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RESEARCH ARTICLE

Growing maize while biological remediating a multiple metal-contaminated soil: a promising solution with the hyperaccumulator plant *Sedum alfredii* and the earthworm *Amynthas morrisi*

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Abstract

Aims This study was aimed to investigate the effect of earthworm *Amynthas morrisi* on the metal bioaccumulation by two plant species, *Sedum alfredii* Hance (Sedum) and maize (Zea mays L.), in a co-cropping system and possible influencing pathways, and assess the remediation potential of all combination of these organisms to identify the best option.

Methods In this study, an eight-week microcosm experiment was conducted to investigate the main and interactive effects of the earthworm *Amynthas morrisi*, the hyperaccumulator plant *Sedum alfredii* Hance (Sedum), and maize (*Zea mays* L.) on C and N forms, and microbial characteristics and diethylene triamine penta acetic acid (DTPA) extractable metals of a soil heavily contaminated by multiple metals (i.e., Cd, Zn, Pb, and Cu). In addition, plant growth and metal accumulation were evaluated and the possible influencing pathways of metal accumulation by the two plant species were assessed. Finally, a remediation strategy was proposed based on the amounts of metals removed by sedum and maize.

Results The soil quality index achieved after eight weeks of experiments, was best with Sedum, and worst in the control with no plants and no earthworms. A path analysis suggests that earthworms exerted strong effects on plant metal accumulation by changing plant growth, with soil microbes playing a mediating role. The association of Sedum and

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Maize significantly increased the Zn concentration and decreased the Pb and Cu concentrations in the aboveground parts of Sedum compared with Sedum alone, whereas Sedum decreased the Zn, Cd, Pb, and Cu concentrations in the aboveground parts of maize compared with maize alone treatment. PCA showed that when maize was co-cropped with Sedum, metal transfer from its roots to the aboveground parts decreased.

Conclusions The Earthworm + Sedum + Maize treatment displayed the most effective Cd and Zn removal, indicating that the combined introduction of *A. morrisi* and *S. alfredii* can effectively remediate soils co-contaminated by Cd and Zn in maize cropping systems.

Keywords Earthworm · Contaminated soil · Crop plant · Co-cropping system · Zoo remediation

Introduction

Metal pollution of cultivated lands is attracting worldwide attention because it is increasingly recognized as a major threat to the environment and human health (Cai et al. 2019). Phytoremediation is a promising technique to reduce metal pollution because of its low cost, relative simplicity of operations, and absence of secondary pollution (Lasat 2000; McGrath and Zhao 2003). However, the phytoremediation efficiency is generally low due to the slow growth and low biomass of hyperaccumulator plants. Therefore, existing phytoremediation techniques need to be improved, in particular in areas where soils need to be quickly decontaminated. Co-cropping- the association of a crop plant and a hyperaccumulator plant in the same field (Knorzer et al. 2009), is an interesting alternative that allows agricultural production and phytoremediation at the same time. However, this approach is challenging because it requires to maximize metal accumulation in the hyperaccumulator plant while minimizing the metal content of the crop plant. Adding earthworms to the co-cropping technique could help to tackle this challenge.

It is recognized that earthworms improve plant growth (Brown et al. 1999; Scheu 2003), which may be due to their effects on soil structure, organic matter, nutrients, and microorganisms (Lavelle et al. 2020, Zhang et al. 2009; Jacquiod et al. 2020). Earthworms increase metal uptake by plants and metal concentrations in plants according to a meta-analysis of 1185 pairwise comparisons from 42 peer reviewed papers (Sizmur and Richardson 2020). Changes in pH, dissolved organic carbon, redox condition, and microbial activities and increase in metal bioavailability due to earthworm activities are the major reasons for the increased heavy metal mobility in soil (Zhang et al. 2016b, 2020). Nevertheless, it is still unclear whether earthworms increase plant metal accumulation by improving metal availability or by improving plant growth with more nutrients and higher microbial activities. It remains unclear how earthworms influence metal bioaccumulation by crop plants as well. Previous studies showed that earthworms stimulate metal uptake by hyperaccumulator plant species in monocropping systems (Ji et al. 2020; Kaur et al. 2018; Jusselme et al. 2013). However, they did not assess the effect of earthworms on metal bioaccumulation by crop plants. In addition, these previous studies used artificially polluted soil instead of polluted field soil. The effect of earthworm introduction on metal removal from contaminated soils in field with co-cropping systems remains unclear.

Therefore, this study aimed to evaluate the main and interactive effects of the earthworm *Amynthas morrisi* and the hyperaccumulator Sedum, separately or in association, on the growth and metal accumulation of crop maize growing in a multiple metal-contaminated soil. *Sedum alfredii* Hance is a Zn/Cd hyperaccumulator and Pb accumulator native to China. Studies showed that in about 12 to 21 days it accumulates 9,000–11,000 mg·kg^{-1} Cd in leaves when grown in soils with 45–67 mg·L^{-1} of Cd (Yang et al. 2004; Zhou and Qiu 2005), 19,674 mg·kg^{-1} of Zn in the aboveground parts when grown in an 80 mg·L^{-1} Zn solution (Yang et al. 2002), and 514 mg·kg^{-1} Pb in shoots when grown in a 160 mg·L^{-1} Pb solution (He et al. 2002). Previous studies have mainly focused on the physiological characteristics of Sedum and its metal hyperaccumulation mechanisms related to plant root exudates, special organs, functional gene and so forth (Lu et al. 2009; Li et al. 2013, 2018; Zhang et al. 2016a). Some studies investigated the effects of rhizospheric microbes on the heavy metal phytoextraction efficiency of Sedum and suggested that synthetic chelators (e.g., ethylene diamine tetra acetic acid (EDTA), S, S-ethylene diamine disuccinic acid (EDDS), diethylene triamine penta acetic acid (DTPA)) and low molecular weight organic acids can be added to improve phytoextraction efficiency (Liu et al. 2008; Wu et al. 2007a, 2020; Huang et al. 2008; Xiong et al. 2008; Sun et al. 2009; Wang et al. 2009). However, no study has been conducted so far to evaluate the effect of earthworm activity on the growth and heavy metal removal characteristics of Sedum in co-cropping systems. In our study, soil DTPA-extractable metals, C and N forms, and microbial properties were evaluated. Plant height, biomass, and metal uptake of Sedum and maize were determined. We conducted a path analysis to understand the effect of earthworms on metal accumulation in Sedum and maize, and the relationship between metal accumulation and plant growth, soil C and N forms, microbial characteristics, and metal availability. Finally, a strategy for the remediation of multiple metal-contaminated soils is proposed. We hypothesized that maize would grow better and accumulate less heavy metals when co-cropped with Sedum in the presence of *A. morrisi*.

