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REGULAR ARTICLE

Enhanced Cd-Zn-Pb-contaminated soil phytoextraction by *Sedum alfredii* and the rhizosphere bacterial community structure and function by applying organic amendments

Wenhao Yang · Shanshan Wang · Wuzhong Ni · Christopher Rensing · Shihe Xing

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Abstract
Background and aims The phytoextraction efficiency of metal contaminated soil needs to be improved. Organic amendment can be used to enhance the effectiveness of phytoextraction. However, studies on how organic amendment influence the rhizosphere microbial community of hyperaccumulators are still scarce.
Methods Two kinds of spent mushroom substrates (H and T) and their biochars (HB and TB) were used to facilitate the phytoextraction by *Sedum alfredii* Hance. The phytoextraction efficiency was monitored by measuring Cd, Zn and Pb extraction and the subsequent changes of soil microbial biomass, activity and diversity (indicators of soil health and functioning) .
Results Compared to the control, the H and T amendments increased *S. alfredii* Cd uptake by 92% and 73%, and Zn by 106% and 67%, respectively. Organic amendments increased the microbial biomass and activities and substantially changed bacterial composition and diversity of rhizosphere soil. The relative abundances of some rhizosphere beneficial genera such as *Promicromonospora, Acidibacter, Roseiflexus, Microbispora, Kribbella* and *Streptomyces* were significantly increased under the H and T treatments.
Conclusions Spent mushroom substrates enhanced metal extraction of *S. alfredii* and improved soil health by increasing microbial biomass, activity and changing microbial community composition thus providing an effective option to facilitate phytoextraction of metal contaminated soil.

Keywords Cd-Zn-Pb-contaminated soil · Spent mushroom substrates · Biochar · Assisted phytoextraction · Metal extraction · Microbes

Introduction

Phytoextraction, the use of hyperaccumulators to remove metals from soil, is a low cost and environmentally

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W. Yang · S. Wang · S. Xing (✉)
Fujian Provincial Key Laboratory of Soil Environmental Health and Regulation, College of Resource and Environment, Fujian Agriculture and Forestry University, Fuzhou 350002, People's Republic of China
e-mail: fafushx@126.com

W. Yang · S. Wang · S. Xing
Key Research Laboratory on Soil Ecosystem Health and Regulation, Fujian Provincial University, Fuzhou 350002, People's Republic of China

W. Ni
College of Environmental and Resource Sciences, Key Laboratory of Agricultural Resource and Environment of Zhejiang Province, Zhejiang University, Hangzhou 310058, People's Republic of China

C. Rensing (✉)
Institute of Environmental Microbiology, College of Resources and Environment, Fujian Agriculture and Forestry University, Fuzhou 350002, People's Republic of China
e-mail: rensing@fafu.edu.cn

friendly method for the clean-up of metal-contaminated soils (van der Ent et al. 2013; Wood et al. 2016). Although phytoextraction has been extensively studied, in practice, application of this technique usually displays low efficiency and is time-consuming due to the poor bioavailability of metals in soils and the low biomass yield of hyperaccumulators (Burges et al. 2017; Liang et al. 2017). In fact, metal-contaminated soil from mining or industrial sites usually has unfavorable conditions such as poor physical structure, low organic matter and low mineral nutrient content (Mendez and Maier 2008; Pratas et al. 2013; Barbafieri et al. 2018), which may inhibit plant establishment, growth, and subsequent phytoextraction. Therefore, many authors have highlighted the need to optimize the phytoextraction process using appropriate management practices. Recently, the use of organic amendments to facilitate the phytoextractionhas been proposed and tested. Indeed, several studies have shown that applying organic amendment to soil can increase the phytoextraction efficiency directly by increasing soil metal availability (Zhang et al. 2015; Chu et al. 2017), biomass and metal concentrations of hyperaccumulators (Rees et al. 2015; Álvarez-López et al. 2016; Moameri and Khalaki 2017). What is more, organic amendments were shown to display many beneficial effects on soil physico-chemical properties such as pH, electrical conductivity (EC), organic matter and nutrients (Wu et al. 2012; Fellet et al. 2014).

Biochar has attracted attention as a strategy to increase soil carbon sequestration, soil fertility and improve plant growth (Tan et al. 2017). Biochar has high surface area and is highly alkaline and has been widely studied for the immobilization of soil heavy metals (Ahmad et al. 2016; Mohamed et al. 2017). The use of biochar seems counterproductive for the purpose of phytoextraction. For example, a decreased phytoextraction of Cr, Cu and Zn by *Carpobrotus rossii* (Haw.) Schwantes after biochar addition was reported by Zhang et al. (2015). Houben and Sonnet (2015) also showed that biochar derived from miscanthus straw decreased Cd uptake by *Agrostis capillaris*. L. (*A. tenuis* Sibth.). However, some available reports showed contrasting results. Gong et al. (2019) showed that the tea waste biochar increased Cd accumulation and translocation in *Boehmeria nivea* (L.) Gaudich. Rue et al. (2019) reported that biochar increased the phytoextracted Ni by hyperaccumulator *Alyssum murale* Waldst. & Kit. Thus, the effects of biochar on plant metal uptake depended on several factors including types and properties of biochars, soils, plants and elements. Therefore, more investigations are needed in order to understand how biochar could affect the efficiency of metal phytoextraction.

