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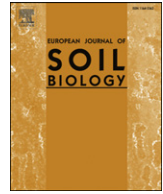
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Original article

Effects of multiple heavy metal contamination and repeated phytoextraction by *Sedum plumbizincicola* on soil microbial propertiesJinping Jiang^a, Longhua Wu^{a,*}, Na Li^a, Yongming Luo^a, Ling Liu^a, Qiguo Zhao^a, Lei Zhang^b, Peter Christie^c^a Key Laboratory of Soil Environment and Pollution Remediation, Institute of Soil Science, Chinese Academy of Sciences, Nanjing 210008, China^b Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China^c Agri-Environment Branch, Agri-Food and Biosciences Institute, Newforge Lane, Belfast BT9 5PX, UK

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ABSTRACT

The threat of heavy metal contamination to food and human health in south and east China has become a public concern as industrial development continues. The aims of this study were to investigate the influence of repeated phytoextraction over a two-year period by successive crops of the Zn and Cd hyperaccumulator *Sedum plumbizincicola* on multiple metal contaminated soils and to assess recovery of soil quality. Total and NH₄OAc-extractable Zn and Cd concentrations were significantly reduced in planted soils compared to unplanted soils. Microbial biomass C (C_{mic}), basal respiration and microbial quotient (qM) were significantly and positively correlated and soil metabolic quotient (qCO₂) was negatively correlated with heavy metal concentrations in unplanted soils ($P < 0.05$). However, C_{mic}, basal respiration and qM values increased significantly after phytoremediation by five crops over two years compared to unplanted soil. Urease, β -glucosidase, neutral phosphatase and arylsulfatase activities also increased significantly with decreasing heavy metal contents and hydrolase activity was enhanced in planted soil ($P < 0.05$) compared to the unplanted control. The data indicate the capacity of *S. plumbizincicola* to extract Zn and Cd from contaminated soil and also that phytoremediation had beneficial effects on soil microbial and hydrolase activities, with the metal phytoextraction procedure restoring soil quality.

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1. Introduction

Heavy metals are natural constituents of the Earth's crust but human activities have drastically altered their geochemical cycles and biochemical balance in the biosphere [17]. In recent decades the development of industry and agriculture and activities such as mining and smelting of metal ores, industrial emissions and applications of agrochemicals and fertilizers have all contributed to elevated levels of heavy metals in soils of the Yangtze River Delta in east China and soil metal pollution has become an environmental issue of great public concern [22]. Heavy metals are non-biodegradable and therefore display long-term persistence in aquatic and terrestrial ecosystems. They are potentially harmful to all biota and tend to accumulate in the food chain so that heavy metal contamination represents one of the most pressing threats to water and soil resources and to human health [47].

It is therefore necessary to test and select the most appropriate remediation methods for soils contaminated by heavy metals. Compared with conventional chemical and physical treatment methods, phytoremediation may provide a cost-effective, long-lasting and aesthetic solution for remediation of metal polluted soils [34] and is a new field of environmental remediation that has entered an active development phase during last decade. In recent years the use of repeated phytoextraction (a technology based on the utilization of metal hyperaccumulating plant species that have the capacity to accumulate, translocate and tolerate high concentrations of metals over the complete growth cycle [13]) in managing contaminated sites has attracted a considerable interest [25]. Although hyperaccumulator plants (species that have the capacity to accumulate large quantities of metals from the surrounding soil in their aerial tissues) have been extensively studied for metal phytoextraction and more than four hundred species are known to be metal hyperaccumulators [6], their deployment for the phytoremediation of metal polluted soils presents constraints because the plants tend to be relatively small in general, have slow rates of biomass production, and lack any established cultivation, pest management or harvesting practices [14,44]. Consequently, there is considerable interest in searching

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for and breeding fast-growing and high biomass hyper-accumulators and studying and establishing improved agronomic practices to optimize phytoremediation efficiency.

Sedum plumbizincicola has a remarkable capacity to extract zinc (Zn) and cadmium (Cd) from polluted soils in south and east China and field experiments have indicated the potential of *S. plumbizincicola* for Zn and Cd phytoextraction [45,46]. Although there is much interest in increasing the heavy metal extraction capacity and biomass yields of hyperaccumulators to improve remediation efficiency, their influence on soil quality during the period of phytoremediation has been rarely investigated. The goal of remediating heavy metal contaminated soil is not only to remove metals but also to restore the capacity of the soil to perform or function according to its potential [21]. Thus relevant indicators are urgently needed to assess and monitor soil quality after remediation [1]. Several studies have been carried out on soil quality changes in heavy metal polluted soils after repeated phytoextraction [14,20,30], but experimentally contaminated soils have usually been used and this leads to difficulties interpreting the results because of differences from field soils subjected to long-term metal stress. In addition, short-term phytoremediation is inadequate for assessment of long-term changes in soil quality. It is therefore desirable to study soil quality dynamics during long-term phytoremediation *in situ* and selection of appropriate indicators is key to assessment of soil quality. Biological indicators related to the size, activity and diversity of soil microbial communities are becoming increasingly popular because of their sensitivity to changes in the soil and their capacity to provide information that integrates multiple environmental factors [1].

It is well known that heavy metals can lead to decreased soil quality because metals affect the growth, morphology and metabolism of soil microorganisms through functional disturbance, protein denaturation and destruction of cell membrane integrity [27]. We therefore hypothesized that soil quality can partly recover when metals are removed from contaminated soils by repeated extraction with the Zn and Cd hyperaccumulator *S. plumbizincicola* and that biological indicators of soil quality can be valid monitoring tools. The objectives of the present study were therefore to investigate the effects of repeated phytoextraction by six successive crops of a hyperaccumulator on a range of biological indicators of soil quality in a pot experiment over a two-year time period.