Materials and methods

Experimental design

Eight treatments were set up as follows: (1) Control: no plant or earthworm; (2) Sedum only; (3) Maize only; (4)Sedum and Maize; (5)Earthworm only; (6) Earthworm and Sedum;(7) Earthworm and Maize;(8) Earthworm, Sedum and Maize. Each treatment was replicated 3 times (24 pots).

The experiment was conducted from March to May in Guangzhou, China (113°17’ E, 23°8’ N), and the average daily temperature was approximately 23^{o}C during the experiment. Each experimental pot was filled with 900 g of soil and 100 g of organic amendment that were mixed thoroughly. We used 5 cm lower diameter, 11 cm upper diameter, 13.5 cm height pots. In treatments with Sedum, two 10 cm-long shoots of sedum were planted in each pot. In treatments with Maize, three maize seeds were sown in each pot and thinned to one at the three-leaf stage. Soil moisture was always kept at field capacity in each pot. After two weeks of plant growth, healthy adult earthworms with clitella (averaging 0.4 g·individual^{-1}), which had been depurated for 7 days, were introduced at approximately 4 g·kg^{-1} soil to the relevant treatments.The quantity of earthworm was fit to the local planting-earthworm breeding system in fields (2–5 g·kg^{-1} soil). 95% of the introduced earthworms survived in the treatments, although slightly non-significant decreases in biomass were observed at the end of the eight-week experiment (STable 1).

Soil, earthworm, plants, and organic amendment

The soil used in this study was collected from the upstream area of the Yanghe River basin. The area had been subjected to 40 years of pollution by metals (Cu/Cd/Pb/Zn) from the mine wastes of the Dabaoshan opencast mine in Guangdong Province, South China (24°30′ N, 113°45′ E). Soil samples were taken from the upper 20 cm of three paddy fields, air-dried, mixed thoroughly, and sieved to <2 mm. The soil had a high clay content of 40%, a low pH of 4.2, an organic C content of 15.3 g·kg^{-1}, a total N content of 1.46 g·kg^{-1}, and a C:N ratio of 10.5. The soil was contaminated by multiple metals. Total Cd, Zn, Pb, and Cu contents were 0.639 ± 0.056, 405 ± 29.0, 439 ± 8.4, and 394 ± 10.2 mg·kg^{-1}, respectively, which were 2.13, 2.02, 6.27, and 7.88 times, respectively, higher than their respective acceptable limits for arable soils in China, according to the national standard GB15618-2018.

Clitellate adults of *A. morrisi*, a local epiendogeic earthworm species (Beddard, 1892), were collected from an uncontaminated field (24°18′ N, 113°23′ E) and cultured in an uncontaminated soil for one month at 25 ºC in the laboratory before use. Sedum was taken from an old mine site in the Zhejiang Province of China (Yang et al. 2002; He et al. 2002; He 2003) and grown for later use. Maize variety Yunshi-5 was provided by the Guangdong Academy of Agricultural Sciences.

The organic input in this study was obtained by mixing air-dried, sieved (<2 mm) coco coir and cattle dung at a mass ratio of 4:1. The organic C, total N, and C:N ratio were 50.33%, 0.66%, and 72, respectively, for coco coir and 15%, 0.6%, and 25, respectively, for cattle dung.

Laboratory analyses

After eight weeks, plant height and shoot diameter were measured. The plants were harvested, separated into roots, shoots, and leaves, washed with distilled water, oven-dried at 65 ºC for 72 h, and pulverized before analysis. Plant samples were ashed in a muffle furnace at 500 ºC for 5.5 h, dissolved with 6 $\text{mol}\cdot\text{L}^{-1}$ HCl, adjusted to 25 mL with distilled water, and analyzed for Cu, Cd, Pb, and Zn concentrations by atomic absorption spectrophotometry.

Soil aggregates, from all treatments except the control, were separated manually (Velasquez et al. 2007), air-dried, crushed by hand, and sieved to <2 mm before analysis. Soil pH was measured at a soil: water ratio of 1:2.5. Organic C was determined by the dichromate digestion method, and total N was measured by Kjeldahl digestion (Sparks et al. 1996). Available N was determined using the method described by Sparks et al. (1996). Soil respiration rate was determined by measuring CO_2 evolution during 7-day incubation (Bekku et al. 1997). After 7-day incubation, dissolved organic C (DOC) was measured according to Dai et al. (2004). Microbial biomass C was determined using the chloroform fumigation extraction method (Vance et al. 1987). Available metals were extracted with DTPA according to Lindsay and Norvell (1978) and quantified with an atomic absorption spectrophotometer (Unicam, UK).

Calculations

The transfer of a heavy metal from soil to roots and from plant roots to aboveground parts were evaluated with the R/S and A/R ratios, respectively, and calculated as follows:

$$\text{R/S} = \frac{\text{Heavy metal concentration in roots}}{\text{DTPA} - \text{extractable heavy metal concentration in soil}}$$

$$\text{A/R} = \frac{\textit{Heavy metal concentration in plant aboveground parts}}{\textit{Heavy metal concentration in plant roots}}$$

Relative concentration of heavy metals in plant aboveground parts was evaluated with the A/T ratio, which was calculated as:

$$\text{A/T} = \frac{\textit{Heavy metal accumulation in plant aboveground parts}}{\textit{Heavy metal accumulation in the whole plant}}$$

Remediation efficiency is defined as the time taken by a remediation system to decrease the concentration of a pollutant to below its acceptable limit. When soil pH is ≤ 5.5, the acceptable limits of soil total Cd, Zn, Pb, and Cu are 0.3, 200, 70, and 50 $\text{mg}\cdot\text{kg}^{-1}$, respectively, for arable lands in China according to the national standard GB15618-2018. The remediation efficiency ($\text{efficiency}_{\text{metal}}$) of a treatment was calculated as:

$$\text{Efficiency}_{\text{metal}} = \frac{[(C_{total\ metal} - C_{limit}) \times 1.00]}{[(P_{Sedum\ aboveground\ parts} + P_{maize\ aboveground\ parts}) \times (C_{total\ metal} \times 1.00)] \div 56}$$

where $C_{\text{total metal}}$ is the total concentration of a metal in soil at the beginning of the experiment ($\text{mg}\cdot\text{kg}^{-1}$), C_{limit} is the metal acceptable limit for arable soils in China according to the national standard GB15618-2018 ($\text{mg}\cdot\text{kg}^{-1}$), $P_{\text{Sedum aboveground parts}}$ is the percentage of the metal in Sedum aboveground parts at the end of the experiment (%), $P_{\text{maize aboveground parts}}$ is the percentage of the metal in maize aboveground parts at the end of the experiment (%), and 56 is the duration of the experiment (day).