Spent mushroom substrate (SMS) is a by-product after the cultivation of edible mushrooms. China has a large edible mushroom industry, which produces about 75% of the global annual mushroom production (Lou et al. 2017). Large amounts of SMS were generated which would be a waste of resources if dumped directly into the environment and could also result in environmental pollution (Rajput et al. 2009). Thus, finding sustainable solutions for the treatments of SMS has become an increasing concern. In recent years, SMS has been used in agriculture, horticulture and environmental amelioration (Steffen et al. 1994; Marin-Benito et al. 2009; Zhang et al. 2012). Soil application is an effective way for the sustainable recycling of SMS. Previous studies have shown the benefits of using SMS as a soil amendment, including increasing of organic matter and available nutrients (Medina et al. 2012; Peregrina et al. 2012) and improving soil physico-chemical properties (Stewart et al. 1998; Courtney and Mullen 2008). However, few studies have considered the use of SMS and their biochar to increase the phytoextraction efficiency.

Although much work has been conducted on organic amendments assisted phytoextraction, most of these have focused on metal uptake by hyperaccumulators. The ultimate goal of remediating metal-contaminated soil is not only to remove metals from soil but restore soil functionality (Epelde et al. 2014). The basic soil functions include the decomposition of organic matter, storing and transforming of soil mineral elements and production (Volchko et al. 2014). Soil microorganisms play important roles in many soil processes, such as organic matter transformation and nutrient cycling (Ciarkowska et al. 2014). Thus, soil microbial properties, which provide information on the biomass, activity and structure, are useful indicators for the evaluation of soil function (Gómezsagasti et al. 2012). On the other hand, pollutants such as toxic metals have negative effects on soil microbial properties, thus the recovery of soil function during the phytoremediation processes can be assessed by monitoring microbial properties (Epelde et al. 2009; Cui et al. 2016; Burges et al. 2017). Organic amendments (including biochar) could modify the microbial properties in the contaminated

sites without plants (Zhang et al. 2016; Nie et al. 2018). However, to the best of our knowledge, little is known about the effects of organic amendments on rhizosphere microbial properties of hyperaccumulators during phytoextraction, especially the microbial community structure as assessed by high-throughput sequencing technology. Since improvements in microbial properties are essential to the success of remediation, it is necessary to explore the changes of the microbial community in organic amendment assisted phytoextraction.

Sedum alfredii Hance, originating from a mining site in Zhejiang Province in Southeast China, is a vegetatively propagated perennial of the *Crassulaceae* family. It has been reported to be a Zn-Cd hyperaccumulating and Pb accumulating plant species (Yang et al. 2002; Yang et al. 2014), which has potential for usage in phytoextraction of metal contaminated soil. In this study, we conducted a greenhouse experiment to comprehensively evaluate the effects of organic amendments on phytoextraction efficiency, and on both metal uptake and soil function improvements. Finally, the correlation between microbial and physicochemical properties of the rhizosphere soil were analyzed. We hypothesized that SMS and SMS-based biochar could increase the phytoextraction efficiency by increasing *S. alfredii* growth and metal uptake as well as improving rhizosphere soil microbial properties, such as biomass, activity and structure. Soil microbial community structure was analyzed using next generation sequencing technology (Illumina MiSeq). This study intended to explore the feasibility of using SMS and SMSB to facilitate the phytoextraction of Cd-Zn-Pb-contaminated soil by *Sedum alfredii*.

Materials and methods

Soil and plant used for this study

Soil used for the experiment was collected from the top 15 cm of a Pb-Zn mine in Zhejiang province (N 29°13′, E118°47′), China. After collection, soil was air-dried and sieved. Some selected soil properties are shown in Table 1. Soil basal chemical properties were analyzed following Bao (2000). Soil pH was measured by a pH electrode with a soil/Milli-Q water ratio of 1:2.5 (*w*/*v*). Soil total carbon (TC) and total nitrogen (TN) were measured using an elemental analyzer (LECO TruMac, USA). Soil available nitrogen (AN) was determined using the micro-diffusion technique. Available phosphorus (AP) was extracted by $NaHCO_3$ and determined by the molybdenum-blue colorimetric method. Soil total Cd, Zn and Pb were determined by ICP-MS (Agilent 7500a, USA) after digest with HNO3-HClO4-HF.

The seedlings of *S. alfredii* were obtained from a Pb/Zn mine site in Quzhou in Zhejiang Province (N 29°13′, E118°47′), China. The plants have been grown in uncontaminated soil for more than 3 generations to eliminate the influence of the formerly acquired Cd, Zn and Pb in the plants.

Organic amendments preparation

The organic amendments used in this study were two different spent mushroom substrates (SMS) and their biochars (SMSB). The SMSs chosen in this study were collected from two edible mushroom factories, which were built for two different kinds of edible mushrooms. One was used to produce *Hypsizygus marmoreus* (Peck) H. E. Bigelow (H), and the other was used to produce *Tremella fuciformis* Berk (T). After collection, the SMS were dried, smashed and sieved to a size of <2 mm. Then part of them were made for biochar and others were used for the experiment. The pyrolysis of SMS was carried out in a fixed stainless steel furnace. The furnace was purged continuously with high purity N_2 gas at a flow rate of 2 L/min before pyrolysis and at 0.5 L/min to produce O_2 free conditions during pyrolysis. The temperature was heated at a rate of 10 °C/min with the final temperature being at 500 °C. The temperature was maintained for 2 h. Once the pyrolysis process was completed, the furnace was shut off and the produced biochar was allowed to cool down overnight. The biochars of H and T were recorded as HB and TB, respectively. Some basic properties of the SMS and SMSB are shown in Table 1. The pH of raw feedstocks and their biochar samples sieved at 2 mm were analyzed in a suspension (1:10 *w*/*v*) in deionized water by a pH electrode after equilibrated for 1 h (Fidel et al. 2017). TN, TC, AN and AP were analyzed following the methods of Bao (2000), and the details were shown in "*Soil and plant used for this study*".

