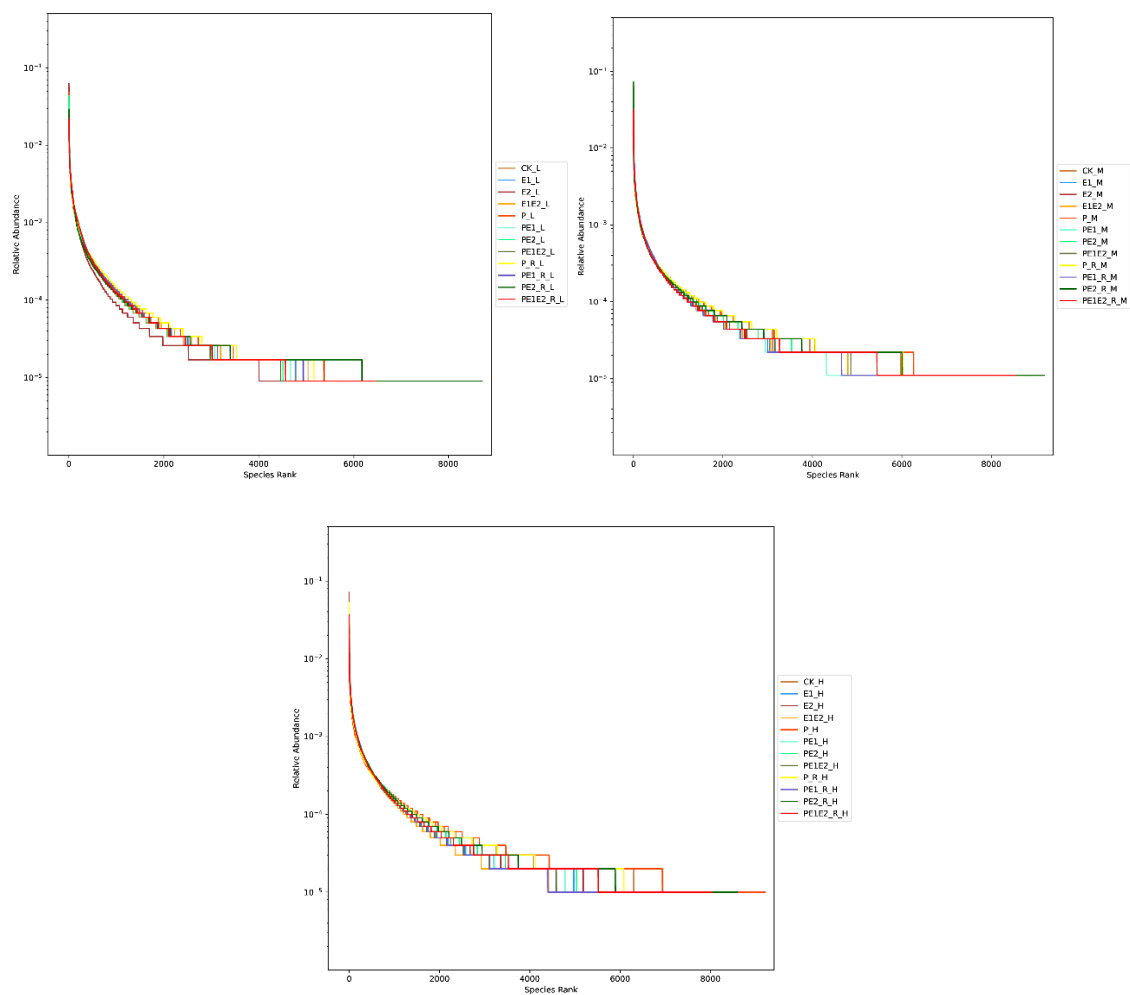


Fig. S2. Rank Abundance curves of bacteria for the bulk and rhizosphere soil samples.

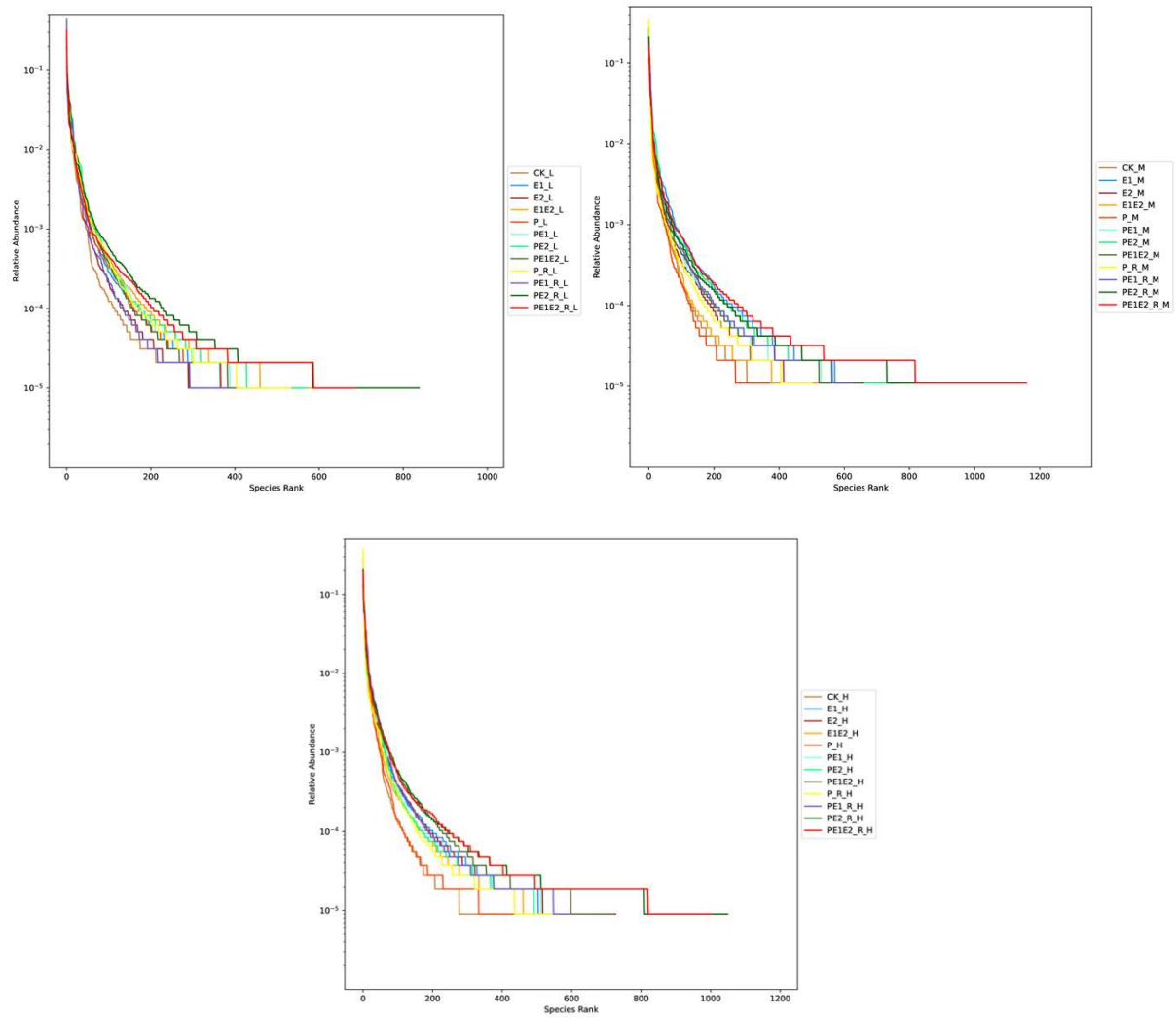


Fig. S3. Rank Abundance curves of fungi for the bulk and rhizosphere soil samples.

CONCLUSION GÉNÉRALE ET PERSPECTIVES

1. Conclusion générale

Grâce à des expériences en pot dans des conditions de laboratoire, le potentiel de bioaugmentation de *Sedum plumbizincicola* agissant avec différentes espèces de vers de terre sauvages a été évalué à différents niveaux de contamination ; en outre, les mécanismes sous-jacents ont été révélés en termes de communautés microbiennes du sol. Nous avons émis l'hypothèse que (i) les vers de terre pourraient favoriser la croissance de *S. plumbizincicola* et améliorer l'efficacité de la phytoextraction des métaux, sous l'influence de la préférence pour l'injection, des habitudes de creusement et des stratégies de survie des différentes catégories écologiques de vers de terre, et du stress lié aux métaux. (ii) l'effet de plusieurs espèces de vers de terre est différent de celui d'une seule espèce de ver de terre sur l'efficacité de la phytoremédiation. (iii) les vers de terre peuvent modifier le microbiote du sol et l'abondance microbienne associée à la résistance aux métaux et au cycle des nutriments, en aidant *S. plumbizincicola* dans des conditions de stress métallique et de stérilité. Les résultats du chapitre II ont démontré que les vers de terre sauvages peuvent favoriser la croissance de *S. plumbizincicola* et montrer des effets d'augmentation spécifiques à l'espèce. Dans les chapitres III et IV, nous avons constaté que les effets de bioaugmentation des espèces de vers de terre combinées étaient similaires ou légèrement inférieurs à ceux des espèces individuelles. Les communautés de bactéries et de champignons ont été fortement influencées par l'activité des vers de terre, jouant un rôle vital dans la transformation des métaux. Le microbiote clé favorisant l'absorption du Cd et du Zn par *S. plumbizincicola* a changé avec les niveaux de contamination du sol, passant à un microbiote dont les fonctions contribuent principalement aux cycles biogéochimiques et à la résistance aux métaux dans les niveaux fortement contaminés.

(1) Effets de bioaugmentation de trois catégories écologiques de vers de terre sauvages sur la phytoextraction par *S. plumbizincicola* dans des sols contaminés par le Cd et le Zn dans le sud de la Chine

En établissant le système sol minier contaminé - *S.plumbizincicola* - vers de terre sauvages, nous avons démontré (chapitre II) que l'introduction de vers de terre favorisait de manière significative la croissance vigoureuse de *Sedum plumbizincicola*, améliorant à la fois la robustesse de la plante et l'efficacité de la phytoextraction du sol. Les quantités de Cd et de Zn absorbées se sont révélées significativement plus élevées dans l'inoculation de *S. plumbizincicola* avec les vers de terre épigés *A. cortices*, anéciques *A. aspergillum* et *M. guillelm* que dans l'inoculation avec l'endogé *A. robustus* et la seule culture de *S. plumbizincicola*. En outre, les différentes espèces de vers de terre (*A. aspergillum* et *M. guillelm*) appartenant à la même catégorie écologique ont eu des effets différents sur la biodisponibilité des métaux dans les zones sensibles (rhizosphère, paroi du terrier et moulages). Il existe un compromis entre la minéralisation des nutriments et la résistance au stress dans les différentes catégories écologiques. Nous avons révélé que les facteurs dominants pour améliorer l'efficacité de la phytoremédiation étaient la biomasse aérienne, suivie des facteurs de transfert et du phosphore disponible, puis de l'azote alcali-hydrolysable.