Statistical analysis

Data were analyzed using SAS 8.0 (SAS institute Inc.) and the ADE-4 package in R (Thioulouse et al. 1997). T-test was applied to compare earthworm biomass between treatments. One-way and two-way ANOVA were used to compare aggregate production, soil chemical and biological properties, soil DTPA-extractable metal concentrations, metal concentrations and accumulations in plants, metal removal indicators, and total available metal percentages in soil and plant systems among the treatments. Kruskal-Wallis rank sum test was used instead of one-way ANOVA, and Scheirer-Ray-Hare test was used instead of two-way ANOVA when variables did not have a normal distribution or/and homoscedasticity condition was not observed. Duncan's test was used to distinguish the differences when one-way ANOVA test was significant. Residual normality and homoscedasticity were verified using Kolmogrov-Smirnov and Bartlett tests, respectively.

Principal component analysis (PCA) in ADE-4 package (Thioulouse et al. 1997) was used to describe general trends. A discriminant analysis coupled with a Monte Carlo permutation test allowed to statistically compare treatments using tables with multiple

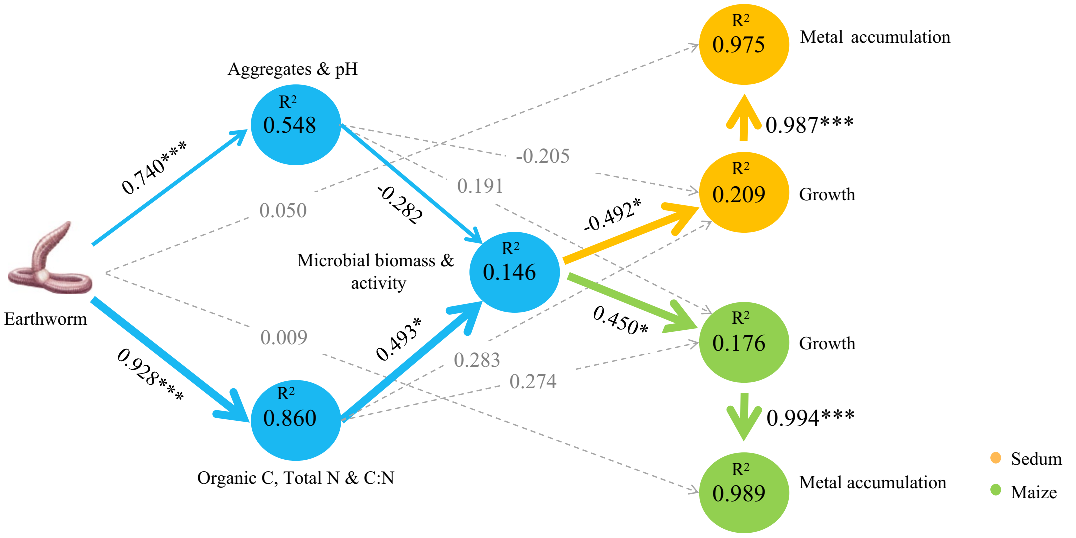


Fig. 1 Pathway analysis of earthworm contributions to metal accumulation in plants. *The latent variable "Microbial biomass and activity" represents changes of microbial biomass C and respiration rate respectively

variables. Soil pH, organic C and total N contents, aggregate production, available N, DOC, microbial biomass C, soil respiration rate, DTPA-Zn, DTPA-Cu, DTPA-Cd, and DTPA-Pb were chosen as soil quality variables in this study. For the first two axes of the PCA, the variables with the highest contributions were selected. The values of each variable set, adjusted to a range from 0.1 to 1.0 via homothetic transformation, were multiplied by their respective weight factors and summed, giving soil quality index I as: $I = F_1 \cdot (\alpha_1 a + \beta_1 b + \gamma_1 c \ldots) + F_2 \cdot (\alpha_2 a + \beta_2 b + \gamma_2 c \ldots)$, where F_1 and F_2 are the percentages of variance explained by axis 1 and axis 2 of the PCA, respectively, α_i, β_i, and γ_i represent the contributions of the variables to the formation of their respective axe i(i=1 or 2), and a, b, and c are the values of the selected variables on their corresponding axes (Velasquez et al. 2007). In this case, a general subindicator of soil quality was calculated.

Partial least squares (PLS) path analysis was performed using Smart PLS 3.0, which was used to explain the direct and indirect effects of earthworms on metal accumulation in these two plants. Earthworm was considered as an exogenous variable. Soil organic C, total N, the ratio of C and N, aggregate contents, pH value, microbial biomass C and respiration rate were used as endogenous variable in our study to build the model based on current knowledge of the impact of earthworm and other key environmental factors on plant growth and then metal accumulation in plant. Plant height and total biomass as endogenous variables reflected plant growth, and metal concentration in the entire plant as an ultimate response variable represented plant metal accumulation. We assumed that earthworms had a direct or indirect impact on soil characteristics, which subsequently affect plant biomass and/or metal accumulation in plants.

Results

Influencing pathway of earthworm on metal accumulation in plants

Path analysis generally was performed to analyze how earthworms affected metal accumulation in Sedum and maize (Fig. 1). The path coefficients of soil C and N forms, microbial biomass C and activity, the growth of plants, and metal accumulation in plants were significant ($P < 0.05$). Earthworms had an indirect effect on metal accumulation in plants. The introduction of earthworms influenced soil microbial biomass and activity by directly speeding up soil C and N transformation, which influenced plant growth and eventually stimulated metal accumulation in the plants. Reliability and validity indexes of the PLS are shown in STable 2.

Heavy metal concentration and accumulation in plants

Metal concentrations in Sedum and maize

Both the introduction of earthworms and the addition of a second plant species in the co-cropping system affected the metal uptake of Sedum and maize (SFig. 4, STables 3 and 4).