2. Materials and methods

2.1. Soil characteristics and sample preparation

The experiment was conducted from 5 April 2006 to 5 April 2008 in the Institute of Soil Science, Chinese Academy of Sciences, Nanjing. The pot experiment was conducted with soil collected from the top 15 cm of the soil profile of an agricultural field adjacent to a copper smelter near Hangzhou city [22], Zhejiang province, east China. Two soil samples with contrasting degrees of heavy metal contamination were collected, namely a less polluted soil (S1) collected about 150 m from the pollution source and a more polluted soil (S4) collected only 10 m from the copper

smelter. Immediately after collection the two samples were air-dried at room temperature and passed through a 2-mm sieve. Portions of the air-dried samples were then mixed together to give two additional intermediate contamination levels, S2 (2 parts S1 and 1 part S4) and S3 (1 part S1 and 2 parts S4). Selected physico-chemical properties of the soil, a Typic Agri-Udic Ferrosol [19], are shown in Table 1.

The contaminated soils were stored in the dark for one month at 20% humidity and 20 °C. Soil (1.5 kg oven-dry weight) was placed in each plastic pot (16 cm upper diameter, 11 cm basal diameter and 14 cm high) and fertilized with 100 mg kg⁻¹ of N (as NH₄NO₃) and P and K (as KH₂PO₄) before planting. Two treatments for every metal contamination level (i.e. UP, unplanted and P, planted with 5 plants of *S. plumbizincicola* per pot) were set up with five replicates per treatment. Five successive crops of the hyperaccumulator were grown over a two-year time period in a double glazed greenhouse located in Nanjing (32°00' N, 118°48' E) at an altitude of 12 m above sea level within the northern subtropical monsoon climatic zone. During the experiment the maximum, minimum and average temperatures were 35, 15 and 19–31 °C, respectively. The shoots of the first, second, third, fourth, fifth and sixth crops were harvested in July 2006, November 2006, April 2007, July 2007, November 2007 and April 2008. Five seedlings about 3 cm in height were then transplanted into each pot. Throughout the experiment the plants were watered periodically from below as needed and fertilized before the next growth season with the same fertilizers as described above.

2.2. Soil sampling and laboratory procedures

At the end of the experiment the soil in each pot was sampled by collecting five cores using a 2-cm stainless steel soil corer and the five cores were combined to give a composite sample which was divided into two portions after carefully removing the surface litter and fine roots. One portion of fresh soil sample was passed through a 2-mm sieve, sealed using a plastic bag, and stored at 4 °C in a refrigerator prior to determination of microbial properties. Microbial properties and enzyme activity assays were conducted within four weeks of collecting the soil samples. The other portion was air-dried and ground to pass a 0.15-mm sieve for subsequent physico-chemical analysis.

2.3. Soil physico-chemical properties

Soil pH was measured with a glass electrode at a soil:water ratio of 1:2.5. Available phosphorus (P) was extracted with 0.5 M NaHCO₃ by the Olsen method [5]. Total P was determined by H₂SO₄/HClO₄ digestion and analyzed by the molybdenum blue method [26]. Soil organic C (C_{org}) was determined by the Walkley–Black method [37] and total and available nitrogen by Kjeldahl digestion and distillation [7]. Available K was determined by flame photometer after extraction with 1 M NH₄OAc [32]. Total K and total metal concentrations were determined using atomic absorption spectrophotometry (AAS: Varian SpectraAA 220 FS, 220Z, Varian, Palo Alto, CA) following aqua-regia digestion [35]. The extractable

Table 1
Nutrients and 'total' (aqua-regia) metal concentrations in soil used in the pot experiment.

Soil	pH (1:2.5)	C _{org} (g kg ⁻¹)	Total N (g kg ⁻¹)	Total P (g kg ⁻¹)	Total K (g kg ⁻¹)	Total Cd (mg kg ⁻¹)	Total Zn (mg kg ⁻¹)
S1	6.47 ± 0.1	42.2 ± 0.5	3.75 ± 0.05	0.24 ± 0.02	21.6 ± 0.1	1.11 ± 0.14	321 ± 8
S2	6.95 ± 0.4	37.6 ± 2.3	3.19 ± 0.23	0.22 ± 0.03	22.3 ± 0.1	5.82 ± 0.35	2367 ± 11
S3	7.13 ± 0.2	33.7 ± 1.0	2.84 ± 0.09	0.24 ± 0.01	24.4 ± 1.3	10.6 ± 0.5	4343 ± 276
S4	7.24 ± 0.2	29.1 ± 0.6	2.21 ± 0.06	0.22 ± 0.02	22.9 ± 1.1	15.3 ± 0.6	6499 ± 324

C_{org}: total organic carbon. Values are means ± 1 SD.

Table 2
Soil nutrients in pots at the end of the two-year phytoextraction experiment.