(2) Effet de différentes espèces de vers de terre sauvages individuelles et combinées sur le potentiel de phytoextraction de *S. plumbizincicola*, et réponses des communautés microbiennes du sol

Sur la base des résultats du chapitre II, *A. cortices* épigée et *A. aspergillum* anécique ont été sélectionnés pour des tests supplémentaires, à la fois individuellement et en combinaison, dans le système *Sedum plumbizincicola* afin d'explorer les effets de plusieurs espèces de vers de terre

sur l'efficacité de la phytoremédiation et de révéler les mécanismes sous-jacents permettant d'optimiser les processus de biorémédiation. Dans l'ensemble, la combinaison d'espèces de vers de terre avec *S. plumbizincicola* facilite la survie d'*A. cortices* et d'*A. aspergillum*. La biomasse de *S. plumbizincicola* montre une augmentation stable sous l'action des vers de terre. En détail, la biomasse des tissus aériens et des racines a montré une augmentation stable chez *S. plumbizincicola* inoculé avec *A. cortices*, *A. aspergillum*, et la combinaison des deux, atteignant 0,17-3,68 fois et 1,36-4,64 fois plus élevés que ceux de *S. plumbizincicola* seul. En termes d'espèces de vers de terre, la biomasse des différents tissus et le facteur de transfert dans *S. plumbizincicola* + *A. cortices* + *A. aspergillum* étaient similaires ou légèrement inférieurs à ceux de *S. plumbizincicola* + *A. cortices* et *S. plumbizincicola* + *A. aspergillum*. Un effet de dilution marqué a été observé dans différents tissus à un niveau de contamination élevé, en particulier à des niveaux de contamination moyens et élevés. Néanmoins, l'activité des vers de terre a encore amélioré l'efficacité de la phytoremédiation. Les quantités d'absorption de Cd et de Zn ont augmenté jusqu'à 435%, 540%, 315%, et 167%, 207%, 130% dans *S. plumbizincicola* + *A. cortices* + *A. aspergillum*, *S. plumbizincicola* + *A. cortices*, et *S. plumbizincicola* + *A. aspergillum*, respectivement. Les quantités les plus élevées d'absorption de Cd et de Zn ont atteint $124 \pm 10,3$ µg et $5,09 \pm 0,34$ mg par 1,5 kg de sol dans *S. plumbizincicola* + *A. aspergillum* et *S. plumbizincicola* + *A. cortices*, respectivement. La mobilité et la biodisponibilité des métaux du sol ont augmenté sous l'action des vers de terre avec le temps de culture, facilitant le transport des métaux du sol vers la solution du sol et finalement vers les racines des plantes. Les vers de terre ont affecté l'absorption du Cd et du Zn par *S. plumbizincicola* en améliorant l'environnement du sol, la mobilité et la biodisponibilité des métaux, ainsi qu'en renforçant les interactions avec la communauté microbienne du sol dans les sols en vrac et dans la rhizosphère.

En outre, la communauté microbienne des sols en vrac et de la rhizosphère a été étudiée pour comprendre l'impact de l'activité des vers de terre sur la composition et la structure microbiennes, et pour identifier les prédictors microbiens clés de l'absorption du Cd et du Zn par *S. plumbizincicola* à différents niveaux de contamination. Nous avons constaté que l'activité des vers de terre augmentait de manière significative l'abondance des phyla dominants et l' α et la β diversité microbienne. En présence de vers de terre, davantage de taxons biomarqueurs de bactéries et de champignons ont augmenté dans la rhizosphère, en particulier à des niveaux de contamination moyens et élevés, les fonctions contribuant principalement à la décomposition de la matière organique du sol, au cycle du carbone et de l'azote, à la survie dans un environnement soumis à un stress métallique, et affectant indirectement le transfert de métaux, etc. Les modèles d'équation structurelle ont révélé que les activités des vers de terre avaient un effet positif et (in)direct sur la quantité de Cd et de Zn absorbée par *Sedum plumbizincicola* en influençant les propriétés du sol et la diversité microbienne dans la rhizosphère et les sols en vrac. Le microbiote clé de l'amélioration de l'absorption du Cd et du Zn par *S. plumbizincicola* a changé avec les niveaux de contamination du sol. En détail, les genres dominants *Fusarium*, *Desulfotobacterium*, *Pseudeurotium*, *Cellulomonas*, *Georgenia*, *Edaphobacter*, *Anaerosporeobacter*, *Sorangium* ont été remplacés par les genres *Defluviicoccus*, *Methyloceanibacter*, *Microlunatus*, *Skermanella*, *Aridibacter*, *Myxococcus*, *Intrasporangium*, *Rubrobacter*, *Defluviicoccus*, *Stenotrophobacter*, *Puia*, et la famille Vicinamibacteraceae et Caldilineaceae, dans les sols fortement contaminés par les métaux. En résumé, les vers de terre participent à la réponse à l'absorption de Cd et de Zn par *S. plumbizincicola*, en façonnant le microbiote fonctionnel du sol, en particulier ceux qui sont liés au cycle des nutriments. Cette étude fournit une base théorique pour comprendre les effets combinés et le potentiel de plusieurs organismes dans la remédiation des sols.

2. Perspectives

Dans la suite de la recherche, il serait intéressant de se concentrer sur : (1) une analyse approfondie des mécanismes sous-jacents permettant d'améliorer la phytoextraction d'un point de vue métabolomique ; (2) les performances en cours d'utilisation de la phytoremédiation assistée par bioaugmentation.

(1) Analyse approfondie des mécanismes sous-jacents de l'amélioration de la phytoextraction du point de vue de la métabolomique

Cette étude a démontré que l'amélioration de l'extraction des métaux par *Sedum plumbizincicola* est significativement influencée par des processus liés au microbiote. Le microbiote dominant dans les sols en vrac et dans la rhizosphère, affecté par l'activité des vers de terre, joue un rôle crucial dans la mobilisation des métaux et l'absorption par les racines. Les mécanismes sous-jacents sont probablement liés au mucus et aux excréments des vers de terre, qui interagissent avec les sécrétions extracellulaires microbiennes, les exsudats racinaires et les métaux présents dans le sol. Ces interactions ont un impact direct sur la biodisponibilité des métaux et sur la facilité avec laquelle les métaux sont absorbés par les racines des plantes. Les métabolites peuvent aider à comprendre les processus biologiques et leurs mécanismes de manière plus intuitive et plus efficace. À l'avenir, une exploration plus approfondie de ces mécanismes à l'aide d'approches métabolomiques permettra de comprendre comment ces interactions biologiques peuvent être exploitées pour améliorer l'efficacité de la phytoextraction dans les environnements contaminés.

(2) Performance in situ de la phytoremédiation assistée par bioaugmentation

Au cours des expériences en pots, les effets de l'augmentation n'ont pas toujours eu une bonne performance, ce qui peut être attribué aux dommages causés aux racines. Il est donc essentiel d'optimiser la densité des vers de terre à des niveaux appropriés pour maintenir l'équilibre entre l'aération du sol, le cycle des nutriments et la mobilisation des métaux sans avoir d'impact négatif sur l'écosystème du sol. En outre, le but ultime de la phytoremédiation assistée par bioaugmentation est d'appliquer cette technologie à des sols réellement contaminés dans des environnements naturels. Comme nous le savons, les effets de bioaugmentation des vers de terre sauvages peuvent montrer des performances différentes sur le terrain et en laboratoire. En outre, les conditions de terrain sont plus propices à la croissance et à la survie des vers de terre sauvages. Il est possible qu'il puisse augmenter davantage l'absorption de Cd et de Zn par *S. plumbizincicola*. Il est donc essentiel d'appliquer cette technologie de remédiation assistée par bioaugmentation sur le terrain.

GENERAL CONCLUSION & PERSPECTIVES

1. General conclusion

Through pot experiments under the laboratory conditions, the bioaugmentation potential of *Sedum plumbizincicola* acting with different species of wild earthworms was assessed under different contaminated levels; moreover, the underlying mechanisms were revealed in terms of soil microbial communities. We hypothesized that (i) earthworm could promote the growth of *S. plumbizincicola* and enhance metal phytoextraction efficiency, influenced by the injection preference and digging habits and survival strategies of different ecological categories of earthworms, and metal stress. (ii) the effect of multiple earthworm species is different from that of single earthworm species on phytoremediation efficiency. (iii) earthworms can modify the soil microbiota and microbial abundance associated with metal resistance and nutrient cycling, assisting *S. plumbizincicola* in metals-stress and unfertile conditions. The results of chapter II demonstrated that wild earthworms can promote the growth of *S. plumbizincicola* and show species-specific augmentation effects. In chapter III and IV, we found that the bioaugmentation effects of combined earthworm species were similar to, or slightly lower than, those of individual species. Bacteria and fungi communities were strongly influenced by earthworm activity, playing a vital role in metal transformation. The key microbiota enhancing Cd and Zn uptake by *S. plumbizincicola* changed with soil contaminated levels, shifting to microbiota with functions primarily contributing to biogeochemistry cycles and metal resistance in highly contaminated levels.

(1) Bioaugmentation effects of three ecological categories of wild earthworms on phytoextraction by *S. plumbizincicola* in Cd and Zn contaminated soils of South China

By establishing the mining contaminated soil - *S. plumbizincicola* - wild earthworm system, we demonstrated (Chapter II) that the introduction of earthworms significantly promoted the vigorous growth of *Sedum plumbizincicola*, enhancing both plant robustness and soil phytoextraction efficiency. Cd and Zn uptake amount shown significantly high in inoculation *S. plumbizincicola* with earthworm epigeic *A. cortices*, anecic *A. aspergillum* and *M. guillelm* than inoculation with endogeic *A. robustus* and only cultural *S. plumbizincicola*. In addition, the different earthworm species (*A. aspergillum* and *M. guillelm*) belonging to same ecological category exhibited different effects on metal bioavailability in hotspots areas (rhizosphere, burrow wall and casts). There was a trade-off between nutrient mineralization and stress resistance among different ecological categories. We revealed that the dominant factors for improving phytoremediation efficiency were the aboveground biomass, followed by the transfer factors and available phosphorus, and then alkali-hydrolysable nitrogen.

(2) Effect of individual and combined different wild earthworm species on the phytoextraction potential of *S. plumbizincicola*, and responses of soil microbial communities

Based on the finding of Chapter II, epigeic *A. cortices* and anecic *A. aspergillum* were selected for further testing, both individually and in combination, within the *Sedum plumbizincicola* system to explore the effects of multiple earthworm species on phytoremediation efficiency and reveal the underlying mechanisms to optimize bioremediation processes. In totally, combining earthworm species with *S. plumbizincicola* facilitate the survival of *A. cortices* and *A. aspergillum*. The biomass of *S. plumbizincicola* show stable increase under earthworm activities.