Zn concentration in maize in all organs decreased in all treatments, except in the roots in the Sedum + Maize treatment and in the aboveground parts in the earthworm + maize treatment (SFig. 4, STable 3). The maximum decrease of Zn concentration by 25.1% occurred in the aboveground parts of maize. The lowest Zn concentration in Sedum was in the treatment with only Sedum, while the highest concentrations were in the co-cropping treatments (SFig. 4a, STable 4).

Changes of Cd concentration in maize were very small, less than 1 $mg \cdot kg^{-1}$(SFig. 4b, STable 3). The concentration of Cd in Sedum increased in all plant organs in the earthworm + Sedum + maize treatment (SFig. 4b, STable 4). Opposite results were observed in the treatment with earthworms but not maize (SFig. 4b, STable 4). In the Sedum + Maize treatment, the Cd concentration of Sedum increased in the roots but decreased in the aboveground parts.

Pb tended to accumulate more readily in maize than in Sedum (SFig. 4c, STables 3 and 4). In maize, it mainly accumulated in the roots, and its concentration decreased slightly in the aboveground parts. The presence of earthworms and Sedum decreased the accumulation of Pb in the above ground parts of maize. Pb concentration in Sedum decreased in all treatments but the Earthworm + Sedum treatment where increases of 1–10 $mg \cdot kg^{-1}$ were observed.

The Cu concentration in maize decreased greatly in the roots (by more than 8 $mg \cdot kg^{-1}$), but increased in the aboveground parts in the earthworm + maize treatment (SFig. 4d, STable 3). High Cu concentrations were observed in Sedum, but with rather variable patterns (SFig. 4d, STable 4): concentration in roots increased in the co-cropping treatments but decreased in the mono-cropping treatment. In contrast, Cu concentration in plant aboveground parts decreased in the co-cropping treatments but increased in the mono-cropping treatment with Sedum but no earthworms.

Metal accumulation in Sedum and maize

The effect of different treatments on the biomass and rates of accumulation in different parts of the two plants resulted in rather variable rates of total accumulation in the plants. Maize accumulated more Zn in the earthworm + maize treatment (SFig. 4a and STable 5). Zn was readily accumulated by Sedum, especially in the aboveground parts (SFig. 4a and STable 6), in the Sedum + maize, earthworm + Sedum, and earthworm + Sedum + maize treatments as compared with the Sedum mono-cropping treatment.

A rather different and less clear pattern of Cd accumulation was observed. When co-cropped with Sedum, in the absence of earthworms, maize accumulated less Cd than in treatments with earthworms (SFig. 4b, STable 5). Accumulation of Cd in Sedum was high in the earthworm + Sedum + maize treatment but low in the earthworm + Sedum and Sedum + maize treatments (SFig. 4b, STable 6).

More Pb was accumulated in the roots of maize in the co-cropping treatments or the earthworm + maize treatment than in the maize mono-cropping treatment. In contrast, Pb accumulation in the aboveground parts decreased in the co-cropping treatments but increased slightly in the earthworm + maize treatment compared with the maize mono-cropping treatment(SFig. 4c, STable 5). Pb accumulation in Sedum decreased in the Sedum + maize and earthworm + Sedum + maize treatments but increased in the aboveground parts in the earthworm + maize treatment compared with the Sedum mono-cropping treatment (SFig. 4c, STable 6).

Maize accumulated less Cu in both roots and aboveground parts when it was co-cropped with Sedum or when earthworms were present. Earthworms tended to stimulate Cu accumulation in