Growth experiment

A growth experiment was performed to compare the influence of different organic amendments on growth and metal uptake of *S. alfredii*, as well as on soil

Table 1 Selected chemical and physical characteristics of the soil and organic amendments

Characteristics	Soil	T	H	TB	HB
pH	7.7	6.0	6.3	10.4	10.4
Total Carbon g kg^{-1}	10.1	490	410	623	499
Total nitrogen g kg^{-1}	0.5	26.1	20.4	29.0	24.1
Total Phosphorus mg kg^{-1}	2.6	363	1031	390	979
Total Cd mg kg^{-1}	25.7	nd	nd	nd	nd
Total Zn mg kg^{-1}	5108	31.2	30.9	77.8	77.3
Total Pb mg kg^{-1}	13,940	nd	nd	nd	nd

CK, without any amendments; T, addition of spent mushroom substrates for Tremella; H, addition of spent mushroom substrates for *Hypsizygus marmoreus* (Peck) H. E. Bigelow; HB, addition of the biochar of spent mushroom substrates for *Hypsizygus marmoreus* (Peck) H. E. Bigelow; TB, addition of the biochar of spent mushroom substrates for Tremella

microbial properties. Totally, five treatments were carried out consisting of treatment without any amendments (CK) and treatment with addition of different amendments, H, biochar of H (HB), T and biochar of T (TB). The organic amendments were mixed with the mine soil before filling the pots. Biochars (HB and TB) were applied into the soil at a rate of 30 g kg^{-1}. The yield of the biochar was 40%, thus, to ensure the amount of SMS was the same as the amount of SMS that the biochar was derived from, the application rates of SMS were 30/0.4 = 75 g/kg soil.

Healthy and equal-sized plant shoots were chosen and pre-cultivated hydroponically for 3 weeks to grow roots in basic nutrient solution. Then the re-rooted seedings were transplanted to the pots for the experiment. The plants were grown in plastic pots with 20 (diameter) × 10 (height) cm, with drainage holes drilled at the base of the containers. In each pot, there was 1 kg of soil or soil-amendment mixture in total. To separate the rhizosphere soils, a rhizobag technique was used in this study. In this method, a cylindrical rhizobag (diameter 5 cm, height 7 cm) was made of water permeable nylon with a mesh size of 50 μm. Then the rhizobag was placed in the center of the pot. About 200 g soil was put into the rhizobag and the plant was grown in the rhizobag. The soils in the rhizobag were treated as rhizosphere soil. The growth experiment was arranged in randomized block design and performed in triplicate. From June–December 2017, *S. alfredii* were grown in a naturally lit greenhouse at 20–26 °C with a 16 h/8 h day/night cycle (Hou et al. 2017b). The humidity was kept at about 60–70%. The pots were irrigated with deionized water to maintain 60% of soil water holding capacity (WHC) by weight every two days. After 180 d of growth, the plants and the rhizosphere soils were sampled. Soil sampling was completed in plastic bags put into an ice box and then transported to the laboratory. Soil samples were divided into two parts: one portion was air-dried for the analysis of chemical properties, some fresh soil samples were stored at 4 °C for microbial biomass and enzyme activity analysis, and the remaining were frozen rapidly and stored at −80 °C for DNA extraction.

Plant and soil chemical analyses

The harvested plant samples were separated into roots and shoots, and then washed thoroughly with tap water, rinsed with deionized water. The fresh weight, plant height and root length were recorded. Then the plant samples were oven-dried and ground into fine powder. For Cd, Zn and Pb analysis, 0.2 g of plant shoots and roots were digested using a mix of HNO_3 and $HClO_4$ (4:1). Blank determination was performed using the same procedure. Concentrations of Cd, Zn and Pb in the digests were determined using ICP-MS (Agilent 7500a, USA). A standard plant reference material (GSB1–10) were used through all the digestion and analysis process as the quality assurance-quality control protocol (accuracies within 100 ± 10%).

Soil basal chemical properties were analyzed following Bao (2000), and the details were shown in "*Soil and plant used for this study*". Soil DOC (dissolved organic carbon) was extracted following the method of Jones and Willett (2006). And DOC in the solutions was determined using TOC analyzer (Shimadzu TOC-V CPH, Japan). Soil bioavailable Cd, Pb and Zn were extracted by DTPA (0.005 mol L-1 DTPA, 0.1 mol L^{-1} triethanolamine (TEA), and 0.01 mol L^{-1} $CaCl_2$ at pH 7.3) with a 1:5 soil/water ratio. The solutions were shaken in an end-over-end shaker for 1 h, and the concentrations of Cd, Pb and Zn in the extracts were determined using ICP-MS (Agilent 7500a, USA). The blank determination was performed using the same procedure. All these results were expressed on a dried soil weight basis.