In details, the biomass of aboveground tissues and roots showed a stable increase in *S. plumbizincicola* inoculated with *A. cortices*, *A. aspergillum*, and combination of both, reaching 0.17-3.68 folds and 1.36-4.64 folds higher than those in *S. plumbizincicola* alone. In terms of earthworm species, the biomass of different tissues and transfer factor in *S. plumbizincicola* + *A. cortices* + *A. aspergillum* were similar to or lightly lower than those in *S. plumbizincicola* + *A. cortices* and *S. plumbizincicola* + *A. aspergillum*. A marked dilute effect was observed in different tissues in high contaminated level, particular in medium and high contaminated levels. Nevertheless, earthworm activity still improved the phytoremediation efficiency. Cd and Zn uptake amounts increased by up to 435%, 540%, 315%, and 167%, 207%, 130% in *S. plumbizincicola* + *A. cortices* + *A. aspergillum*, *S. plumbizincicola* + *A. cortices*, and *S. plumbizincicola* + *A. aspergillum*, respectively. The highest Cd and Zn uptake amounts reached $124 \pm 10.3 \mu\text{g}$ and $5.09 \pm 0.34 \text{ mg}$ per 1.5 kg soil in *S. plumbizincicola* + *A. aspergillum* and *S. plumbizincicola* + *A. cortices*, respectively. Soil metal mobility and bioavailability increased under earthworm activity with cultivation time, facilitating metal transport from soil to soil solution and ultimately to plant roots. Earthworms affected Cd and Zn uptake by *S. plumbizincicola* by improving the soil environment, metal mobility and bioavailability, as well as enhancing the interactions with the soil microbial community in bulk and rhizosphere soils.

Furthermore, the microbial community in both bulk and rhizosphere soils was conducted to understand the impact of earthworm activity on microbial composition and structure, and to identify key microbial predictors of Cd and Zn uptake by *S. plumbizincicola* at varying contamination levels. We found that earthworm activities significantly increased the abundance of the dominate phyla and the microbial α and β diversity. In the presence of earthworms, more biomarker taxa of bacteria and fungi increased in the rhizosphere, especially in medium and high

contamination levels, the functions mainly contributing to the soil organic matter decomposition, carbon and nitrogen cycling, survival in metal-stress environmental, and indirectly affecting metals transfer, etc. Structural equation models revealed that earthworm activities had a positive and (in)direct effect on Cd and Zn uptake amount of *Sedum plumbizincicola* through influencing the soil properties and the microbial diversity in rhizosphere and bulk soils. The key microbiota of enhance Cd and Zn uptake by *S. plumbizincicola* changed with soil contaminated levels. In details, the dominant genus *Fusarium*, *Desulfitobacterium*, *Pseudeurotium*, *Cellulomonas*, *Georgenia*, *Edaphobacter*, *Anaerosporobater*, *Sorangium* were replaced by genus *Defluviicoccus*, *Methyloceanibacter*, *Microlunatus*, *Skermanella*, *Aridibacter*, *Myxococcus*, *Intrasporangium*, *Rubrobacter*, *Defluviicoccus*, *Stenotrophobacter*, *Puia*, and family Vicinamibacteraceae and Caldilineaceae, in high metals contaminated soils. In summary, earthworm take part in the response to Cd and Zn uptake by *S. plumbizincicola*, by shaping soil functional microbiota, especially those related to nutrient cycling. This study provides a theoretical foundation for understanding the combined effects and potential of multiple organisms in soil remediation.

2. Perspectives

In the further research, it would be interesting to focus on: (1) an in-depth analysis of the underlying mechanisms for enhancing phytoextraction from a metabolomics perspective; (2) the in suit performance of bioaugmentation-assisted phytoremediation.

(1) In-depth analysis of the underlying mechanisms for enhancing phytoextraction from a metabolomics perspective

This study has demonstrated that the enhancement of metal extraction by *Sedum plumbizincicola* is significantly influenced by processes related to microbiota. The dominant

microbiota in bulk and rhizosphere soils, affected by earthworm activity, play a crucial role in metal mobilization and root uptake. The underlying mechanisms are likely linked to earthworm mucus and excreta, which interact with microbial extracellular secretions, root exudates, and metals in the soil. These interactions directly impact metal bioavailability and the ease with which metals are absorbed by plant roots. Metabolites can help to understand the biological processes and their mechanisms more intuitively and effectively. Moving forward, a deeper exploration of these mechanisms using metabolomic approaches will provide insights into how these biological interactions can be further leveraged to improve the efficiency of phytoextraction in contaminated environments.

(2) In situ performance of bioaugmentation-assisted phytoremediation

During the pot experiments, the augmentation effects did not always have a good performance, which may be attributed to root damage. Hence, optimizing earthworm density to appropriate levels is essential for maintaining the balance between soil aeration, nutrient cycling, and metal mobilization without negatively impacting the soil ecosystem. Moreover, the ultimate goal of bioaugmentation-assisted phytoremediation is to apply this technology to real contaminated soils in natural environments. As we know, the bioaugmentation effects of wild earthworms may show different performance in field condition with in laboratory condition. In addition, the field condition more fit for wild earthworm growth and survival. Maybe it can make a greater augmentation effects on Cd and Zn uptake by *S. plumbizincicola*. Therefore, it is critical to apply this bioaugmentation-assisted remediation technology to fields.

Annexes

Published Papers

- [1] **Jia, L.**, Liu, Q., Chen, S., Liu, K., Chen, Y., Motelica-Heino, M., Zhong, H., Zhang, M., Tibihenda, C., Lavelle, P., Dai, J., Zhang, C. (2024). Individual and combined effects of earthworm and *Sphingobacterium* sp. on soil organic C, N forms and enzyme activities in non-contaminated and Cd-contaminated soil. *European Journal of Soil Biology*, 120, 103576. DOI: 10.1016/J.EJSOBI.2023.103576.
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Conferences

- [1] Soil Biology and Soil Health (Hangzhou City, Zhejiang Province, China), 04.2023, Zhejiang Province, China.
- [2] 12e Symposium International sur l'Ecologie des Vers de Terre. 06.2022, Rennes, France.
- [3] 100 years of Soil Science, 05. 2024, Florence, Italy. Best poster.



Original article

Individual and combined effects of earthworms and *Sphingobacterium* sp. on soil organic C, N forms and enzyme activities in non-contaminated and Cd-contaminated soil

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ARTICLE INFO

Handling editor: Céline Pelosi

Keywords:

Earthworm

Sphingobacterium sp.

Cd-contaminated soil

Carbon and nitrogen forms

Enzyme activities

ABSTRACT

Earthworms and *Sphingobacterium* sp. are known for their strong organic compound decomposition ability and wide distribution in soil. However, interactions of soil organic matter decomposition with soil properties and whether microbial species such as *Sphingobacterium* sp. could assist earthworms in carbon and nitrogen transformation in soil remain poorly understood. Earthworms (*Eisenia fetida*, *Amyntas gracilis*) and *Sphingobacterium* sp. were introduced in non-contaminated and cadmium-contaminated soils under controlled laboratory conditions for 20 days. We examined their individual or combined effects on carbon and nitrogen forms and related enzyme activities to assess their influence on soil C and N cycling. Individual *Sphingobacterium* sp. inoculation led to significantly decreased organic carbon (SOC) contents, reducing it by 16.5% in non-contaminated soil and by 3.77%, in Cd-contaminated soil. It resulted in an increased microbial biomass carbon (MBC) contents, reaching $1685 \pm 292 \text{ mg} \cdot \text{kg}^{-1}$ in non-contaminated soil. Individual introductions of *E. fetida* and *A. gracilis* caused a decline in SOC content in non-contaminated soil, but increased significantly dissolved organic carbon (DOC) and alkali-hydrolysable nitrogen (AN) contents by 75.8%, 53.6% and 32.9%, 20.9%, respectively. In contrast, in Cd-contaminated soil, only the significant combined effects of earthworms and *Sphingobacterium* sp. were linked to significant increase in SOC contents, raising by 7.22% and 9.64% in *E. fetida* + *Sphingobacterium* sp. and *A. gracilis* + *Sphingobacterium* sp. treatments, respectively. In non-contaminated soil, the combined effects of earthworm and *Sphingobacterium* sp. further increased DOC and AN content by 212%, 134% and 31.3%, 25.4% in the treatments of *E. fetida* + *Sphingobacterium* sp. and *A. gracilis* + *Sphingobacterium* sp., respectively; the highest ratios of DOC to SOC and AN to total Nitrogen (TN) were found in the earthworm + *Sphingobacterium* sp. treatments as well. In non-contaminated soil, *Sphingobacterium* sp. and earthworms mainly influenced β -glucosidase (BG), urease (URE), N-acetyl- β -D-glucosaminidase (NAG) activities and fluorescein diacetate hydrolysis (FDA) hydrolysis, while in Cd-contaminated soil, they mainly influenced invertase (INV), NAG, URE, and protease (PRO) activities. Principal component analysis indicated that in non-contaminated soil, the earthworm activities dominated the mineralization processes of soil carbon and nitrogen, and *Sphingobacterium* sp. can intensify this process when it was inoculated in soil along with earthworms. Furthermore, both earthworm species increased C and N levels by elevated INV and PRO activities in combined inoculation. However, in contaminated soil, the impact of earthworm inoculation on soil C stabilization showed a species dependent pattern. *E. fetida* reduced C mineralization by decreasing URE activities, while *A. gracilis* enhanced C stabilization by increasing INV activities and decreasing PRO activities. In conclusion, earthworms played a key role in enhancing C and N mineralization in non-contaminated soil and promoting C stabilization in contaminated soil. Both earthworm species followed similar strategies in the former process but adopted different strategies in the latter. When introduced individually, *Sphingobacterium* sp. was able to promote mineralization in both soils,

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primarily assisting earthworms in improving carbon and nitrogen mineralization in non-contaminated soil but hindering these processes in Cd-contaminated soil. These findings provide insights into the combined effects of earthworms and microorganisms on carbon and nitrogen cycling.

1. Introduction

Soil serves as both an important source and sink for terrestrial carbon and nitrogen in the biosphere. Various soil organisms play essential roles in soil carbon and nitrogen cycles [1,2]. The interactive processes involving soil organisms and restoration of soil carbon and nitrogen stocks in sustainable soil management have garnered widespread attention [3,4]. However, soil metal pollution is increasingly emerging as a serious environmental and social concern, potentially resulting in changes in the interactions among soil organisms and affecting soil carbon and nitrogen cycles [5]. It is of great ecological importance to understand the interactive relationships among soil organisms in the context of the carbon and nitrogen cycle, particularly under non-contaminated and metal-contamination stress.