Soil	pH (1:2.5)	C _{org} (g kg ⁻¹)	Total N (g kg ⁻¹)	Total K (g kg ⁻¹)	Total P (g kg ⁻¹)	Av-N (mg kg ⁻¹)	Av-P (mg kg ⁻¹)	Av-K (mg kg ⁻¹)
P1	6.02 ± 0.21	32.3 ± 0.48	3.45 ± 0.06	18.3 ± 0.3	0.73 ± 0.03	153 ± 15	42.2 ± 3.5	32.8 ± 4.4
P2	6.33 ± 0.15	30.4 ± 0.41	3.08 ± 0.06	18.5 ± 1.2	0.71 ± 0.03	136 ± 4	21.7 ± 3.5	32.7 ± 2.2
P3	6.74 ± 0.16	28.7 ± 0.74	2.75 ± 0.08	19.0 ± 0.6	0.69 ± 0.04	111 ± 9	20.9 ± 4.0	33.9 ± 4.9
P4	6.91 ± 0.12	24.8 ± 0.76	2.27 ± 0.02	19.6 ± 1.8	0.73 ± 0.04	98.4 ± 10.0	42.3 ± 5.5	29.5 ± 3.6
UP1	6.38 ± 0.24	32.2 ± 0.47	4.06 ± 0.05	21.4 ± 0.5	0.53 ± 0.04	143 ± 7	8.20 ± 0.40	33.0 ± 2.4
UP2	6.78 ± 0.19	30.0 ± 0.27	3.47 ± 0.13	22.1 ± 0.2	0.52 ± 0.02	141 ± 8	8.60 ± 0.40	50.2 ± 4.9
UP3	7.03 ± 0.11	26.2 ± 0.34	3.18 ± 0.09	22.5 ± 0.7	0.52 ± 0.01	124 ± 10	12.0 ± 0.7	75.4 ± 7.8
UP4	7.15 ± 0.13	21.9 ± 0.28	2.61 ± 0.06	24.5 ± 0.3	0.51 ± 0.02	123 ± 8	13.1 ± 1.3	128 ± 13

C_{org}: total organic carbon; Av-N: available N; Av-P: available P; Av-K: available K; P: planted soil; UP: unplanted soil. Values are means ± 1 SD.

fraction of metals was obtained using 1 M NH₄OAc (pH 5.0) [33] and determined by AAS.

2.4. Soil basal respiration

Basal respiration was determined by measuring the CO₂ evolved during 40 days of incubation at 28 °C in which 20 g of each soil sample at 60% of water-holding capacity was placed in a hermetically sealed polyethylene flask with a vial containing 10 ml 0.1 M NaOH and a vial containing 10 ml distilled water in the dark. The NaOH was titrated with 0.05 M HCl every 5 d and replaced with 10 ml 0.1 M NaOH. The metabolic quotient (*q*CO₂) was also calculated by dividing the CO₂-C released from the soil sample in 1 h by the microbial biomass C content [4].

2.5. Soil microbial biomass and related parameters

The fumigation-extraction method [43] was used to determine soil microbial biomass C (C_{mic}) and the concentration of C_{mic} in the extractant was determined using an automated N/C analyzer (Model 2100, Jena Corporation, Germany). A K_{EC} factor of 0.38 [38] was used to convert the C content to microbial biomass C. The soil microbial quotient (*q*M) was calculated as the ratio of C_{mic} to C_{org} and was expressed as mg C_{mic} mg⁻¹ C_{org} [3].

2.6. Enzyme assays

Arylsulfatase (*p*-nitrophenol (*p*-NP) μg g⁻¹ h⁻¹) and neutral phosphatase (*p*-NP μg g⁻¹ h⁻¹) activities were assayed according to the methods of Tabatabai [42], β-glucosidase activity (glucose mg g⁻¹ h⁻¹) by the method of Tabatabai [41] and urease activity (NH₄⁺-N μg g⁻¹ h⁻¹) according to Nannipieri et al. [36]. All enzyme assays were carried out using incubation at 37 °C. The concentration of *p*-NP produced in the arylsulfatase and neutral phosphatase activities was calculated from a *p*-NP curve after subtraction of the absorbance of the controls at 410 nm wavelength. The NH₄⁺ produced by urease activity and the glucose by β-glucosidase activity were calculated from a nitrogen curve and glucose curve after subtraction of the absorbance of the controls at wavelengths of 578 and 508 nm. Product was calculated from a glucose curve after subtraction of the absorbance of the controls at 508 nm. The reagents *p*-nitrophenyl phosphate disodium (PNPP), potassium 4-nitrophenyl sulfate, 3,5-dinitrosalicylic acid, trihydroxymethyl

aminomethane, malic acid, citric acid and urea were all analytical grade and were purchased from Shanghai Chemical Reagent Company.

2.7. Statistical analysis

Data are presented on oven-dry (105 °C) basis as arithmetic mean values per pot ± 1 SD, or in the case of the biological data, mean values ± 1 standard error of the mean (SEM). Data were analyzed by one-way analysis of variance using the SPSS version 13.0 for Windows statistical package. Regression analysis was used to study the relationships between soil metal concentrations and soil biological or physico-chemical parameters and correlation coefficients were calculated. Principal components analysis (PCA) was performed separately on microbial property and enzyme activity data.

3. Results

3.1. *S. plumbizincicola* yields and heavy metal uptake

The plants grew well in soil with all four levels of heavy metal contamination throughout the course of the experiment and showed no visual symptoms of phytotoxicity. Their aboveground biomass yields after 24 months are shown in Table 3, with the largest biomass 92.2 ± 2.1 mg DM per pot in P3 and the lowest biomass 65 ± 1.9 mg DM per pot in P1. The amounts of Zn and Cd accumulation in aboveground fractions of *S. plumbizincicola* were greater in P3 and P4 than P1 and P2 (Tables 4 and 5). Furthermore, the average Zn and Cd concentrations in the aboveground parts of the plants increased with increasing heavy metal concentrations in the soil. However, the efficiency of plant heavy metal uptake decreased with decreasing heavy metal concentrations in the soil (data not shown). As expected, over the four pollution levels, *S. plumbizincicola* showed a remarkable ability to take up Zn and Cd from polluted soils and translocate them to the aboveground parts.

3.2. Effect of *S. plumbizincicola* on soil physico-chemical characteristics

After two years of phytoremediation by the hyperaccumulator the pH value in the planted contaminated soils was between 0.24 and 0.45 units lower than in the unplanted contaminated soils

Table 3
Aboveground biomass of *S. plumbizincicola* at different soil contamination levels (g pot⁻¹).