Soil microbial biomass carbon, basal respiration and enzyme activity analyses

Soil microbial biomass carbon (MBC) was measured by the fumigation-extraction method (Vance et al. 1987). The organic C concentration was determined using an automated total organic C analyzer (Shimadzu TOC-V CPH, Japan). Soil basal respiration rates were determined by measuring CO_2evolved during 24 h incubation (Vogeler et al. 2008). Soil alkaline phosphatase activity was determined by a colorimetric method (Tan et al. 2018), and the enzymatic activity was expressed as μg phenol g^{-1} dry soil h^{-1}.Soil urease activity was measured following the method of Cang et al. (2009), which was expressed as μg ammonia g^{-1} dry soil h^{-1}. Soil invertase activity was determined according to the method described by Guan (1986), and the result was expressed as mg glucose g^{-1} dry soil 24 h^{-1}.

DNA extraction, and 16S rRNA gene amplification

Soil total DNA was extracted from 500 mg soil by Fast DNA SPIN extraction kits (MP Biomedicals, Santa Ana, CA, USA), following the manufacturer's instructions. The extracted DNA concentrations were determined by the NanoDrop ND-2000 spectrophotometry (NanoDrop Technologies, Thermo Scientific, USA). Bacterium-biased primers 515F (5'-GTGCCAGC MGCCGCGGTAA-3′) and 907R (5'-CCGTCAAT TCMTTTRAGTTT-3′) with barcodes were used to amplify the bacterial 16S rRNA genes V4–V5 region (Hou et al. 2017b). All the PCR amplification reactions were performed in triplicate with mixture containing 4 μL of 5 × Reaction Buffer, 2 μL of dNTPs (2.5 mM), 1 μL of each primer (5 μM), 0.25 μL of High-Fidelity DNA Polymerase (Takara Biotechnology Co., Ltd., Japan), and 10 ng DNA Template. Amplification conditions included an initial denaturation stage of initial denaturation at 98 °C for 2 min, followed by 25 cycles consisting of denaturation at 98 °C for 15 s, 50 °C for 30 s, 72 °C for 30 s, with final extension at 72 °C for 5 min. After PCR amplification, the products were purified using a Qiagen QIA quick Gel Extraction kit (Qiagen Gmbh, Germany), and then pooled in a single tube to get equimolecular concentration (10 ng μL^{-1}) for pyrosequencing, which was performed on an Illlumina MiSeq platform with MiSeq Reagent Kit v3 at a Personal Biotechnology Co., Ltd. (Shanghai, China).

Quantification of total bacterial abundance in soil

Real-time quantitative polymerase chain reaction (qPCR) was performed to determine 16S rRNA gene copy numbers (Hou et al. 2017b), which were determined using the same primer as for pyrosequencing. The qPCR was carried out using a CFX96 Optical Real-Time Detection System (Bio-Rad Laboratories, Inc. Hercules, CA). Each qPCR reaction was carried out in a 20 μL volume having 1 μL of template DNA, 0.5 μM of each primer and 10 μL of *Premix ExTaq*™. All the amplifications were conducted under the following conditions: an initial denaturation step at 95 °C for 30 s, then followed by 40 cycles of amplification (30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C). Serial dilutions of a known amount of DNA were used as standard curves. Each DNA sample was performed in triplicate. Reaction with water instead of DNA were used as the negative control.

Pyrosequencing data processing

The raw data generated from Illumina Miseq sequencing were processed using QIIMEv1.8.0 (Caporaso et al. 2010). Chimeric sequences were identified and removed using the UCHIME (Edgar et al. 2011). The remaining sequences were clustered into operational taxonomic units (OTUs) using the UCLUST program at the 97% similarity level. Alpha diversity metrics were reckoned for soil samples using diversity indices (Shannon, Simpson), which were generated based on the gained OTUs. The principal coordinate analysis (PCoA) based on Bray–Curtis distances was performed using R. To compare the dominating classified genera (relative abundance >1%), a heatmap analysis was performed through R. Hierarchical cluster dendrograms (with Bray-Curtis distance dissimilarities) were generated to compare the bacterial community structures across all the soil treatments through QIIME.

Statistical analysis

Data of plant biomass, metal concentrations, soil chemical properties and soil microbial properties such as microbial biomass, basal respiration and enzyme activities were presented as mean ± standard error of the mean based on three replicates. The differences between each treatment were performed using one-way analysis of variance (ANOVA) followed by Tukey's test ($P <$

0.05). These statistical analyses were conducted using Excel 2016 and SPSS18.0. Redundancy analysis (RDA) of the correlation between the bacterial community and environmental variables was performed using Canoco 5.0.

Results

Plant growth and metal uptake by Sedum alfredii

As shown in Table 2, in general, treatment with organic amendments increased biomass production of *S. alfredii* compared to the untreated controls (CK). Compared to the CK, the T, H, TB and HB increased the dry weights of the shoots by 41%, 91%, 32% and 49%, respectively. The height of *S. alfredii* showed no difference but the root length was significantly increased in the biochar treatments (TB and HB) compared to CK.