As "soil ecosystem engineers", earthworm can degrade soil organic matter and significantly influence the forms and contents of soil carbon and nitrogen [6]. Earthworm's preferential feeding and concentration processes have a substantial impact soil carbon and nitrogen cycling [7]. Their bioturbation activities, including burrowing, feeding, and excretion (casts and surface mucus), affects the soil carbon and nitrogen distribution within soil profile [8–11]. These effects result from complex mutualism interactions between earthworms and microorganisms [7,12,13]. Previous studies have established connections between earthworms and rhizosphere growth-promoting bacteria or specific arbuscular mycorrhizal fungi to enhance nutrient availability or nitrogen storage in soil [14,15]. Moreover, the collaboration of indigenous earthworms and gut bacteria has been employed to remediate metal contaminated soil and has shown superiority compared to chemical amendments [16]. Likewise, combining earthworms with dominate gut bacteria taxa to enhance organic matter decomposition is also advantageous, because it is more readily accepted by earthworms. In our previous studies, *Sphingobacterium* sp. was identified as a dominant bacterial taxon in the earthworm gut [17]. *Sphingobacterium* sp., a gram-negative bacterium, exhibits a wide range of nutrient sources, high adaptability, and a broad ecological distribution [18–20]. In practice, it has been used to decompose agricultural waste and degrade organic pollutants (e.g. alkanes and polycyclic aromatic hydrocarbons) in soil [21–26]. Therefore, this decomposition ability of earthworms probably derives from symbiotic microorganisms in their guts. Considering that earthworms and *Sphingobacterium* sp. impact organic matter decomposition and the latter was dominant bacterial taxon in earthworm gut, a synergistic relationship may exist, enhancing soil organic matter decomposition.

However, under environment stress, such as metal contamination, the activities and complex interactions of them may be suppressed. Specifically, metal contamination can induce biomass loss, reduce survival rate, cause irreversible physiological damage, decrease the stability of gut bacterial network, and dampen its ecological functions [27–29]. Similarly, the inhibition effects also impact soil microorganisms, leading to reduced soil enzyme functions critical for organic matter cycling [30]. In addition, different earthworm species and ecological categories have varying effects on soil organic matter degradation and soil C and N cycles [31]. The species of the experimental model earthworm *E. fetida* and local wild epigeic species may have different survival strategies that affect microbial biomass or activities, resulting in their different performances in soil carbon and nitrogen cycles in non-contaminated and Cd-contaminated soil.

Soil C and N cycles involve a series of biological and biochemical processes. Soil carbon and nitrogen forms, dissolved organic carbon (DOC), alkali-hydrolysable nitrogen (AN), microbial biomass carbon and nitrogen (MBC and MBN) have been considered as the key

indicators for assessing the status of the soil carbon and nitrogen cycles. The ratios of these forms can further provide a more intuitive representation of the overall mineralization or sequestration of carbon and nitrogen. Besides, soil enzymes are metabolic drivers for soil organisms, and their activity can, to some extent, reflect the changes in the soil microbial community [32], and the intensity of soil organic matter decomposition and nutrient transformation [33]. Studies on the influence of enzymes on carbon and nitrogen transformation have mainly focused on β -glucosidases (BG) and invertase (INV), which are involved in the decomposition of labile cellulose, sucrose, and carbohydrate polymers. N-acetyl-glucosaminidase (NAG), urease (URE), and proteases (PRO) are involved in the hydrolysis of chitooligosaccharide, urea and protein. These enzymes mainly participate in terminal catalytic reactions or intra-systemic enzymatic reactions, which can reflect the level of carbon and nitrogen metabolism in soils [34]. Furthermore, fluorescein diacetate hydrolysis (FDA) is an enzyme that can indicate the total microbial activity in the soil [35]. Therefore, the effects of earthworm inoculation on soil carbon and nitrogen cycles can indirectly be assessed by studying the changes in soil enzyme activities related to carbon and nitrogen cycles.

Given that *Sphingobacterium* sp. is the dominant bacterial taxon in the earthworm gut, we hypothesized that *Sphingobacterium* sp. and earthworm could individually promote soil organic matter decomposition and, when combined, exert reinforcing effects. These effects are negatively impacted by metal stress and influenced by the survival strategies of different earthworm species; Additionally, *Sphingobacterium* sp. and earthworms may play different roles in this combined process, with *Sphingobacterium* sp. stimulating microbial activities in the earthworm gut.

This study aimed to investigate the effects of different earthworm species (*Eisenia fetida* and *Amyntas gracilis*) and exogenous *Sphingobacterium* sp. on the transformation of soil carbon and nitrogen under non-contaminated and Cd-contaminated stresses. The specific research objectives were (1) to analyze and compare the effects of *Sphingobacterium* sp. and the two earthworm species, when inoculated alone and in combination, on the carbon and nitrogen forms and related enzyme activities in non- and Cd-contaminated soils. (2) to elucidate the effects of Cd pollution stress on this process and the underline mechanisms. (3) to clarify the interaction and roles of *Sphingobacterium* sp. and earthworms in these processes. This study can provide a theoretical basis for insight into the combined effects of earthworms and microorganisms on soil carbon and nitrogen cycling processes, the biologically driven mechanisms, and offer suggestions for adjusting soil remediation practices and their further sustainable management.

2. Materials and methods

2.1. Soil, earthworms, and strain

Two representative soils from South China were selected: non-contaminated soil (Soil I) and soil contaminated with metals from mining areas (Soil II). Soil I was collected from the tree garden of South China Agricultural University (23°9'N, 113°21'E); Soil II was collected from a paddy field (24°30'N, 113°45'E) about 6 km downstream of the opencast mining area of the copper-sulfur ore, limonite and lead-zinc mine in Dabao Mountain, northern Guangdong Province. The basic physicochemical properties of soils are listed in Table 1.

Earthworm species *Eisenia fetida* (*E. fetida*, epigeic species) and *Amyntas gracilis* (*A. gracilis*, epigeic species) were selected. *A. gracilis* was collected from South China Agricultural University, while *E. fetida*

were laboratory-raised earthworms. After two weeks of acclimation in the laboratory, earthworms with similar individual weights and a mature clitellum were chosen for the experiment.

Sphingobacterium sp. was purchased from BeNa Culture Collection (BNCC). CM0827 *Propionibacterium* sp. medium, which consisted of tryptone 15 g, L-cystine 0.5 g, yeast extract 5 g, sodium thioglycolate 0.5 g, glucose 5 g, agar 0.7 g, sodium chloride 2.5 g, resazurin 0.001 g, distilled water 1 L, was employed to cultural *Sphingobacterium* sp. and was set at pH 7.0. The strain was incubated at a constant temperature of 30 °C for 2 days before inoculation into the soil.

2.2. Experimental design

A two-factor (inoculation with *Sphingobacterium* sp. and different earthworm species (*E. fetida*, *E. A. gracilis*, *A*)) experimental design was employed in non-contaminated (Soil I, C) and Cd-contaminated (Soil II, S) soils. Six treatments were set up for each soil, with three replicates for each treatment and 36 pots in total.

Soil I: (1) Soil I (C); (2) Soil I + *Sphingobacterium* sp. (CS); (3) Soil I + *E. fetida* (CE); (4) Soil I + *A. gracilis* (CA); (5) Soil I + *Sphingobacterium* sp. + *E. fetida* (CSE); (6) Soil I + *Sphingobacterium* sp. + *A. gracilis* (CSA).

Soil II: (1) Soil II (S); (2) Soil II + *Sphingobacterium* sp. (SS); (3) Soil II + *E. fetida* (SE); (4) Soil II + *A. gracilis* (SA); (5) Soil II + *Sphingobacterium* sp. + *E. fetida* (SSE); (6) Soil II + *Sphingobacterium* sp. + *A. gracilis* (SSA).

Each pot was filled with 200 g of soil, which was passed through a 2 mm mesh. The pots have dimensions of caliber × bottom diameter × height of 75 × 53 × 90 mm and a microporous bottom. Soil moisture was adjusted to 60 % of the maximum field water holding capacity and stabilized for 48 h. Each pot was inoculated with 3 g of earthworms, approximately 8 individual *E. fetida* (~0.37 g·ind⁻¹) or 5 individual *A. gracilis* (~0.60 g·ind⁻¹). One milliliter of *Sphingobacterium* sp. solution (OD₄₀₅ value of 1.45) was added to the soil surface in CS, CSA, CSE, SS, SSA, and SSE treatments. To prevent earthworms from escaping, the top and bottom of each pot were covered with a fine nylon mesh with a 1 mm pore diameter. The temperature in the laboratory was maintained at 25 °C.

After 20 days, the earthworms were hand-picked, and soil samples were collected. A portion of the fresh soil was refrigerated at 4 °C for the analysis of microbial characteristics and microbial biomass carbon and nitrogen. The remainder was thoroughly air-dried, ground, and sieved for the analysis of soil physical and chemical properties. Earthworms were counted and weighed during sampling.

2.3. Soil properties, carbon and nitrogen forms, enzyme activities

Soil pH was measured using a glass electrode in a 1:2.5 (w/v) soil/water suspension. Soil clay, soil organic carbon (SOC), alkali-hydrolysable nitrogen (AN), and total nitrogen (TN) were analyzed by the pipette [36], dichromate oxidation [37], alkali N-proliferation [38] and Kjeldahl [39] methods, respectively. Microbial biomass carbon and nitrogen (MBC and MBN) were determined via the chloroform fumigation-extraction method [39,40]. Dissolved organic carbon (DOC) content was extracted with deionized water (1:5) and determined [41]. β-glucosidase, protease, and N-acetyl-β-D-glucosaminidase activities were all determined by colorimetric assay. Urease, invertase, and fluorescein diacetate hydrolytic activities were determined by sodium phenolate colorimetric assay, titration, and colorimetric using

fluorescein methods [42], respectively.

2.4. Total and DTPA extractable cadmium content

Total cadmium (Cd) in the soils was extracted by microwave digestion with aqua regia (HNO₃:HCl (v/v) = 1:3). The diethylene-triaminepentaacetic acid (DTPA) extractable form was regarded as bioavailable metals and toxic. DTPA-extractable Cd was extracted with DTPA extractant at a Soil:DTPA extractant ratio of 5:25 (g·mL⁻¹), which included a solution of 5 mM DTPA and 10 mM CaCl₂. The extracted suspensions were analyzed for total cadmium (Cd) and DTPA-extractable contents using inductively coupled plasma-mass spectrometry (ICP-MS, 7700ce, Agilent Technologies, Santa Clara, CA, USA) [43]. GBW07455 (GSS-26) was employed as certified reference material for soil analyses, and the measured concentrations were within 98% of the reported certified concentrations for Cd. Quality control measures included three repetitions for each soil and three blanks for each batch to ensure the quality of the analysis.