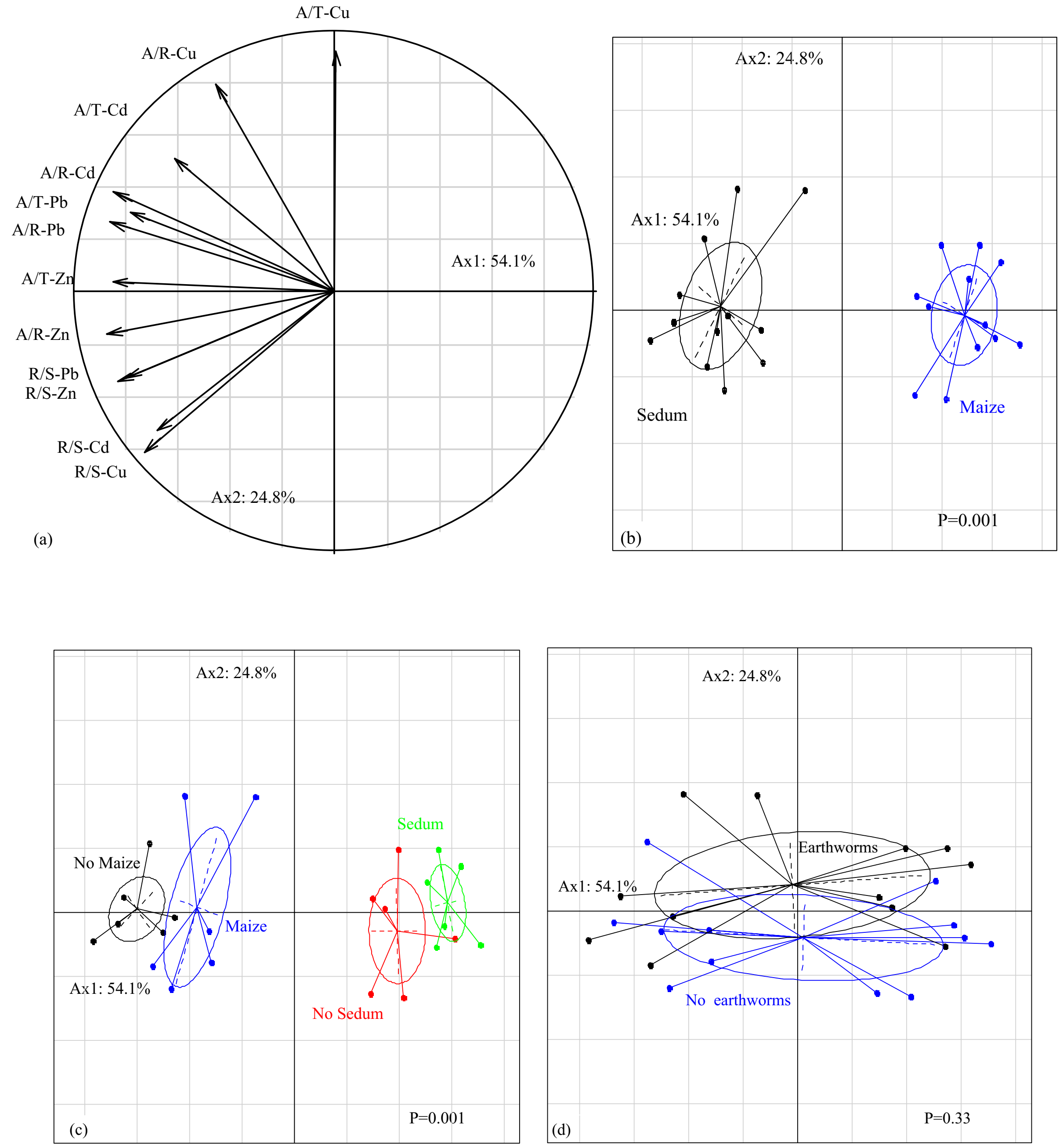


Fig. 2 Principal component analysis of metal transfer from soil to the aboveground parts of plants in the treatments. **a** Correlation circle of metal transfer in treatments in components F1 and F2. **b** Score plot of treatments with Sedum and maize. **c** Score plots of treatments planted with Sedum alone or together with maize and of treatments planted with maize alone or together with Sedum. **d** Score plot of treatments with and without earthworms

the aboveground parts of mono-cropped maize (SFig. 4d, STable 5). Cu accumulation in the whole plants of Sedum increased in the co-cropping and earthworm + Sedum treatments as compared with the Sedum mono-cropping(SFig. 4d, STable 6), with a remarkable 76.4% increase in roots of the co-cropping systems, while accumulation decreased in the aboveground parts.

Metal removal by earthworm and plants

Figure 2 shows the PCA results of metal removal by Sedum and maize from the soil to roots (R/S), metal transfer from roots to the aboveground parts (A/R), and the proportion of metal accumulation in the aboveground parts (A/T). The first axis (54.1% of total inertia explained) ranked the treatments according to general metal transfer intensities (Fig. 2b). Sedum, which accumulated more metals and exhibited larger A/R and A/T ratios, was located on the left end side of axis 1. Sedum removed less metals when it was co-cropped with maize, and similarly, maize accumulated less metals when it was co-cropped with Sedum ($P=0.001$, Fig. 2c). The second axis (24.8% of total inertia explained) ranked the metals according to the metal-accumulating plant parts and metal transfer. The A/R and A/T ratios, that measure transfer to the above ground plant parts, were at the positive end of the axis while the R/S ratios that measure transfer from soil to roots, were at the other end of the axis with higher values for Cu and Cd (Fig. 2a). The presence of earthworms seemed to enhance, though not significantly, the removal of metals from the soil to roots (i.e., larger R/S values), the transfer of metals from the roots to aboveground parts (i.e., larger A/R values), and the accumulation of metals in the aboveground parts of plants (i.e., larger A/T values).

The metal removal indicators showed that Cd and Zn were more effectively removed than Pb and Cu in the co-cropping or earthworm + plant treatments (SFig. 4 and STables 7 and 8). More Zn was removed from the soil to the roots of Sedum in the treatments with maize or/and earthworms (SFig. 4a). In treatments with earthworms, Zn removal from the soil to the roots of maize decreased, and the proportion of Zn in the aboveground parts increased. Cd removal from the soil to the roots of Sedum increased, but the A/R ratio of Cd concentration and the A/T ratio of Cd accumulation decreased in the co-cropping treatments. The opposite was found in the earthworm + Sedum treatment as earthworms enhanced the flux of Cd from roots to above ground parts. Similar results were found for maize. Pb removal from the soil to the roots of Sedum decreased in the co-cropping treatments compared with the Sedum mono-cropping treatment (SFig.4c). The A/R and A/T ratios decreased in the co-cropping treatments as well. The presence of earthworms in the earthworm + Sedum treatment tended to decrease Pb removal from the soil to Sedum roots and resulted in increased A/R and A/T ratios. Pb removal from the soil to the roots of maize increased in the earthworm + maize treatment and especially in the co-cropping treatments compared with the maize mono-cropping treatment. The A/R and A/T ratios decreased in both the earthworm + maize treatment and the co-cropping treatments compared with the maize mono-cropping treatment. Cu removal from the soil to the roots of Sedum increased in the co-cropping treatments, but Cu removal from the soil to the roots of maize decreased in both the earthworm + maize treatment and the co-cropping treatments (SFig.4d). In the earthworm + co-cropping system, Cu transfer from the roots to aboveground parts decreased in Sedum but increased in maize.

Soil DTPA-extractable metal contents and earthworm-phytoremediation

Soil DTPA-extractable metal contents

The DTPA-extractable Cd, Zn, Cu, and Pb contents of soil aggregates in the treatments with plants or/and earthworms decreased as compared with the control bulk soil (SFig. 1a-d and STable 9). The largest decrease in the DTPA-Cd and DTPA-Zn contents of aggregates were found in the Sedum + Maize treatment (40.2% and 51.6%, respectively). The largest decreases in DTPA-Cu and DTPA-Pb were found in the Sedum only treatment (18.6% and 14.9%, respectively). The interaction between maize and Sedum significantly decreased the DTPA-extractable Cd and Zn in soil aggregates. DTPA-Pb increased in all the treatments with both earthworms and plants compared with those with earthworms or plants only.

Meal balance and earthworm-phytoremediation

The percentages of available metals in aggregates were calculated to evaluate the overall effects of plants and earthworms on soil remediation based on comparison with bulk soil in control treatment (STable 10a). In general, the aggregates had higher percentages while the bulk soil had lower percentages of available metals in the treatments with earthworms than in the treatments without ($P < 0.05$). For the soil systems, total available Cd and Zn decreased substantially, especially in the cropping treatments with the presence of earthworms. Total metal uptake by plants was significantly different between treatments as well (STable 10b). The highest percentage of Cd uptake by plants was found in the earthworm + Sedum + maize treatment, which had higher plant uptake of Zn, Pb, and Cu ($P < 0.05$, STable 10b). The highest percentages of available metals, especially Cd and Zn, in the plant-soil systems were found in the earthworm + Sedum + maize treatment ($P < 0.05$). Metal accumulation in the aboveground parts of plants is commonly used to evaluate the efficiency of phytoremediation.

In this study, high accumulations of Cd and Zn in the aboveground parts of plants were found, especially in the earthworm + Sedum + maize treatment. It took the earthworm + Sedum + maize treatment the shortest time to decrease the total Cd and Zn to below their respective acceptable limits in this study (60 days for Cd and 152 days for Zn). In addition, maize cultivated in this association exhibited lower accumulation of pollutants in above ground parts.