Treatment	2006		2007		2008	Total biomass over two years
	5/7	15/11	15/4	5/7	15/11	
P1	9.45 ± 0.5	14.2 ± 1.2	9.11 ± 1.1	10.0 ± 0.5	10.2 ± 0.9	65.0 ± 0.8
P2	12.8 ± 0.3	17.1 ± 0.9	14.2 ± 0.8	12.7 ± 0.4	12.5 ± 0.8	84.9 ± 0.8
P3	15.3 ± 0.1	14.2 ± 1.1	15.9 ± 0.8	12.1 ± 0.5	18.0 ± 1.6	92.2 ± 0.9
P4	14.5 ± 0.6	13.2 ± 0.8	8.96 ± 1.50	13.3 ± 0.3	24.1 ± 1.4	84.4 ± 2.4

Values are means ± SEM.

Table 4Zn accumulation in aboveground parts of *S. plumbizincicola* at harvest of 5 consecutive crops and total metal removed (mg pot⁻¹).

Treatment	2006		2007		2008	Total metal removed	
	5/7	15/11	15/4	5/7	15/11		
P1	60 ± 15	60 ± 8	23 ± 5	20 ± 6	15 ± 1	35 ± 5	213 ± 20
P2	110 ± 12	212 ± 62	190 ± 44	206 ± 45	202 ± 29	254 ± 21	1174 ± 47
P3	134 ± 28	189 ± 37	196 ± 28	211 ± 24	256 ± 33	252 ± 32	1238 ± 45
P4	215 ± 65	169 ± 34	117 ± 38	217 ± 12	300 ± 50	145 ± 13	1163 ± 65

Values are means ± SD.

($P < 0.05$). Total C contents were not significantly different between P1 and UP1 and between P2 and UP2, but were significantly higher in planted than unplanted soils for treatments P3, P4, UP3 and UP4 (Table 2). The trends in total N and K were similar in planted and unplanted pots, i.e. values in unplanted soils were significantly higher than planted soils. Differences in available K were similar to total N and K between planted pots and unplanted pots, except that there was no significant difference between P1 and UP1 (Table 2). However, total P and available P contents were significantly higher in planted than unplanted pots and available N did not change significantly over the 24 months except in P4 and UP4 (Table 2).

The concentrations of total Zn and Cd were significantly lower ($P < 0.05$) in soil recovered from planted pots than from unplanted pots (Fig. 1) at all four heavy metal pollution levels. The plants took up between 97 (S1) and 1752 (S3) mg Zn and from 0.89 (S1) to 9.97 (S4) mg Cd kg⁻¹ soil after 24 months (Fig. 1). Similar trends occurred in 1 M NH₄OAc-extractable Zn and Cd, with values decreasing significantly in the planted contaminated soil (Fig. 1). There was clearly no significant difference in total heavy metal content between the original contaminated soil and the unplanted contaminated soil included in the experiment for two years (Table 1 and Fig. 1).

The number of years required for phytoremediation was estimated by establishing an equation for the specific experimental conditions. According to the Chinese Environmental Quality Third Standard for soils (GB 15618-1995) (the minimum concentrations of Cd and Zn are 0.3 mg kg⁻¹ and 300 mg kg⁻¹, respectively), in S1, S2, S3 and S4 remediation of Cd would be complete after 1.5, 3.5, 4.5 and 6.0 years, respectively, but remediation of Zn would not be achieved until phytoextraction was practiced for 0.5, 7.0, 13.0 and 24.0 years.

3.3. Effect of *S. plumbizincicola* on soil microbial characteristics

C_{mic} and qM of the soil with different heavy metal pollution levels are shown in Fig. 2. Significant differences in C_{mic} were found among the pollution levels ($P < 0.05$), and C_{mic} decreased with increasing soil heavy metal concentrations (Fig. 2). The highest C_{mic} values were found in P1 and UP1, with 291 and 265 mg C kg⁻¹ in planted and unplanted pots, respectively, the polluted treatments with the lowest quantities of heavy metals. Furthermore, C_{mic} values in the four polluted treatments increased significantly after two years of phytoremediation and increased by 10.2, 30.2, 99.9 and 118%, respectively. Changing trends in qM were similar to C_{mic} with increasing heavy metal concentrations and the values were

higher in planted than unplanted soil (Fig. 2). However, the ratios were not significantly different between the higher pollution levels (S2, S3 and S4) of both planting treatments nor between planted and unplanted pots at lower pollution levels (S1 and S2) (all $P > 0.05$). The values of the ratio varied from 0.64% in P3 to 0.90% in P1, with an average of 0.73% in planted pots, and from 0.35% in UP3 to 0.82% in UP1, with an average of 0.53% in unplanted pots, respectively (Fig. 2). In general, higher ratio values occurred in planted than unplanted pots and this difference was significant ($P < 0.05$).

Soil basal respiration was determined to evaluate biological activity for the production of mineral nutrients. The results showed a significant decrease with increasing soil heavy metal concentrations (Fig. 2). During the first 40 days of incubation, 1.75 and 1.96 times CO₂-C were respired, respectively, in polluted soils collected from P1 and P4 planted pots compared with unplanted pots UP1 (0.657 mg CO₂-C kg⁻¹ soil h⁻¹) and UP4 (0.366 mg CO₂-C kg⁻¹ soil h⁻¹). Analysis of variance indicates that there were significant differences between planted and unplanted pots. As for soil metabolic quotient (qCO_2) at the end of the experiment, the values showed increase trends with increasing pollution levels in unplanted pots (Fig. 2) but there were no significant differences among the values for planted pots and the values ranged between 3.95 and 4.31 μg CO₂-C (kg C_{mic})⁻¹ h⁻¹ in planted soil and from 2.48 to 4.51 μg CO₂-C (kg C_{mic})⁻¹ h⁻¹ in unplanted soil.