The T addition significantly increased shoot Cd concentration of *S. alfredii*, while TB and HB decreased shoot Cd concentration of *S. alfredii* (Fig. 1). Concentrations of Zn in the shoots of *S. alfredii* were also significantly increased with T amendments, while they generally decreased under H, TB and HB treatment. For Pb, only TB significantly increased the shoot concentration, while H show a decreased effect. In addition, the total extracted metal by *S. alfredii* was calculated as the metal concentrationin the shoot × shoot biomass. Compared to the CK, both T and H significantly increased the total Cd extraction by *S. alfredii* by 92% and 73%, respectively. Compared to the CK, the T, H, TB and HB treatment increased the shoot Zn extraction by 106%, 67%, 12% and 15%, respectively. All the organic amendments significantly increased Pb accumulation compared to the CK. Compared to the CK, the T, H, TB and HB increased the shoots Pb extraction by 33%, 37%, 55% and 45%, respectively.

Soil physicochemical properties after plant growth

There were highly significant variations in soil pH as a result of applying different organic amendments (Table 3). The rhizosphere pH values were significantly decreased in the SMS treatment (T) compared to the CK rhizosphere soil. While compared to the CK, the rhizosphere pH values were significantly increased with the incorporation of biochars (HB and TB). Soil TC, TN, available P and K significantly increased by the presence of organic amendments (Table 3). The H and T significantly increased soil DOC and available N compared to the CK (Table 3). The DTPA Cd and Zn were significantly increased under the T treatment, which were increased by 40% and 31%, respectively. The HB treatment significantly decreased soil DTPA Cd and Pb. The DTPA Pb was also significantly lower in the H treatment than the CK (Table 3).

Soil microbial biomass and activity

After 180 d of growth, T and H addition significantly increased the soil microbial biomass as indicated by both MBC and bacterial 16S rRNA gene copy numbers (Table 4). Although not significant, the biochar treatments also increased soil MBC. Basal respiration rate and enzymes activities (urease, phosphatase and invertase) were used to serve as the indicators of microbial activity in this study. All the organic amendments significantly increased basal respiration rates. Compared to the CK, the T, H, TB and HB treatments increased the respiration by 92%, 90%, 45% and 65%, respectively. The activities of urease, phosphatase and invertase were

Table 2 Influence of organic amendments addition on growth of *S. alfredii*

Treatment	Fresh weight (g plant^{-1})		Dry weight (g plant^{-1})		Plant Height (cm)	Root length (cm)
	Shoot	Root	Shoot	Root		
CK	4.39 ± 0.34d	0.25 ± 0.01b	0.28 ± 0.04c	0.03 ± 0.00b	19.4 ± 2.0a	8.0 ± 1.2b
T	6.94 ± 0.04b	0.37 ± 0.01a	0.40 ± 0.02b	0.05 ± 0.00a	16.2 ± 1.8a	9.2 ± 0.4b
H	8.90 ± 0.11a	0.36 ± 0.01a	0.54 ± 0.02a	0.05 ± 0.00a	16.5 ± 1.5a	8.8 ± 0.2b
TB	5.91 ± 0.38c	0.28 ± 0.05ab	0.37 ± 0.02b	0.04 ± 0.01ab	16.4 ± 1.0a	12.0 ± 0.4a
HB	6.71 ± 0.03b	0.35 ± 0.04ab	0.42 ± 0.04b	0.05 ± 0.00a	17.1 ± 1.0a	11.7 ± 1.0a

Different letters in the same column indicate significant differences at $P < 0.05$