Plant growth

Growth of maize

Earthworms significant increased plant height (3.6–15.2%), shoot diameter (18.0–32%), shoot biomass (14.7–17.8%), aboveground (8.90–13.4%), and total biomass (7.14–12.2%) of maize (SFig. 2, $P < 0.05$). A significant interaction effect of Sedum and earthworms on the height of maize was found (STable 11), which showed earthworm increased the height of maize but Sedum decreased it. No significant differences were observed in maize root biomass and shoot:root biomass ratio among treatments ($P > 0.05$).

Growth of Sedum

Shoot, aboveground and total dry weights of Sedum were significantly different between treatments, while plant height, leaf and root dry weights were not significantly different between treatments (SFig. 3 and STable 12). The presence of earthworms increased Sedum shoot biomass in the mono-cropping and co-cropping treatments by 7.2% and 8.1%, respectively, as compared with the corresponding earthworm-free treatments. The shoot biomass of Sedum decreased significantly by 6.7% and 7.5% in Sedum + Maize and Earthworm + Sedum + Maize, respectively ($P < 0.05$). The biomasses of aboveground parts and whole plant of Sedum were higher in the treatments with earthworms than in the other treatments ($P < 0.05$), and the shoot:root biomass ratio also increased in the presence of earthworms ($P = 0.04$).

Soil quality evaluation and soil properties

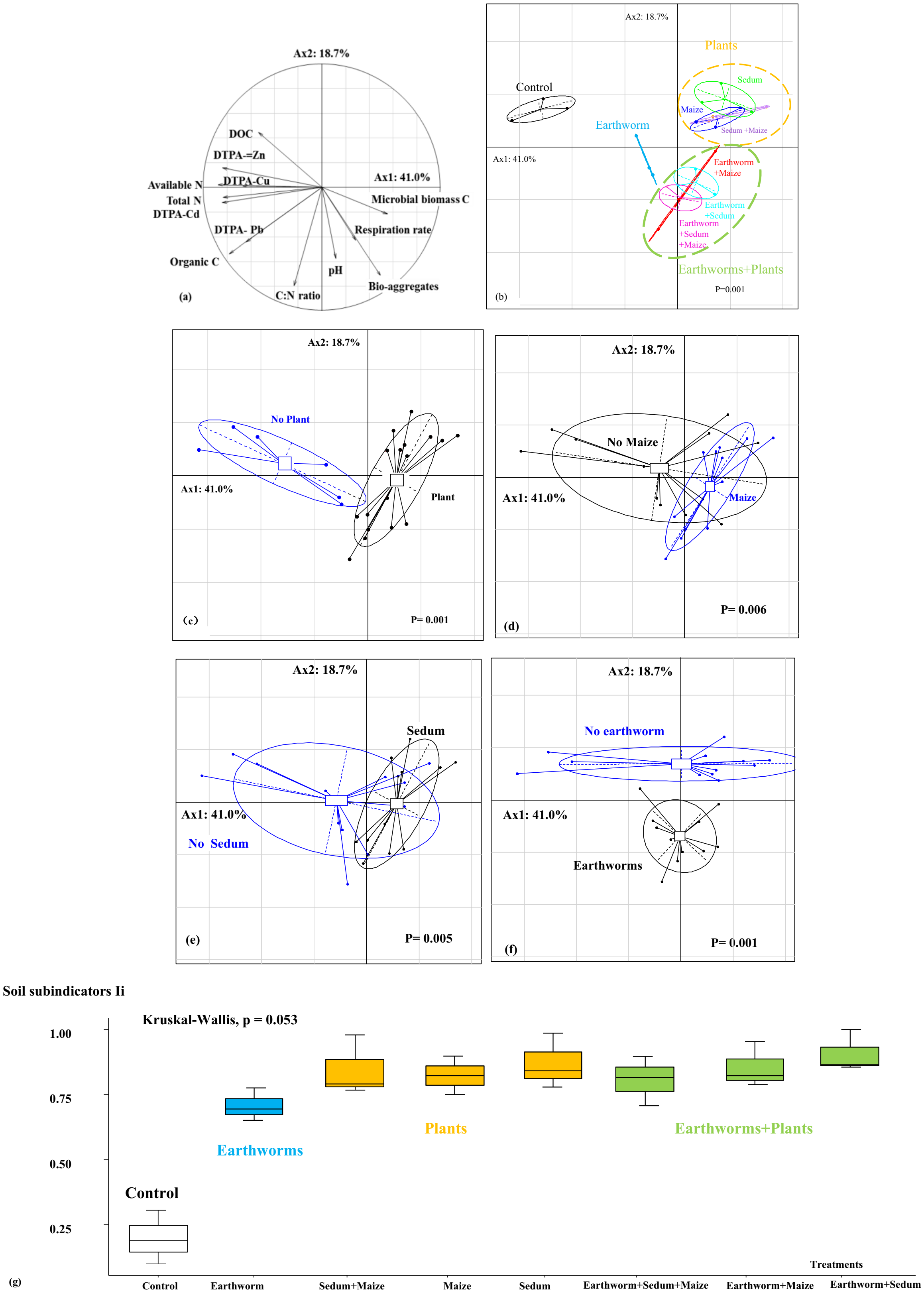
Soil quality evaluation

In general, enhanced mineralization of organic matter in the presence of plants and/or earthworms resulted in decreases in all variables related to this attribute and a correlative increase in microbial biomass C and soil macroaggregates.

The PCA of soil properties showed the significant effect of earthworms, plants, earthworms and plants together on soil properties (Fig. 3). The first axis (41.0% variance explained) expressed the negative effects of plants (maize and Sedum) on soil total N, available N, organic C, DOC, DTPA-Zn, DTPA-Cu, DTPA-Cd, and DTPA-Pb and positive effects on soil macroaggregate formation and microbial biomass C (maize: $P = 0.006$; Sedum: $P = 0.005$; Fig. 3c, d, e). The second axis (18.7% variance explained) separated the treatments according to soil pH and C:N ratio (Fig. 3a, f). It mainly represented the effects of earthworms ($P = 0.001$). Significant differences were observed among the treatments (Fig. 3a, b, $P = 0.001$). Both the plants and earthworms improved soil quality through creating bio-aggregates, improving soil biological properties (e.g., microbial biomass C, respiration rate), and decreasing DTPA-extractable metals. The lowest values of soil quality indicators were found in the control, whereas the highest occurred in treatments with plants.