Correlation analysis indicates that soil total Zn and Cd in unplanted pots were significantly negatively correlated with C_{mic} ($r = -0.95$, $P \leq 0.05$ and $r = -0.969$, $P \leq 0.05$, respectively) and basal respiration ($r = -0.971$, $P \leq 0.05$ and $r = -0.991$, $P \leq 0.01$, respectively), and were negatively correlated with qM ($r = -0.916$, $P > 0.05$ and $r = -0.944$, $P > 0.05$ respectively), and positively significantly correlated with qCO_2 ($r = 0.96$, $P \leq 0.05$ and $r = 0.982$, $P \leq 0.05$, respectively). However, total Zn and Cd concentrations in planted pots were negatively correlated with C_{mic} ($r = -0.767$, $P > 0.05$ and $r = -0.789$, $P > 0.05$, respectively), basal respiration ($r = -0.895$, $P > 0.05$ and $r = -0.917$, $P > 0.05$, respectively) and qM ($r = -0.482$, $P > 0.05$ and $r = -0.515$, $P > 0.05$, respectively), and were positively correlated with qCO_2 ($r = 0.143$, $P > 0.05$ and $r = 0.209$, $P > 0.05$, respectively).

3.4. Effect of *S. plumbizincicola* on soil enzyme activities

The urease, β-glucosidase, neutral phosphatase and arylsulfatase activities are shown in Fig. 3. The activities of the four hydrolases decreased with increasing pollution level and significant differences

Table 5Cd accumulation in aboveground parts of *S. plumbizincicola* at harvest of 5 consecutive crops and total metal removed (mg pot⁻¹).

Treatment	2006		2007		2008	Total metal removed	
	5/7	15/11	15/4	5/7	15/11		
P1	0.52 ± 0.03	0.52 ± 0.05	0.12 ± 0.03	0.10 ± 0.02	0.05 ± 0.01	0.11 ± 0.02	1.42 ± 0.22
P2	1.61 ± 0.21	2.21 ± 0.20	1.14 ± 0.25	0.75 ± 0.16	0.56 ± 0.08	0.46 ± 0.10	6.73 ± 0.68
P3	3.34 ± 0.45	3.10 ± 0.56	1.83 ± 0.21	1.61 ± 0.14	1.55 ± 0.46	1.07 ± 0.21	12.50 ± 0.92
P4	4.39 ± 0.54	3.40 ± 1.03	1.43 ± 0.46	1.71 ± 0.18	2.10 ± 0.38	0.97 ± 0.23	14.00 ± 1.30

Values are means ± SD.

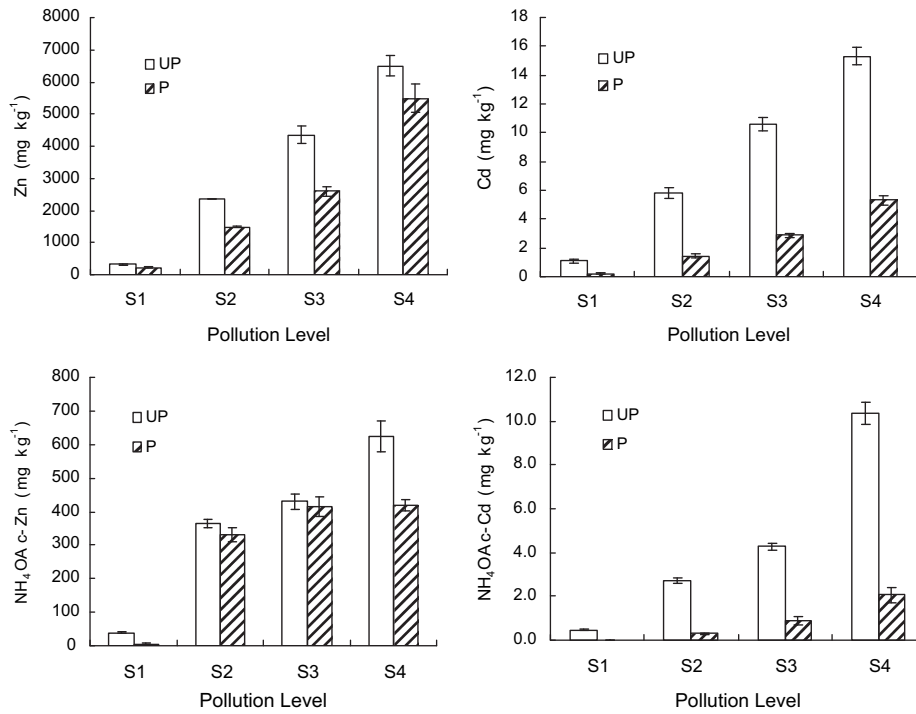


Fig. 1. Total Zn and Cd concentrations and soluble (1 M NH₄OAc extractable) Zn and Cd concentrations in unplanted soil and in soil planted with *S. plumbizincicola* at different heavy metal pollution levels. Two treatments: UP: unplanted pots, and P: pots planted with *S. plumbizincicola*. Values are means \pm SD.

were found among pollution levels (with the exception of urease activity in planted pots) (Fig. 3). In addition, these hydrolytic enzyme activities showed similar trends and were significantly higher in planted than in unplanted soil after two years of phytoremediation. As seen in Fig. 3, the largest values of enzyme activities were found in

S1 pots with lower heavy metals concentrations and the lowest values in S4 with higher heavy metals concentrations in both planted and unplanted pots.