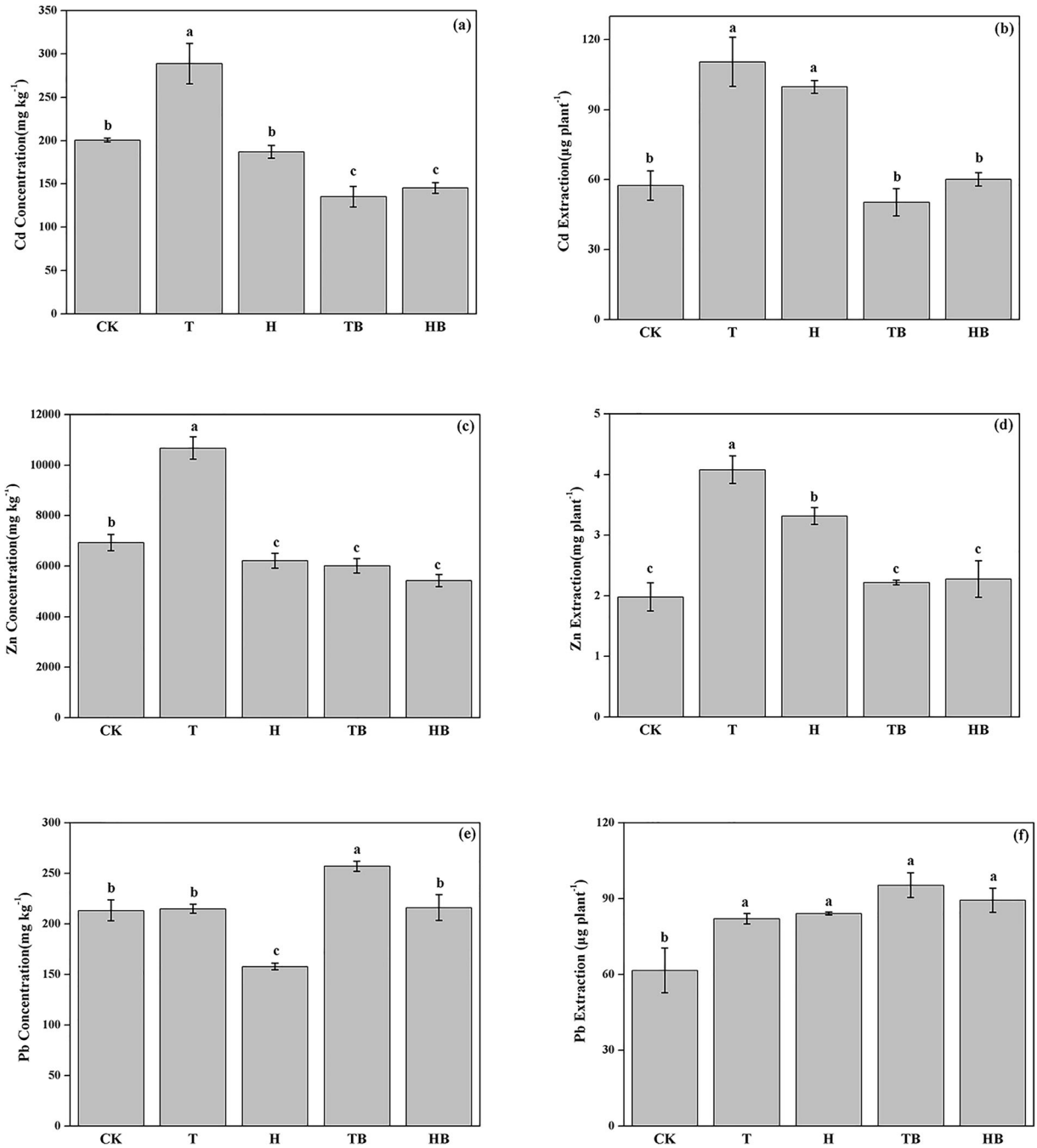

Fig. 1 Cd, Zn, and Pb concentrations (**a**, **b**, **c**) and extractions (**d**, **e**, **f**) in the shoots of *S. alfredii.* CK, without any amendments; T, addition of spent mushroom substrates for *Tremella fuciformis* Berk; H, addition of spent mushroom substrates for *Hypsizygus marmoreus* (Peck) H. E. Bigelow; HB, addition of the biochar of spent mushroom substrates for *Hypsizygus marmoreus* (Peck) H. E. Bigelow; TB, addition of the biochar of spent mushroom substrates for *Tremella fuciformis* Berk. Data representation means ± SE ($n = 3$). Different letters on the error bars indicate significant differences at $P < 0.05$

all significantly enhanced under the T and H treatments. Overall, the biochar treatments did not have significant effects on soil enzyme activities, and HB treatment even decreased phosphatase activity.

Table 3 Influence of organic amendments addition on soil chemical properties

Treatments	pH	TC g kg^{-1}	TN g kg^{-1}	DOC mg kg^{-1}	Available N mg kg^{-1}	Available P mg kg^{-1}	Available K mg kg^{-1}	DTPA Cd mg kg^{-1}	DTPA Zn mg kg^{-1}	DTPA Pb g kg^{-1}
CK	7.70 ± 0.03c	10.0 ± 0.1e	0.48 ± 0.01e	64.0 ± 0.6c	63.9 ± 2.2c	3.3 ± 0.3c	35.6 ± 5.1c	3.23 ± 0.06b	162 ± 2bc	1.58 ± 0.02a
T	7.57 ± 0.01d	43.1 ± 0.4a	2.45 ± 0.03a	282 ± 18b	195 ± 14a	14.0 ± 0.7b	580 ± 5a	4.53 ± 0.03a	212 ± 4a	1.67 ± 0.05a
H	7.75 ± 0.04c	31.9 ± 0.2c	1.92 ± 0.01b	356 ± 13a	148 ± 4b	19.9 ± 1.3a	459 ± 27b	3.37 ± 0.06b	156 ± 6bc	1.31 ± 0.04b
TB	7.92 ± 0.00b	36.6 ± 0.6b	1.63 ± 0.02c	66.6 ± 19.0c	55.5 ± 1.6c	12.5 ± 1.4b	579 ± 19a	3.30 ± 0.06b	166 ± 4b	1.61 ± 0.04a
HB	8.15 ± 0.05a	27.9 ± 0.5d	1.31 ± 0.03d	45.2 ± 1.6c	64.8 ± 2.8c	18.2 ± 0.9a	475 ± 12b	2.92 ± 0.02c	153 ± 3c	1.39 ± 0.05b

Different letters in the same column indicate significant differences at $P < 0.05$

TC, Total carbon; TN, Total nitrogen; DOC, Dissolved organic carbon; Available N, Available nitrogen; Available P, Available Phosphorus; Available K, Available potassium

Microbial community diversity and composition

Soil microbial diversity were estimated by diversity indices (Simpson and Shannon). In this study, the Simpson and Shannon index calculated for 97% OTU clusters were significantly increased under the H and T treatment (Fig. 2), the TB and HB treatments had no significant effect on the microbial diversity.