Soil properties

Significantly more aggregates were formed in the treatments with plants or/and earthworms than in the control (SFig. 1e, $P < 0.05$). The largest amounts of


Ax2: 18.7%
Ax1: 41.0%
DOC
DTPA-=Zn
DTPA-Cu
Available N
Total N
DTPA-Cd
DTPA- Pb
Organic C
C:N ratio
pH
Bio-aggregates
Respiration rate
Microbial biomass C
(a)
Control
Plants
Sedum
Maize
Sedum +Maize
Earthworm
Earthworm +Maize
Earthworm +Sedum
Earthworm +Sedum +Maize
Earthworms+Plants
P=0.001
(b)
No Plant
Plant
P= 0.001
(c)
No Maize
Maize
P= 0.006
(d)
Sedum
No Sedum
P= 0.005
(e)
No earthworm
Earthworms
P= 0.001
(f)
Soil subindicators Ii
Kruskal-Wallis, p = 0.053
1.00
0.75
0.50
0.25
Control
Earthworms
Plants
Earthworms+Plants
Treatments
Control
Earthworm
Sedum+Maize
Maize
Sedum
Earthworm+Sedum+Maize
Earthworm+Maize
Earthworm+Sedum
(g)

◂**Fig. 3** Principal component analysis of soil properties in the different treatments and respective soil quality subindicators values. **a** Correlation circle of variables in treatments in components F1 and F2. **b** Score plot of all treatments. **c** Score plot of treatments with and without plant. **d** Score plot of treatments with and without maize. **e** Score plot of treatments with and without Sedum. **f** Score plot of treatments with and without earthworms. **g** Soil subindicator Ii in the treatments

aggregates were found in the treatments with both plants and earthworms, indicating the contributions of root aggregates and earthworm aggregates and a positive interaction between the earthworms and plants (Sedum or/and maize).

Significant decreases in organic C, DOC, total N, and available N were observed in the treatments with both plants and earthworms compared with the control ($P < 0.05$, SFig. 1g, h, j, k). The organic C content of aggregates decreased by 34.5–36.7% in the treatments with plants but no earthworms and by 7.95–15.0% in all the treatments with earthworms. Similarly, total N decreased by 30.4–32.3% in the treatments with plants but no earthworms and by 18.6–27.0% in all the treatments with earthworms. DOC and available N decreased by 17.5–32.9% and 10.7–34.8%, respectively, in the treatments with plants or/and earthworms.

In addition, higher soil respiration rates and microbial biomass C contents were found in the treatments with plants or/and earthworms, especially in the treatments with maize(SFig. 1l, m). Significant interaction effects of Sedum and maize were found on soil organic C, total N, and available N. Higher concentrations of soil organic C were found in the treatments with earthworms (STable 13, $P < 0.05$). In contrast, no significant differences in the pH of aggregates were found between treatments (SFig. 1f).

Discussion

Metal removal and accumulation in different cropping systems

The effect of earthworms on metal removal from the soils to plants and accumulation in plant tissues differed between the mono-cropping systems and co-cropping systems. In the mono-cropping systems, earthworms exerted a stronger effect on metal removal from the soils to plants and accumulation in plant tissues. The presence of *A. morrisi* increased Pb accumulation in the shoots, leaves, and aboveground parts of Sedum in the earthworm + Sedum treatment (STables 4 and 5), possibly due to the positive effects of earthworms on the organically-bound Pb in the soils with organic matter addition (Zhang et al. 2020). In the meantime, the higher A/T and A/R ratios of Pb reflected that more Pb was transferred from the roots to aboveground parts of Sedum in presence of earthworms and showed the high efficiency of phytoextraction (McGrath and Zhao 2003). Accumulation of Zn, Cd, and Cu in the aboveground parts of maize was promoted by earthworm activity as well (STables 6 and 7), with high A/T and A/R ratios of Zn, Cd and Cu (STable 2 and SFig. 4). A mutualistic relationship between earthworms and soil microorganisms allows digestion of complex soil organic compounds (Barois and Lavelle 1986; Lavelle et al. 1995; Trigo and Lavelle 1993, 1995). Xiong et al. (2008) pointed out that rhizospheric bacteria play an important role in the transfer and accumulation of Zn, Cd, Cu, and Pb in Sedum. Zhang et al. (2016a, b) reported that the earthworm *A. morrisi* increased metal availability by changing microbial activity in a multiple metal-contaminated soil. In this study, we speculated that the increase of heavy metal transfer to plants was probably due to the interaction between earthworms and specific soil microbes. In addition, earthworm mucus in guts and casts is enriched with COOH, N-H, C = O, and COO- functional groups (Silvia et al. 2004), which may increase heavy metal uptake and transport in plants too (Wang et al. 2006; (Zhou et al. 2007; Zhang et al. 2009). To elucidate the mechanisms of how earthworms influence metal uptake and transfer in plants, future studies may need to focus on microbial activities in the rhizosphere and drilosphere, microbial metabolic products, and especially phytohormones that may orientate metal uptake patterns.

The presence of earthworms was expected to maximize metal accumulation in the hyperaccumulator plant while minimizing that in the agricultural crop. However, the earthworms did not have a significant effect on heavy metal accumulation by the plants in the co-cropping system (STables 2–7 and SFig. 4), where the plants had a dominant effect on heavy metal transformations. In the presence of maize, more Cu and Cd were removed from the soil and taken up by the roots of Sedum (STables 3 and 5). Wu et al. (2007b) reported high Zn accumulation in the shoots of Sedum in a co-cropping system. In our experiments, Cu, Cd, Pb, and Zn accumulation in the aboveground parts of maize decreased in the presence of Sedum (STables 4 and 6), possibly due to decreases of heavy metal transfer from the roots to aboveground parts (SFig. 4). The Sedum-maize interaction may have directly (production of phytohormones and allopathic substances) or indirectly (passive uptake of heavy metals due to competition for nutrients) influenced the fate of heavy metals in the co-cropping systems. The path analysis in our study showed that the earthworms had different effects on the growth and metal accumulation of Sedum and maize (Fig. 2 and STable 2). Results also indicated that earthworm inoculation changed soil C and N characteristics, which affected soil microbial biomass and activity and in turn the growth and metal accumulation of plants (Fig. 2 and STable 2). Microbial structure and function in rhizosphere might be related to the selective absorption of metals in *S. alfredii* (Wu et al. 2020; Jacquiod et al. 2020) found that a core microbiota was conserved in the plant-earthworm system and were correlated with an increase in plant growth. Hodson et al. (2023) pointed out that increases of plant growth were due to variations of microbiome brought by earthworm processing of the soil. Therefore, our results suggest that earthworm modulating microbial communities is plant dependent in contaminated soil (Fig. 1). Plant characteristics (i.e. the components of root exudates) may regulate the plant-earthworm interaction at the microbial level. Future studies need to focus more on soil microbial communities in various plants with different earthworm inoculations in contaminated soils.