2.5. Statistics analysis

All data were analyzed and plotted with SPSS statistical software (Version 24.0), Origin 9.0, and R (Version 3.4.3). T-test and variance (ANOVA) analysis were performed. Data were presented as the mean ± SE (standard error). DOC:SOC = DOC/SOC, AN:TN = AN/TN, C: N=SOC/TN, MBC:MBN = MBC/MBN; Microbial quotient carbon (qMBC), qMBC = MBC/SOC; Microbial quotient nitrogen (qMBN), qMBN = MBN/TN.

Principal components analysis (PCA) and Person's correlation analysis were conducted to analyze the relationships of soil carbon and nitrogen forms and ratios (SOC, DOC, MBC, TN, AN, MBN, DOC:SOC, C:N, AN:TN, MBC:MBN, qMBC, qMBN), as well as enzyme activities (BG, INV, URE, NAG, PRO, FDA). The analysis was performed in R version 3.4.3 using the "ade4" and "corrplot" packages.

3. Results

3.1. The contents of carbon and nitrogen forms

The soil carbon form contents are shown in Fig. 1A, B, and C. In non-contaminated soil, both *Sphingobacterium* sp. and earthworms significantly reduced SOC content ($P < 0.05$). Reductions reached 16.7%, 14.8%, 15.6%, 16.7%, and 15.9% in CS, CE, CA, CSE, and CSA treatments, respectively. Furthermore, DOC increased significantly in the presence of earthworms ($P < 0.05$) and was highest in the CSE and CSA treatments at 659 ± 19.2 mg·kg⁻¹ and 430 ± 82.8 mg·kg⁻¹, respectively. Individual inoculation with *Sphingobacterium* sp. significantly increased MBC to 1685 ± 292 mg·kg⁻¹ ($P < 0.05$). However, in all the treatments inoculated with earthworms, there was no significant change in MBC content compared to C treatment, but it was significantly decreased compared to the CS treatment.

SS treatment reduced the SOC content in Cd-contaminated soil, decreasing to 16.0 ± 0.44 g·kg⁻¹. In contrast, SOC increased in all treatments involving earthworms and was significantly higher than that in the S treatment (16.6 ± 0.21 g·kg⁻¹) under the combined effect of SSE (17.8 ± 0.28 g·kg⁻¹) and SSA (17.5 ± 0.50 g·kg⁻¹) ($P < 0.05$). Compared to the control, MBC was slightly decreased in the SS

Table 1
Properties and Cd concentrations of soil.

	pH	Clay (%)	SOC (g·kg ⁻¹)	TN (g·kg ⁻¹)	C:N	Cd (mg·kg ⁻¹)	DTPA-Cd (mg·kg ⁻¹)
Soil I	4.73 ± 0.05	18.6 ± 3.85	27.0 ± 0.37	1.30 ± 0.05	20.8 ± 0.64	0.13 ± 0.15	0.007 ± 0.001
Soil II	4.40 ± 0.02	19.4 ± 0.49	16.6 ± 0.12	1.62 ± 0.05	10.3 ± 0.25	0.64 ± 0.20	0.164 ± 0.000

Note: Soil I and Soil II represents non-contaminated soil and contaminated soil, respectively. SOC, soil organic carbon; TN, total nitrogen; Cd, cadmium. Results are expressed as the mean ± SE (n = 3).

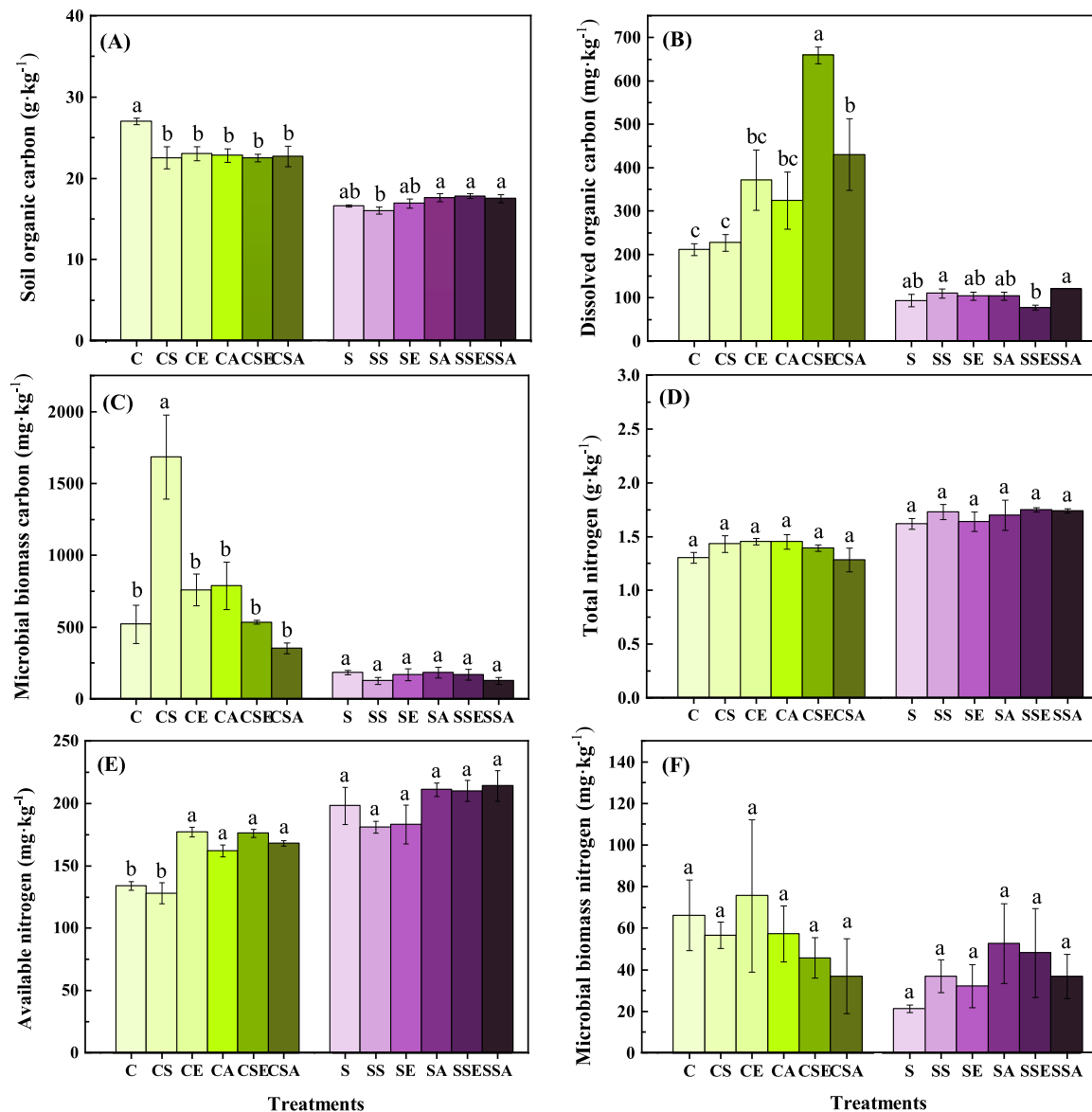


Fig. 1. Soil carbon and nitrogen form characteristics. Results are expressed as the mean value $\pm$ SE ($n = 3$). Different lowercase letters indicate significant differences ($P < 0.05$) among the treatments.

treatment, and the variation was smaller than that in the CS treatment.

The contents of nitrogen forms are displayed in Fig. 1D, E, and F. In non-contaminated soil, earthworm activity significantly increased AN content ($P < 0.05$) by 32.9%, 20.9%, 31.3%, and 25.4% in CE, CA, CSE, and CSA treatments, respectively, while no significant change in the CS treatment was observed. The indexes of nitrogen were not significantly changed in Cd-contaminated soil ($P > 0.05$), but the trend of AN in all treatments was consistent with that in non-contaminated soil. In addition, TN and MBN in both soils did not show significant changes under the influence of *Sphingobacterium* sp. and earthworms.

3.2. The ratios of carbon and nitrogen forms

Soil carbon and nitrogen form ratios are shown in Fig. 2. In general, the trend of ratios in both soils was consistent with those of the corresponding carbon and nitrogen forms. Specifically, the ratio of DOC:SOC followed the same trend as DOC, increasing to 2.93% and 1.94% in the CSE and CSA treatments, respectively. The C:N ratio significantly decreased by 23.6%, 23.6%, 24.0%, 22.6%, and 14.4% in the CS, CE,

CA, CSE, and CSA treatments, respectively. The AN:TN ratio increased significantly in the CE and CA treatments ($P < 0.05$) and further increased in combination treatments. The MBC:MBN ratio was significantly increased in CS treatment and significantly decreased in the SS treatment ($P < 0.05$). In contrast, this ratio significantly decreased in non-contaminated soil ($P < 0.05$) and increased in Cd-contaminated soil when combining *Sphingobacterium* sp. with earthworms. In non-contaminated soil, compared with C treatment, inoculation with *Sphingobacterium* sp. and earthworms individually increased qMBC compared to the C treatment and qMBC was highest in CS treatment. However, qMBC was decreased in the combined treatment. qMBN did not change significantly ($P > 0.05$) in all treatments for both soils.