All the sequences were classified into 26 phyla. As shown in Fig. 3, the ten most abundant phyla that were observed in the rhizosphere soil were *Actinobacteria*, *Proteobacteria*, *Chloroflexi*, *Gemmatimonadetes*, *Acidobacteria*, *Cyanobacteria, Planctomycetes*, *Bacteroidetes*, *Nitrospirae* and *Saccharibacteria*, which accounted for 94.2–98.8% of the total bacterial 16S rRNA gene sequences under all treatments. Variations in the relative contents of phyla were detected between different treatments. Compared to the CK, the T addition increased the relative abundances of *Actinobacteria, Proteobacteria, Planctomycetes* and *Bacteroidetes*, while decreasing the abundance of *Chloroflexi, Gemmatimonadetes, Cyanobacteria* and *Nitrospirae*. The abundances of the phyla *Proteobacteria, Planctomycetes* and *Bacteroidetes* were significantly promoted under the H treatment compared to CK, while decreasing the abundances of*Actinobacteri, Chloroflexi, Gemmatimonadetes* and *Cyanobacteria*. The relative abundances of *Cyanobacteria, Bacteroidetes* and *Nitrospirae* were significantly increased whereas that of *Chloroflexi* was decreased under TB treatment. The relative abundances of *Proteobacteria* and *Nitrospirae* were enhanced by the addition of HB, while the abundance of *Chloroflexi* and *Cyanobacteria* decreased.

A heatmap was generated to visualize the similarities and differences in the microbial community composition between different treatments based on the main genera (>1%). The treatments were classified into two groups based on the abundance of different bacterial genera. It could be found that, the relative abundances of *Dactylosporangium, Kribbella, Streptomyces, Promicromonospora, Nonomuraea, Acidibacter, Roseiflexus, Microbispora* and *Variibacter* were significantly increased under the T treatment compared to CK (Fig. 4). The abundance of *Microbispora, Variibacter, Promicromonospora, Roseiflexus, Acidibacter, Nonomuraea* and *Kribbella* were increased under the H treatment. In the HB treatment, the abundances of *Streptomyces, Variibacter, Sphingomonas, Haliangium* and *Microbispora* was increased. The TB treatment

Table 4 Effects of addition on soil microbial biomass carbon, 16S rRNA gene copy numbers and activities

Treatments	Microbial biomass carbon mg kg^{-1}	16S rRNA gene copy numbers g^{-1} dry soil	Basal respiration mg CO_2-C $kg^{-1}\cdot h^{-1}$	Urease μg ammonia $g^{-1}\cdot h^{-1}$	Phosphatase μg phenol g^{-1} h^{-1}	Invertase mg glucose g^{-1} 24 h^{-1}
CK	49.5 ± 3.2c	5.85E+07 ± 4.29E+06c	5.9 ± 0.3d	6.8 ± 1.2c	58.8 ± 2.4c	0.06 ± 0.00c
T	318 ± 11b	8.32E+08 ± 2.45E+07b	11.4 ± 0.1a	219 ± 12a	140 ± 12b	1.64 ± 0.03b
H	438 ± 14a	1.01E+09 ± 1.09E+08a	11.3 ± 0.6a	134 ± 2b	178 ± 13a	3.20 ± 0.06a
TB	57.6 ± 1.6c	1.32E+08 ± 5.94E+06c	8.6 ± 0.2c	10.9 ± 0.5c	58.6 ± 0.7c	0.10 ± 0.00c
HB	71.6 ± 3.8c	1.41E+08 ± 2.62E+07c	9.7 ± 0.5b	8.8 ± 1.7c	44.5 ± 1.0d	0.08 ± 0.00c

Different letters in the same column indicate significant differences at $P < 0.05$

increased the abundance of *Gossypium_arboreum, Pseudarthrobacter* and *Streptomyces.*

The PCoA based on Bray-Curtis distance of OTUs was used to show the differences in community composition between the different treatments (Fig. 5). PCoA analysis showed that the H and T separated from the CK and the biochar treatments (HB and TB) across the first axis and second axis, and the contribution of PCoA1 and PCoA2 to the bacterial community structure in the rhizosphere soil was 52.21% and 21.20%, respectively. The TB and HB treatments did not separate from the CK treatment. This indicated that the SMS (H and T) exerted significant effects on the bacterial community composition. In addition, the hierarchical clustering (Fig. 6) based on the Bray-Curtis dissimilarity among soil microbial communities also showed that the T and H treatments were classified as one cluster, while the biochar treatments (TB and HB) and control were classified as another cluster. And the HB and TB were classified as one cluster which separated from the CK.

Relationships among the soil microbial and environmental variables

To further evaluate the relative influences of soil chemical properties on soil microbial biomass, activity and community structure (at the phylum level), redundancy analysis (RDA) was conducted (Fig. 7). The results showed a strong separation between the SMS treatments (Hand T) and the CK treatment, which confirmed the PCoA results. In addition, the first two RDA axes explained 98.4% of the total variations, indicating that the environmental properties explained most of the variations of microbial properties between the different treatments. The soils amended with T and H were located at the right margins of the RDA1 axis which was associated with higher DOC, TC, AN, AP, TN, the abundance of *Proteobacteria, Planctomycetes* and *Bacteroidetes,* and lower pH value and the abundance of *Gemmatimonadetes, Chloroflexi, Cyanobacteria* and *Nitrospirae.* Soil MBC, basal respiration, enzyme activities, bacterial 16S rRNA gene copy numbers and the