Correlation analysis shows that total Zn and Cd concentrations in unplanted pots were negatively correlated with urease activity

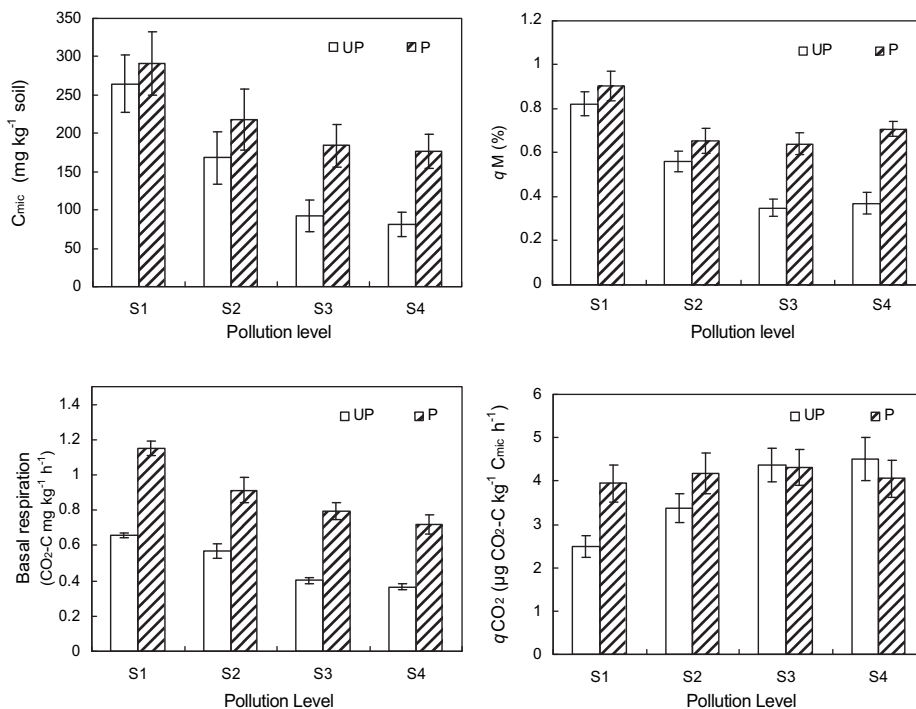


Fig. 2. Effect of phytoremediation on soil microbial properties at different heavy metal pollution levels after 2 years of phytoremediation (5 consecutive crops) in a pot study. Two treatments: UP: unplanted pots, and P: pots planted with *S. plumbizincicola*. qM: microbial quotient; qCO₂: metabolic quotient; C_{mic}: microbial biomass C. Values are means \pm SEM.

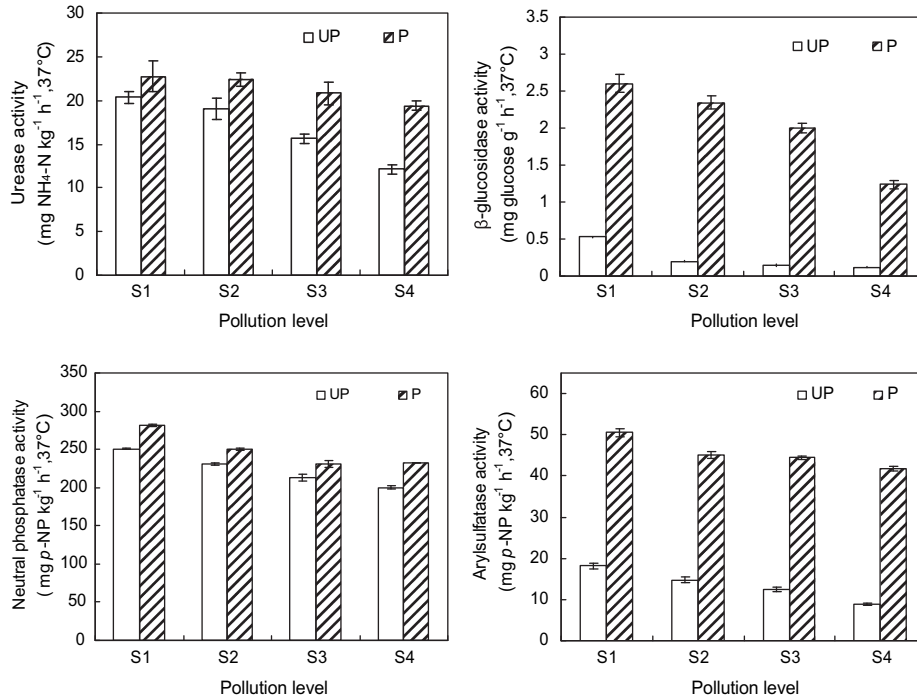


Fig. 3. Effect of treatment on soil enzyme activities at different heavy metal pollution levels, after 2 years of phytoremediation by the hyperaccumulator (5 consecutive crops) in a pot study. Treatments: UP: unplanted pots; and P: pots planted with *S. plumbizincicola*. Data represent mean values of five replicates, and bars represent standard error of mean. p-NP: *p*-Nitrophenol. Values are means ± SEM.

($r = -0.737, P > 0.05$ and $r = -0.746, P > 0.05$ respectively) and β-glucosidase activity ($r = -0.862, P > 0.05$ and $r = -0.873, P > 0.05$ respectively). However, total Zn and Cd concentrations in planted pots were negatively correlated with neutral phosphatase activity ($r = -0.993, P \leq 0.01$ and $r = -0.997, P \leq 0.01$ respectively) and arylsulfatase activity ($r = -0.997, P \leq 0.01$ and $r = -0.985, P \leq 0.05$ respectively). After two years of continuous phytoremediation, total Zn and Cd in planted pots were negatively significantly correlated with urease activity ($r = -0.975, P \leq 0.05$ and $r = -0.996, P \leq 0.01$ respectively) and β-glucosidase activity ($r = -0.989, P \leq 0.05$ and $r = -0.988, P \leq 0.05$ respectively), but total Zn and Cd in planted pots were negatively correlated with neutral phosphatase ($r = -0.797, P > 0.05$ and $r = -0.907, P > 0.05$

respectively) and arylsulfatase activities ($r = -0.834, P > 0.05$ and $r = -0.913, P > 0.05$ respectively).