3.3. Response of soil enzyme activity to inoculated earthworms and *Sphingobacterium* sp

The soil enzyme activities are illustrated in Fig. 3. In non-contaminated soil, inoculation of *Sphingobacterium* sp., *E. fetida*, and *A. gracili*, both individually and in combination significantly decreased

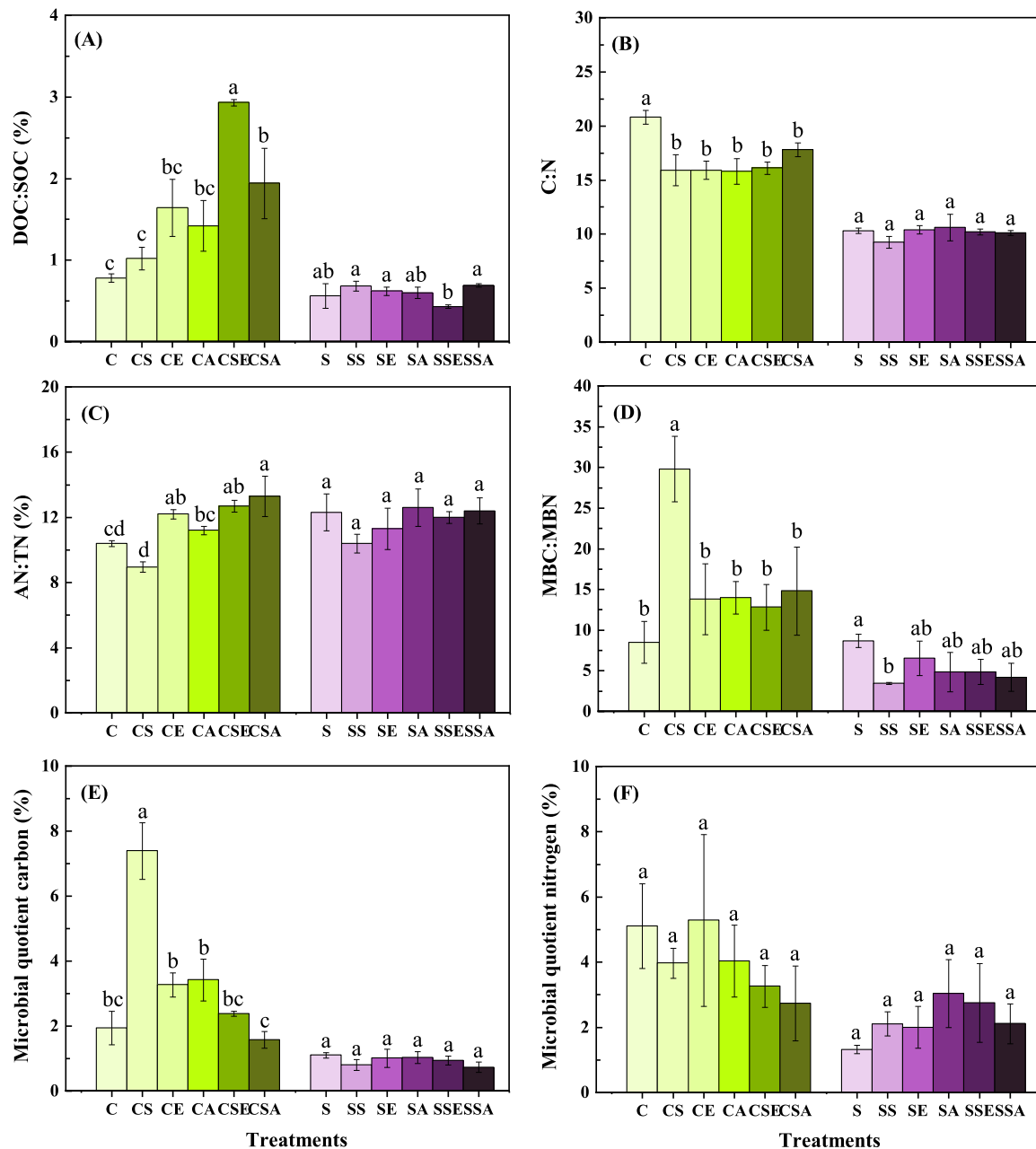


Fig. 2. The ratio of soil carbon and nitrogen forms. SOC, soil organic carbon; DOC, dissolved organic matter; TN, total nitrogen; AN, available nitrogen; C:N, soil organic carbon-to-total nitrogen ratio; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen. Results are expressed as the mean value $\pm$ SE ($n = 3$). Different lowercase letters indicate significant differences ($P < 0.05$) among the treatments.

BG, URE activities and FDA hydrolysis ($P < 0.05$). Additionally, *E. fetida* alone significantly increased NAG activities ($3.86 \pm 0.37 \mu\text{g}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$) while *A. gracilis* had the opposite effects ($P < 0.05$). In Cd-contaminated soil, inoculation of *Sphingobacterium* sp. and *E. fetida* alone and in combination significantly decreased INV, NAG, and URE activities, with *Sphingobacterium* sp. alone significantly increasing PRO activities ($P < 0.05$). Inoculation of *A. gracilis* alone significantly decreased INV, URE activities and increased NAG, PRO activities while increasing INV, NAG, and URE activities when combined with *Sphingobacterium* sp.

3.4. Earthworm survival rate and mean biomass loss

After 20 days, the survival rate and biomass of both earthworm species decreased (Table 2). In terms of survival rate, only the SSE

treatment exhibited a significant decrease of 68.7% ($P < 0.05$). Earthworm biomass decreased by 7.68%–77.2%, with a particularly significant decreasing in SSE treatment ($P < 0.05$). The biomass decreases were higher in Cd-contaminated soil than in non-contaminated soil and was more pronounced in *E. fetida* than *A. gracilis* treatment.

3.5. General effects of the treatments on soil C, N, and related enzyme activity

Principal Component Analysis (PCA) was carried out to identify the overall trends in soil C, N, and enzyme activity in response to *Sphingobacterium* sp. and earthworm activities in non-contaminated and Cd-contaminated soils (Figs. 4 and 5). In non-contaminated soils (Fig. 4 A and B), Axis 1 (27.8% variance explained) was primarily associated with

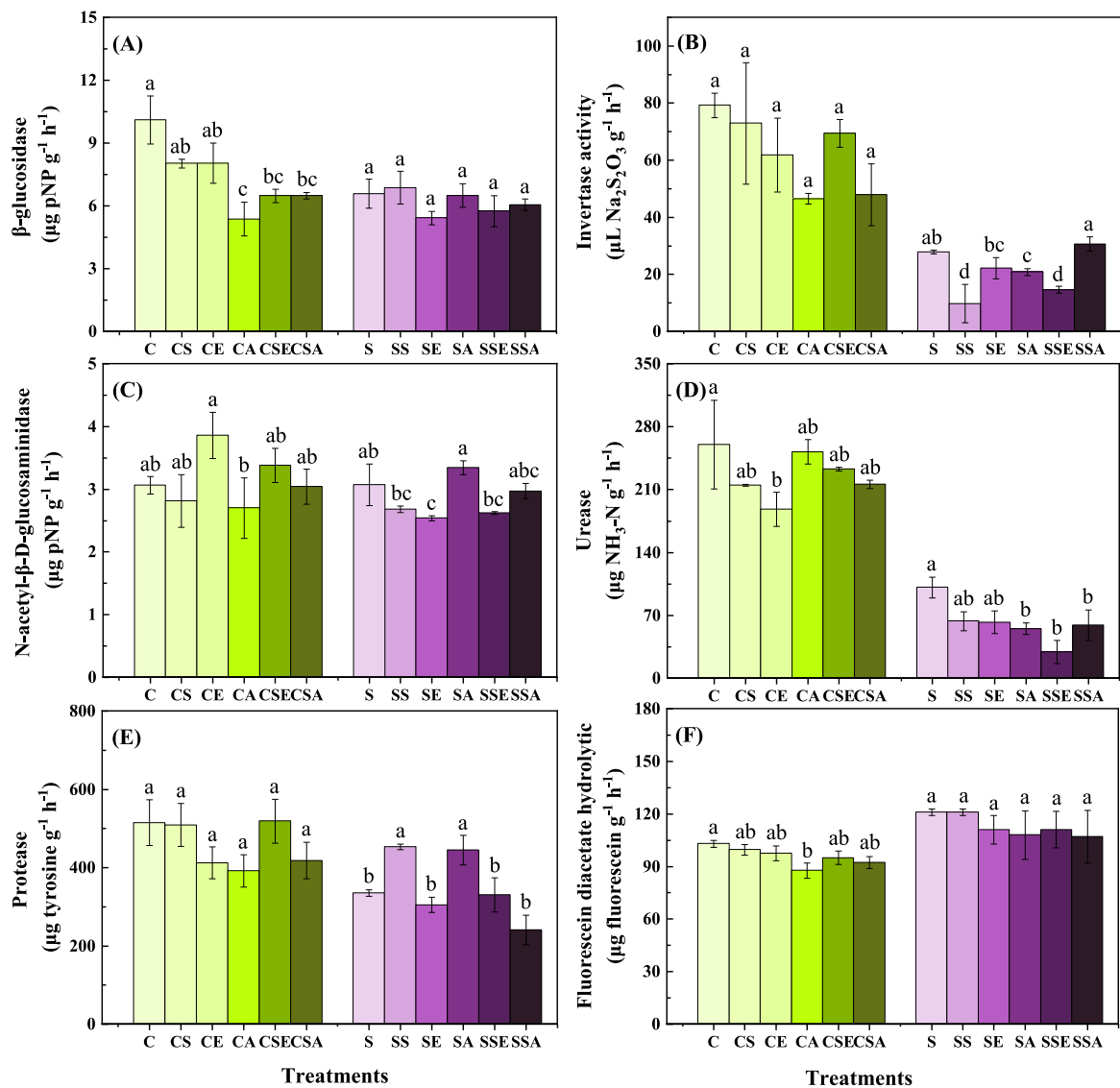


Fig. 3. Characterization of soil enzyme activity. Results are expressed as the mean value $\pm$ SE ($n = 3$). Different lowercase letters indicate significant differences ($P < 0.05$) among the treatments.

Table 2
Survival rate and mean biomass loss of earthworm at the 20th day.

Treatments	Survival rate (%)	Mean biomass loss (%)
CE	95.2 $\pm$ 4.76a	17.4 $\pm$ 13.3b
CA	86.1 $\pm$ 7.35a	7.68 $\pm$ 4.54b
CSE	91.1 $\pm$ 4.49a	19.4 $\pm$ 5.26b
CSA	87.8 $\pm$ 6.19a	13.7 $\pm$ 10.2b
SE	77.8 $\pm$ 11.1a	17.6 $\pm$ 5.22b
SA	100 $\pm$ 0a	18.9 $\pm$ 5.54b
SSE	31.2 $\pm$ 6.10b	77.2 $\pm$ 3.99a
SSA	100 $\pm$ 0a	18.9 $\pm$ 3.27b

Note: Results are expressed as the mean value $\pm$ SE ($n = 3$). The initial letters C and S represents the soil I and soil II. CE, CA, CSE and CSA represents the treatments with *E. fetida*, *A. gracilis*, *Sphingobacterium* sp. + *E. fetida* and *Sphingobacterium* sp. + *A. gracilis* in soil I, while SE, SA, SSE and SSA represents the treatments with *E. fetida*, *A. gracilis*, *Sphingobacterium* sp. + *E. fetida* and *Sphingobacterium* sp. + *A. gracilis* in soil II, respectively. Different lowercase letters indicate significant differences ($P < 0.05$) between different treatments.

DOC, AN, BG, FDA, SOC, MBC, and MBN. Axis 2 (16.4% variance explained) was mainly linked to INV, PRO, and URE. Axis 3 (14.2% variance explained) was mainly related to TN and NAG. Soils with added organisms were significantly differentiated from the control soils (Fig. 4C and D) ($P < 0.001$). In Cd-contaminated soils (Fig. 5A and B), Axis 1 (19.9% variance explained) was mainly related to TN, MBN, MBC, FDA, BG, URE, and NAG. Axis 2 (17.2% variance explained) was mainly related to SOC, AN, PRO, INV. Axis 3 (14.5% variance explained) was mainly related to DOC. There were significant differences among the treatments (Fig. 5C and D) ($P < 0.001$).