Soil quality in different cropping systems

Aggregates of different biological origins (roots or earthworms) are indicators of soil quality (Velasquez et al. 2007). In this study, the earthworms and plant roots produced aggregates, and the highest production occurred in the treatments with plants and earthworms, reflecting their positive additive effects on soil physical structure (SFig. 1). Similarly, in the study of Zangerlé et al. (2011), the combination of two plant species (*Trifolium pratense* and *Plantago lanceolata* L.) and earthworms increased aggregates in an uncontaminated soil. In our experiment, the significant decrease in organic C, total N, DOC, and available N contents in the aggregates of the treatments with plants indicated significant transformation of organic matter and nutrients in the soil-plant systems (SFig. 1). Nutrients were absorbed by the plants and increased plant growth (SFigs. 2 and 3). Aggregates formed by earthworms are stable and the organic C in such aggregates can be conserved for a long time (Lavelle et al. 2020). In our study, the aggregates in the treatments with earthworms were enriched with organic C, total N, and available N, which is commonly reported in the literature (Zhang et al. 2009; Jouquet et al. 2008; Villenave et al. 1999; Van Groenigen 2019) as a result of selective ingestion of organic particles and further physical protection of the non digested fractions (Lavelle and Spain 2001; Lavelle et al. 2020). Aggregates formed by fine roots only generally have rather short life times (Lavelle et al. 2020). The decrease in C content in root aggregates in this study was probably due to the mineralization of organic binding agents by soil microbes (Lavelle et al. 2020). The increases of soil respiration rate and microbial biomass C in aggregates in the treatments with both plants and earthworms were presumably due to the decomposition of plant root exudates and soil organic matters by microbes (Wu et al. 2017; Medina-Sauze et al. 2019), and the variations of microbial community in aggregates of different sizes (Liao et al. 2022).

Plant growth and the most optimized remediation system

Stimulation of plant growth by earthworms has been widely demonstrated and the associated mechanisms have been revealed as well (Blouin et al. 2005; Brown et al. 2000; Van Groenigen et al. 2015). In this work, the increase in shoot and aboveground biomasses of Sedum may be attributed to the presence of earthworms (SFig. 3). The presence of earthworms had a stronger effect on maize than on Sedum growth, significantly increasing the plant height, shoot diameter and biomass, and aboveground and total biomasses of maize (SFig. 2). Earthworms stimulate plant growth mainly by improving N availability (Van Groenigen et al. 2015). Higher shoot:root biomass ratios were observed in the treatments with earthworms, indicating that the higher nutrient availability in the presence of earthworms allows plants to invest less in the belowground parts and more in the aboveground parts

(Brown et al. 1999; Lavelle and Spain 2001). Co-cropping significantly decreased the growth of Sedum and maize compared with mono-cropping (Figs. 4 and 5). However, different results were reported by Wu et al. (2007a), who performed co-cropping in 0.81-m^2 plots. The small volume of soil (0.0004 m^3) in the present study may have induced strong competition for nutrients between Sedum and maize and inhibited their growth.

The decrease in DTPA-extractable metals in aggregates was likely due to plant uptake. However, the available metals in aggregates were higher in the treatments with earthworms than in those without. The earthworm + Sedum treatment showed the highest microbial activity and lowest metal availability. Therefore, it had the highest value of soil quality indicator (0.91) among all treatments (Fig. 3f). The highest percentages of soil available metals in the earthworm-Sedum-maize system indicates that the system can be applied to remediate multiple metal-contaminated soils, especially those polluted by Cd and Zn, while producing food. In addition, the soil was purified effectively in a short time by the earthworm + Sedum + maize system. Phytoremediation technology was known to take a long treatment time (Xu et al. 2019). Our results suggest that the earthworm + Sedum + maize combination improved the remediation efficiency of phytoremediation and could be applied in field. Such earthworm-hyperaccumulator plant-crop systems in soil contaminated by metals may also be developed into a novel ecological farming-breeding model in the future. It could provide multiple benefits on limited arable land: crop production, contaminated-soil cleanup, and earthworm breeding earthworms to provide fodder protein or traditional Chinese medicine.

Neverthless, it is worth mentioning that metal accumulation by plants is influenced by many factors (e.g., plant physiological characteristics and environmental conditions). Under the laboratory conditions in our study, the quick effect of the combined phytoextraction may be due to the large inputs of organic matter and earthworms to the limited mass of soil and a simple model calculation based on aggregates. As most nature hyperaccumulator plants have a limited accumulation ability and usually only hyperaccumulate one or two types of metals (McGrath and Zhao 2003), more hyperaccumulator plants were suggested to be tested in such earthworm-hyperaccumulator plant-crop system in the future. To improve this technology, several key questions need to be answered: which earthworm species, hyperaccumulator plant and crop could be used? What is the approporiate ratio between hyperaccumulator plant and crop? What kind of metal-contaminated soil could be remediated by such systems? How efficient will such systems be in field condition?

Conclusion

Earthworm activity increased the aboveground biomass of the hyperaccumulator *S. alfredii* and the agricultural crop maize in the polluted soil with organic amendment, and the increasing effect was much stronger on maize than on *S. alfredii*. Earthworm activity enhanced metal transfer from the bulk soil to aggregates. The effect of earthworms on metal removal was stronger in the mono-cropping system than in the co-cropping system, which indicates a possible environmental risk of earthworm activity in metal-contaminated agricultural fields with mono-cropping systems. Earthworm inoculation influenced the growth and metal accumulation of plants by influencing soil microbial biomass and activity. In the meantime, plant growth characteristics may influence plant-earthworm interaction by influencing soil microorganisms. Moreover, in the co-cropping system with earthworms, the percentages of soil available metals increased most, indicating that the earthworm + Sedum + maize could be applied to remediate soils contaminated with multiple metals. Co-cropping remediation strategy allows for simultaneous agricultural production and soil remediation, which is of significance in China with a large population but limited arable land. In this study, remediation efficiencies were obtained under laboratory conditions. Future studies should be conducted to evaluate remediation efficiencies in field.

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