3.5. PCA analysis

Ordination biplots for PCA analysis of soil microbial property and hydrolase activity data are shown in Fig. 4A. There were two principal components which took together explained 89.5% of the total variance. The first principal component (PC1) accounted for 69.0% of the total variation in the data and was primarily weighed by the heavy metals, C_{mic}, basal respiration and neutral phosphatase activity. PC2 (20.5% of total variance) was primarily affected by qCO₂. The different positions of the different heavy metal polluted

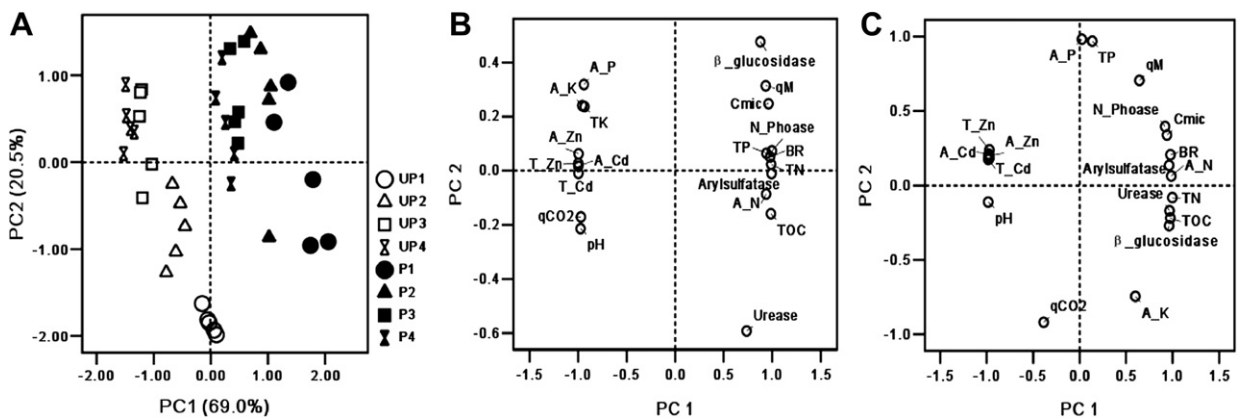


Fig. 4. Principal component analysis (PCA) of nutrients and heavy metals in soil at the end of the pot experiment. (A) Ordination biplot of PCA of soil microbial properties and enzyme activities from different heavy metal polluted soils in unplanted and planted pots; (B) scores and loadings of the microbial and enzyme activities data in relation to soil physical and chemical properties and heavy metals in unplanted pots; (C) scores and loadings of the microbial and enzyme activities data in relation to soil physical and chemical properties and heavy metals in planted pots. Abbreviations, T_Zn: total zinc; T_Cd: total cadmium; A_Zn: soluble (1 M NH₄OAc extractable) zinc; A_Cd: soluble (1 M NH₄OAc extractable); A_K: available K; A_P: available P; A_N: available N; TN: total N; TP: total P; TOC: total organic carbon; C_{mic}: microbial biomass C; qM: microbial quotient; qCO₂: metabolic quotient; BR: basal respiration; urease: urease activity; β-glucosidase: β-glucosidase activity; N_Phose: neutral phosphatase; and Arylsulfatase: arylsulfatase activities.

soils in the plane of the first two principal components indicated that there were large differences in soil quality in these pots. The general soil qualities of different pollution levels were separated from along PC1 and PC2. This also indicates that general soil quality was enhanced by planting with *S. plumbizincicola* and the highest soil quality was showed in P1 according to this ordination.

The loadings of the first two PCs of the microbial property and soil enzyme activity in relation to soil physical and chemical properties under different pollution levels of soil adjacent to a small copper smelter in Zhejiang province for unplanted soils and after phytoremediation are shown in Fig. 4B and C. The biplots given in this figure show that there was considerable change in urease activity, β -glucosidase activity, neutral phosphatase activity, qCO_2 and qM (except for available and total nutrients) between controls and plots after phytoremediation. Urease activity appeared in the same data swarm as available N and total N in phytoremediated soil as shown in Fig. 4C. However, heavy metal concentrations, aryl-sulfatase activity, C_{mic} and basal respiration remained relatively unchanged (Fig. 4B and C) and this factorial axis indicates positive relationships among them. These results were further confirmed by the results of the correlation and regression analysis.

4. Discussion

Six consecutive harvests of *S. plumbizincicola* resulted in significant decreases in soil total Zn and Cd concentrations and also 1 M NH_4OAc -extractable Zn and Cd. The data have also provided further evidence for the potential of *S. plumbizincicola* in Zn and Cd phytoextraction as reported previously [45,46]. This may be due to both its capacity to accumulate high Zn and Cd concentrations in its harvestable parts and the absence of observable phytotoxicity symptoms in soil highly polluted with heavy metals. The DM yield of *S. plumbizincicola* was on average 81.6 g shoot DM pot^{-1} in 1.5 kg of polluted soil each year and there might be scope to further increase yields by various agronomic strategies [28,45].