4. Discussion

4.1. The effects of *Sphingobacterium* sp. on C mineralization

Microorganisms are known to contribute to the mineralization and loss of SOC [44]. *Sphingobacterium* sp. is a type of gram-negative bacteria. Likewise, previous studies [45,46] used *Sphingobacterium* sp. to decompose the agricultural byproducts and to biodegrade organic pollutants. The performance of *Sphingobacterium* sp. in both non-contaminated and Cd contaminated soils indicates its ability to

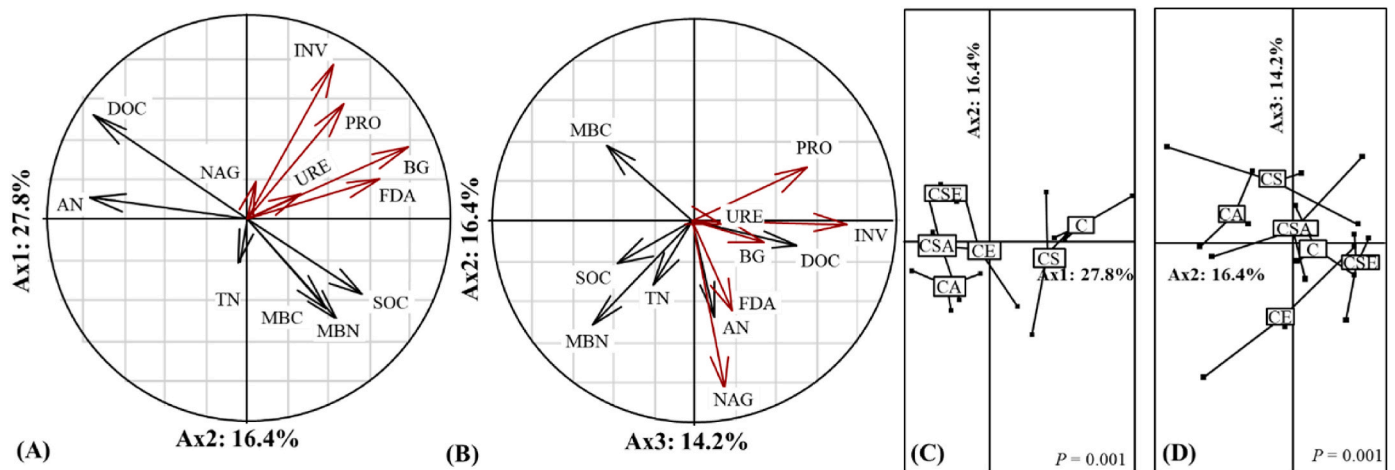


Fig. 4. Principal component analysis (PCA) of the effect of earthworms and *Spingobacterium* sp. on soil carbon and nitrogen fractions and enzyme activities in non-contaminated soil in fractal F1, F2 and F3 planes.

(A) Correlation circles of carbon and nitrogen fractions with enzymes activities in non-contaminated soil in fractal F1 and F2 planes.

(B) Correlation circles of carbon and nitrogen fractions with enzymes activities in non-contaminated soil in fractal F2 and F3 planes.

(C) Projection of experimental points according to treatments with or without earthworm and *Spingobacterium* sp. in non-contaminated soil in fractal F1 and F2 planes.

(D) Projection of experimental points according to classification with or without strain and worm in non-contaminated soil in fractal F2 and F3 planes.

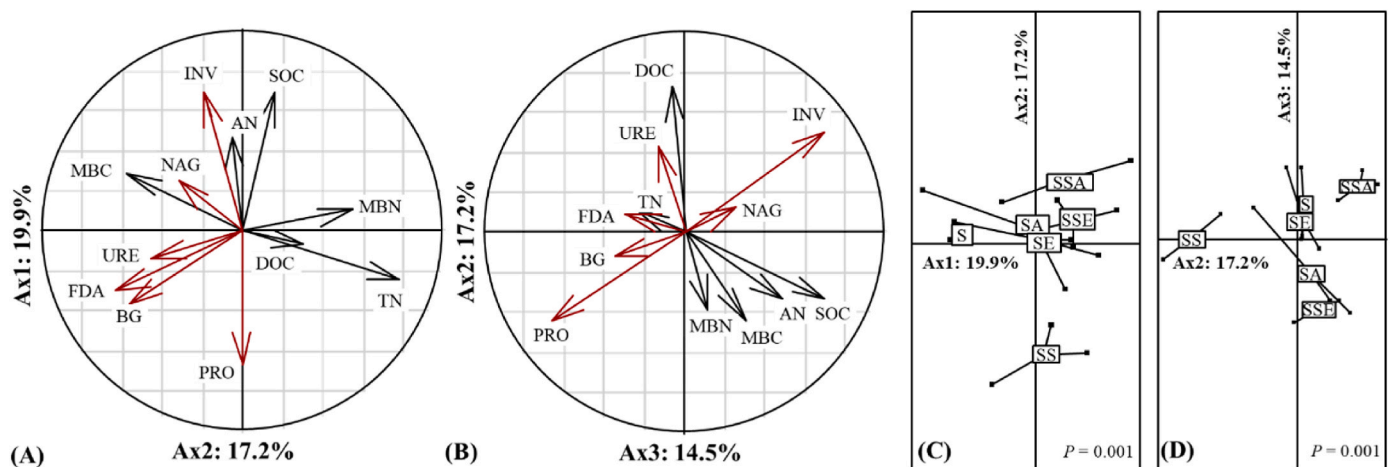


Fig. 5. Principal component analysis (PCA) of the effect of earthworms and *Spingobacterium* sp. on soil carbon and nitrogen fractions and enzyme activities in Cd-contaminated soil in fractal F1, F2 and F3 planes.

(A) Correlation circles of carbon and nitrogen fractions with enzymes activities in Cd-contaminated soil in fractal F1 and F2 planes.

(B) Correlation circles of carbon and nitrogen fractions with enzymes activities in Cd-contaminated soil in fractal F2 and F3 planes.

(C) Projection of experimental points according to treatments with or without earthworm and *Spingobacterium* sp. in Cd-contaminated soil in fractal F1 and F2 planes.

(D) Projection of experimental points according to classification with or without strain and worm in Cd-contaminated soil in fractal F2 and F3 planes.

mineralize carbon and tolerate metal stress. These abilities align with a similar study [47], who demonstrated that the inoculation of *Spingobacterium* sp. can enhance the degradation of organic pollutant residues in Cd (0.30 mg·kg⁻¹) contaminated soil. It also can be inferred from the aforementioned results that these functions of *Spingobacterium* sp. were not dependent on the gut conditions of earthworms. Additionally, the highest microbial biomass carbon content and the microbial quotient carbon in the CS treatment (Figs. 1C and 2E) were due to the inoculation of this strain, its adaption in the soil environment and substantial proliferation. However, this strong adaptive phenomenon was not observed in Cd-contaminated soil, where there is reduced microbial biomass carbon caused by metal stress. For instance, the adaptation process of *Spingobacterium* sp. was observed in the early stage (60 days) in Cd-contaminated soil (0.3 mg·kg⁻¹), with the strain population size gradually increasing after 90 days [47]. Nevertheless, this study only

lasted for 20 days and more time is needed for *Spingobacterium* sp. to complete the adaption processes under metal stress.

4.2. Earthworm survival, growth and species-specific effects on C and N transformation

Earthworm abundance and biomass decreased after 20 days of cultivation in both soils, especially in Cd-contaminated soil, consistent with findings from a previous study [29]. This decline may be attributed to soil nutrient limitation and the toxic effects of high Cd concentration on earthworms [48]. Regarding earthworm species, *A. gracilis* showed higher resistance to environmental stresses than *E. fetida*, probably owing to the different strategies employed by earthworms in metal handling and resistance. In a previous study, a toxicokinetic model was employed, which found that wild earthworm species were more

specialized than *E. fetida* in balancing the trade-off between metal uptake and excretion processes based on their surrounding environment [49].

There are different effects of earthworms on soil C and N transformation in non-contaminated and Cd-contaminated soil. In non-contaminated soil with a high organic carbon content, earthworms alone facilitated soil organic carbon and nitrogen mineralization. This is consistent with the trend of carbon and nitrogen changes observed in vermicomposting [50], presumably through the decomposition of organic matter by specific microorganisms under the unique conditions within the earthworm gut [51]. In addition, it can be found that earthworm activities increased the soil microbial quotient carbon compared to the control, indicating improved efficiency of soil organic matter utilization by microorganisms [52]. However, soil carbon stabilization in Cd-contaminated soil was probably due to earthworms consuming more organic matter to resist metal toxicity [53]. Moreover, it has been proved that gut peristalsis associates partly degraded organic matter with minerals [54] and leads to formation of organic matter-rich aggregates [55,56], which are conducive to SOC sequestration [7, 57–60]. It was concluded that earthworms can slow down SOC mineralization in the presence of more minerals [52]. Therefore, earthworm activities reduced carbon mineralization in mineral-rich Cd-contaminated soil. Furthermore, this effect may also be explained by the fact that contaminated soils contain stable carbon that is not easily mineralized. The positive correlation between β -glucosidase and N-acetyl- β -D-glucosaminidase activity, demonstrated with PCA, also supports the notion that soil microorganisms tend to use the active carbon pool generated by the decomposition of humus formed by litter (Fig. 5A; Fig. S1), and this favors the accumulation of recalcitrant soil carbon pools.

The earthworm species have great impact on biogeochemical cycling through their feeding habits and cast properties [31]. In non-contaminated and Cd-contaminated soil, both *E. fetida* and *A. gracilis* perform similar ecological functions; however, their performance is also influenced by earthworm survival strategies. In environments without metal stress, both species can enhance C and N mineralization. It is worth noting that *E. fetida* has a more pronounced effect on soil carbon and nitrogen mineralization than *A. gracilis* does (Fig. 1B and E; Fig. 2A and C). This could be attributed to the preference of *E. fetida* for ingesting high-quality organic matter. Their castings have a high level of particulate organic matter content and porosity that provides plentiful food and spaces for soil microorganisms [31], thereby accelerating the decomposition of organic matter [61–63]. On the contrary, the wild earthworm *A. gracilis* not only fed on soil organic matter, but also on more mineral particles. In addition, *E. fetida* found it easier to obtain food in non-contaminated soil with a relatively high organic matter content (Table 1). A high carbon to nitrogen ratio was favored for earthworm growth [64], so this species performed better. In contrast, in Cd-contaminated soil, the mineralization processes of soil organic matter slow down. The wild earthworm species *A. gracilis* demonstrated better survival and had more significant effects on C stabilization than *E. fetida* (Table 2, Figs. 1 and 2). The low organic matter content in Cd-contaminated soils limited the decomposition functions of *E. fetida*. Furthermore, earthworms need to consume more food to resist Cd stress. Both species increase their food consumption to feed on more organic matter. In this process, earthworms were compelled to ingest more mineral particles in soils characterized by low organic matter (Table 1) and high soil mineral content. The gut-grazing process facilitated the thorough mixing and binding of organic matter with soil minerals [54]. As for enzymes, the enzymes activity was influenced by earthworm species and metal stress. Likewise, in the previous study, earthworm activities suppressed β -glucosidase activity during the stimulation of C mineralization processes [65]. It was speculated that when soil microorganisms could easily access the readily mineralized C released by earthworms, they would use a smaller portion of the carbon from enzyme-catalyzed decomposition of soil organic carbon. Enzyme

activity only represents the concomitant consequence of earthworm activities but does not serve as the major driver of organic matter decomposition. It is also controlled by multiple factors such as food type, pH, temperature, gut transit time, metal stress, substrates et al., [65–67].

4.3. The combined effects of earthworm and *Sphingobacterium* sp. on the balance between C and N stabilization and mineralization

The combination processes showed that *Sphingobacterium* sp. assisted earthworms in further promoting carbon and nitrogen mineralization in non-contaminated soils (Fig. 4A and C) and carbon stabilization in Cd-contaminated soils (Fig. 5A and C), as reflected by the increased contents of mineralization (DOC, AN) and the stabilization indicators (SOC), respectively. It was also found that inoculated earthworm can process organically polluted water and excess sludge, and *Sphingobacterium*, known for its high ability to degrade organic matter, is one of the most abundant microbes in earthworm castings [68]. Nevertheless, our study demonstrated that external addition of *Sphingobacterium* sp. can help earthworms adapt more easily to environment and exert their ecological functions. Despite having similar effects when used in combination or when being inoculated alone, the strategies of the two species differed in non-contaminated and Cd-contaminated soil (Fig. 4D). Compared with treatments that inoculate earthworms alone, CSA and CSE treatments further activated INV and PRO activities in non-contaminated soil (Figs. 3 and 4D); On the other hand, SSA further activated INV activities and inhibited PRO activities, while SSE further inhibited URE activities (Figs. 3 and 5D). It is well known that soil enzyme activity is mainly derived from microorganisms and can reflect microbial activity [69]. Inoculation of *Sphingobacterium* sp. may stimulate microbial activities in the soil and the earthworm gut in non-contaminated soil [16]. In Cd-contaminated soil, it may represent the natural response of earthworms to the inoculation of *Sphingobacterium* sp. and metal stress. Compared to non-contaminated soil, metal stress induced inhibition of PRO activities in the treatment with *A. gracilis* inoculation and URE activities in the treatment with *E. fetida* inoculation, indirectly demonstrating distinct coping strategies for metal stress among earthworm species. Given that researchers have [70] found that the vermiwash and mucus extracted from earthworms can greatly inhibit the growth of pathogenic fungi, it is also possible that the mucus compounds changed due to metal pollutant stress in this study, such as secretion of specific organic substances to protect itself from physiological damage, while inhibiting the microbial activities. The underlying mechanisms are still not clear and require further research.

In these processes, earthworm activities dominated the soil carbon and nitrogen mineralization processes, while *Sphingobacterium* sp. played a supporting role in combined processes. When the effects of earthworm and *Sphingobacterium* sp. on carbon and nitrogen were consistent, they enhanced carbon mineralization; otherwise they undermined this process. Hence, in non-contaminated soil, carbon mineralization was observed. While in Cd-contaminated soil, carbon storage was more prevalent due to the stronger effect of earthworms on stabilization than *Sphingobacterium* sp. on mineralization. This is further confirmed by PCA which demonstrated that earthworms play a critical role in soil organic carbon decomposition processes, following the same trend as inoculation with earthworm alone and in combination with *Sphingobacterium* sp. (Fig. 4A and C; Fig. 5A and C). Additionally, in this system, a strong interaction existed between earthworms and *Sphingobacterium* sp. Earthworm activities under the combined process dramatically decreased microbial biomass carbon compared to *Sphingobacterium* sp. activities alone. It is suggested that earthworms contribute to maintaining the ecosystem balance and stabilizing the microbial community structure through feeding [71] and regulating the allocation of active organic carbon and dissolved organic carbon pools [57,72]. Therefore, the rapid proliferation of *Sphingobacterium* sp. was prevented in CSE and CAS treatments. It was further confirmed by the significantly lower microbial quotient carbon in treatment CSE and CSA

compared to treatment CS (Fig. 2E). It can be deduced that earthworms may reduce the conversion efficiency of soil microorganisms in utilizing carbon sources into microbial biomass carbon [52] or they can intensely feed and graze on microbes [10,73–75]. Additionally, the different impact of earthworm species may be caused by *E. fetida* being more conducive to the survival and growth of G^- bacterial communities [75], thus promoting *Sphingobacterium* sp. to assist earthworms in carbon mineralization. Based on the above, another underlying factor was postulated that *Sphingobacterium* sp. may assist earthworms in mineralizing soil organic carbon and nitrogen by stimulating the microbial activities in the earthworm gut. The subsequent studies need to focus on the earthworm gut microbial to reveal the internal mechanisms.

5. Conclusion

This study focuses on soil carbon and nitrogen forms and enzyme activities to investigate whether *Sphingobacterium* sp. can assist different earthworm species in influencing soil carbon stabilization and nitrogen cycling under non-contaminated and Cd-contaminated soil. Generally, *Sphingobacterium* sp. inoculation individually facilitated carbon mineralization in both soils, and this function did not depend on the gut conditions of earthworms. In terms of species of earthworms, a species-specific effect was observed with *E. fetida* being more effective in C mineralization than *A. gracilis*. Earthworm activities may have a weak inhibitory effect on enzyme activity during this process. Additionally, Cd pollution stress impacts C and N cycles and results in opposite effects. Earthworms facilitated the mineralization of C and N in non-contaminated soil while promoted C storage in Cd-contaminated soil. Similarly, *Sphingobacterium* sp. assisted earthworms in improving carbon and nitrogen mineralization in non-contaminated soil, with *E. fetida* being more effective than *A. gracilis*. However, they failed to do so in Cd-contaminated soil and promoted carbon storage instead. Therefore, earthworm activity dominates the processes of carbon and nitrogen stabilization and mineralization, with *Sphingobacterium* sp. playing a supporting role. This study provides a clear understanding of the different effects of earthworms and microorganisms on soil carbon and nitrogen cycling in non-contaminated and Cd-contaminated soil. In further studies, the complex interaction mechanisms of earthworms and microorganisms can be designed and tested by some methods of molecular biology or metabolomics methods. Additionally, exploring the interaction of earthworms and microorganisms in soil remediation technology could lead to the adjustment and reconstruction of sustainable soil management practices.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (Grant No. 41201305), Guangdong provincial Natural Science Foundation (Grant No. 2021A1515011543), National Science and Technology Fundamental Resources Investigation Program of China (2018FY100300), along with National Key Research and Development Program of China (2022YFC3703100). The authors gratefully acknowledge financial support from China Scholarship Council.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejsobi.2023.103576>.

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Le rôle de la bioaugmentation des vers de terre sauvages dans la phytoremédiation de *Sedum plumbizincicola* et les mécanismes microbien-médiés dans les sols contaminés par des métaux

La contamination des sols par les métaux lourds, en particulier dans les zones minières abandonnées, représente un défi environnemental majeur. La phytoremédiation, qui utilise des plantes pour assainir les sols contaminés, a émergé comme une solution durable. Cependant, l'efficacité de la phytoremédiation utilisant l'hyperaccumulateur *Sedum plumbizincicola* peut être limitée par une faible production de biomasse et un approvisionnement insuffisant en métaux aux racines. Cette étude visait à évaluer le potentiel de bioaugmentation par des vers de terre sauvages et à identifier les espèces de vers de terre les plus efficaces dans différents sols contaminés par le cadmium (Cd) et le zinc (Zn) dans des conditions de laboratoire contrôlées sur une période de 90 jours. De plus, les mécanismes sous-jacents ont été révélés en termes de communautés microbiennes du sol. Les résultats ont montré que les vers de terre sauvages peuvent promouvoir la croissance de *S. plumbizincicola* et présenter des effets d'augmentation spécifiques à chaque espèce. En outre, nous avons constaté que les effets de bioaugmentation des espèces de vers de terre combinées étaient similaires, voire légèrement inférieurs, à ceux des espèces individuelles. Les communautés bactériennes et fongiques ont été fortement influencées par l'activité des vers de terre, jouant un rôle crucial dans la transformation des métaux. Les microbiotes clés facilitant l'absorption du Cd et du Zn par *S. plumbizincicola* ont changé en fonction des niveaux de contamination des sols, évoluant vers des microbiotes dont les fonctions contribuent principalement aux cycles biogéochimiques et à la résistance aux métaux dans les sols très contaminés. Cette étude offre une base théorique pour comprendre les effets combinés et le potentiel de multiples organismes dans la remédiation des sols.

Mots clés : Cadmium et zinc, *Sedum plumbizincicola*, rhizosphère, drilosphère, bactéries, champignons

The Bioaugmentation Role of Wild Earthworms in the Phytoremediation of *Sedum plumbizincicola* and Microbial-Mediated Mechanisms in Metal-Contaminated Soils

Soil contamination with heavy metals, particularly in abandoned mining areas, poses a significant environmental challenge. Phytoremediation, the use of plants to remediate contaminated soils, has emerged as a sustainable solution. However, the efficiency of phytoremediation using the hyperaccumulator *Sedum plumbizincicola* can be limited by low biomass production and insufficient metal resupply to the roots. This study aimed to evaluate the bioaugmentation potential of wild earthworm and identify the most effective earthworm species in different cadmium (Cd) and zinc (Zn) contaminated soils under controlled laboratory conditions over a period of 90 days. Moreover, the underlying mechanisms were revealed in terms of soil microbial communities. The results demonstrated that wild earthworms can promote the growth of *S. plumbizincicola* and show species-specific augmentation effects. In addition, we found that the bioaugmentation effects of combined earthworm species were similar to, or slightly lower than, those of individual species. Bacteria and fungi communities were strongly influenced by earthworm activity, playing a vital role in metal transformation. The key microbiota enhancing Cd and Zn uptake by *S. plumbizincicola* changed with soil contaminated levels, shifting to microbiota with functions primarily contributing to biogeochemistry cycles and metal resistance in highly contaminated levels. This study provides a theoretical foundation for understanding the combined effects and potential of multiple organisms in soil remediation.

Keywords: Cadmium and Zinc, *Sedum plumbizincicola*, rhizosphere, drilosphere, Bacteria, Fungi