Some studies have indicated that hyperaccumulator species can extract a similar amount of heavy metals in consecutive crops each year [21,31,45]. In the present study the shoot concentrations of Zn decreased with consecutive harvests in less polluted soil (P1) but increased in more heavily polluted soil (P2, P3 and P4) under controlled greenhouse conditions. One possible explanation is the higher accumulation capability of *S. plumbizincicola* for Zn, together with decreasing available Zn concentrations in less polluted soil leading to lower shoot concentrations of Zn, and with the opposite trend in heavily polluted soil. Shoot Cd concentrations decreased significantly with consecutive crops at all four soil pollution levels, perhaps due to a decrease in available Cd concentrations as phytoremediation proceeded. We therefore propose that the soil exchangeable metal concentration may be important factor and Zn and Cd seem to behave differently in this respect. Heavy metal concentrations in the plants may change with changes in growing conditions. It is difficult to predict the number of years required for phytoremediation to be completed because conditions in pot experiments, including the volume of soil explored by the plant roots, are different from field conditions. However, we have estimated the number of years based on the conditions of the present experiment.

The present study showed clear differences in soil pH, total N, total P, total K, available P and available K between planted and unplanted soils due to fertilization and plant uptake in planted treatments during the experiment. Previous studies have indicated that the main driving force for observed decreases in extractable Zn and Cd was plant uptake of the heavy metals [20]. In our study irrigation and fertilization may have contributed to a pH decrease in contaminated soil, but low pH may be a factor resulting in

a compensating replenishment of bioavailable metals from the total metal pools, leading to high concentrations of extractable Zn and Cd after plant uptake. Values of C_{org} differed little in S1 and S2 between planted and unplanted soils but increased significantly in S3 and S4. It has been suggested that the accumulation of organic C in soils with higher heavy metal pollution might be due to the metals impeding mineralization cycles [10,21], especially in soils with high concentrations of NH_4OAc -extractable forms of metals, as in our study.

In heavy metal polluted soil the growth of *S. plumbizincicola* appeared to have a beneficial effect on soil microbial and enzyme activities and, concomitantly, soil quality. Similar trends were found in both C_{mic} and basal respiration values, i.e. microbial properties were enhanced as metal concentrations decreased in agreement with previous studies [11,48]. The presence of the hyperaccumulator resulted in changes in the soil environment and decreases in bioavailable metals. However, some work has suggested that nutrient elements can obscure the effects of heavy metals on soil microbial characteristics [18,23], and other studies have reported that organic matter and nutrients in heavy metal contaminated soils can stimulate soil microorganisms, resulting in increases in their biomass and activity [2,16]. In the present study most soil nutrients (other than phosphorous in planted soils) showed lower concentrations compared with unplanted soils and any nutrient effect may have been weak compared to the effects of the heavy metals on microbial properties. The basal respiration reflected the activity of the soil microflora, which in turn may be related to the biodegradation of organic compounds in the soil [8]. Our results suggest that the rate of biodegradation of soil organic compounds was restricted in soil with high metal concentrations and that this was ameliorated by the presence of the plants.

qM can be interpreted as available substrate and the portion of C_{org} immobilized in microbial cells is a more sensitive index for measuring C_{org} or microbial biomass only [15]. Some studies have shown a negative correlation between qM and soil heavy metal content, with a range from 0.33 to 1.56% and an average of 0.89% in wastewater irrigation areas [48]. However, in the present study the values of qM were relatively low, ranging from 0.37 to 0.82% with an average of 0.53% in unplanted soil and from 0.64 to 0.91% with an average of 0.71% in planted soil. The heavy metal concentrations in our experimental soil were much higher than in the soils studied by Zhang et al. [48]. The value of qCO_2 has been used as an ecophysiological index that reflects the bioenergetic status of microbial biomass [40]. Previous studies have found that when the soil microflora was exposed for a long time to high metal concentrations its qCO_2 increased, indicating that more energy shifted from microbial growth to maintenance [9]. According this view, the increase in qCO_2 value with increasing metal pollution level in our study indicates that the stress exerted by the metals also increased with increasing metal concentration.

Variable effects of heavy metal contamination on soil enzyme activities are due to the metal type, the contamination mode and the contact time [39]. Kandeler et al. [24] found that soil enzyme activities related to N, P, and S cycles could be significantly affected by heavy metal concentrations in soil. However, changes in soil enzyme activities in multiple heavy metal polluted soils after phytoremediation were due to the collective effect of metals, plants and microbes [29]. Liu et al. [30] reported that urease activity recovered in Cd-contaminated soil by phytoremediation. Hernández-Allica et al. [21] found that enzyme activities in soils with multiple heavy metal pollution increased significantly in the presence of *Thlaspi caerulescens* compared to unplanted controls. Similar results were reported by Li et al. [29] in heavy metal polluted soils with *Sedum alfredii* plants. The present study has supported these findings, with activity of the four hydrolases

increasing with decreasing metal concentrations and higher enzyme activities in planted polluted soils than unplanted controls. This stimulatory effect might be due to the presence of extra C from plant root exudates and a decline in the pool of bioavailable metals and the presence of the plants may have contributed to the activation of the biochemical and microbial functionality of the heavy metal polluted soil [21]. In addition, changes in neutral phosphatase activity may have been due in part to fertilization which may have provided excessive phosphorus.

Epelde et al. [13] concluded that the presence of *T. caerulescens* plants exerted a more pronounced effect on soil biological parameters than metal phytoextraction itself and our data support this view. As can be seen in Figs. 2 and 3, although NH_4OAc -extractable Zn and Cd decreased significantly, changes in microbial properties were very small. One explanation is that the presence of plants may have provided additional surfaces for microbial colonization and organic compounds may have been released by the plant roots [12]. Another factor may be stimulation of reproduction of heavy metal resistant bacteria during continuous phytoextraction.

We conclude that *S. plumbizincicola* may have the potential to effectively phytoextract Zn and Cd from soils with multiple metals pollution and contribute to the recovery of soil quality. Soil microbial properties and enzyme activities can be a useful tool to evaluate the success of the heavy metal phytoremediation process. Further studies are required to determine soil quality under long-term heavy metal stress and by longer term phytoremediation in metal polluted agricultural soils.

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