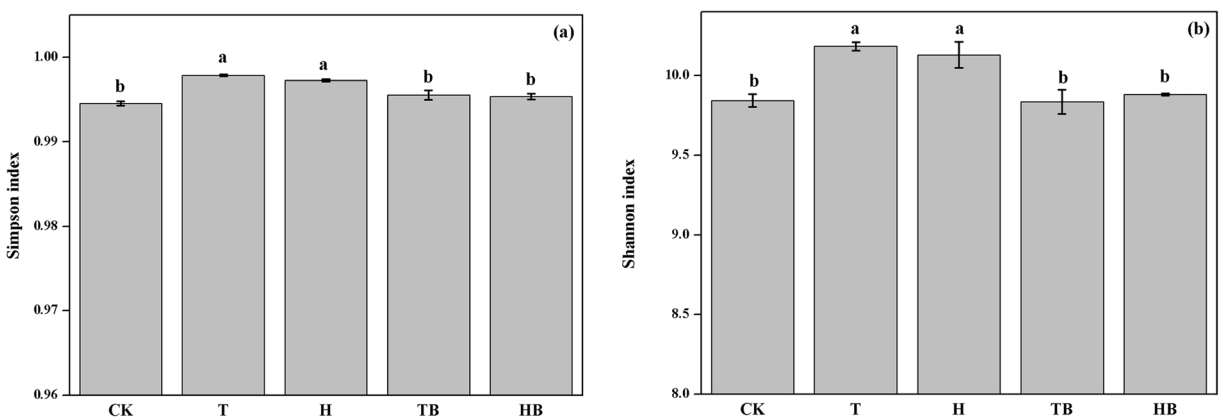

Fig. 2 Bacterial richness and diversity indices under the different treatments. a, Simpson index; b, Shannon index. Data represent means ± SE (n = 3). Different letters on the error bars indicate significant differences at $P < 0.05$

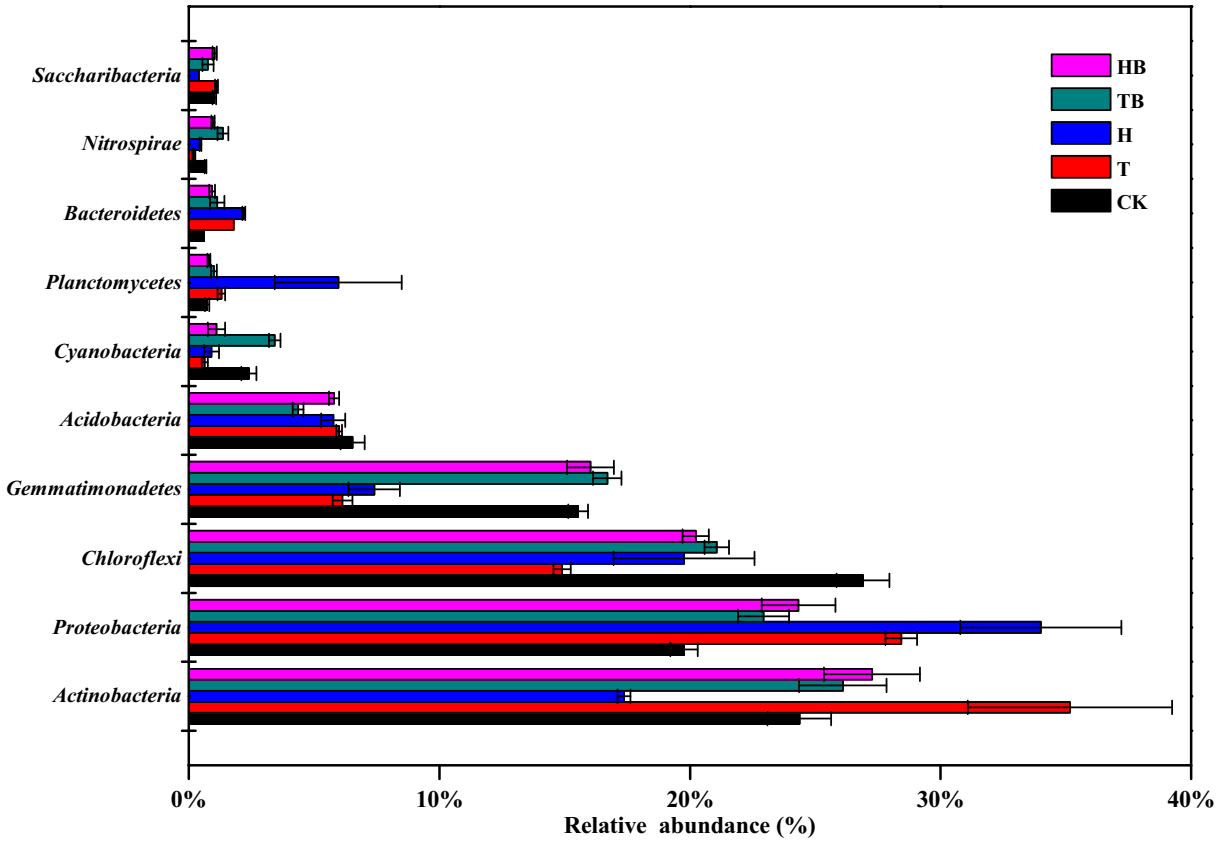

Fig. 3 Relative abundance of bacteria at the phylum level under the different treatments. Data representation means ± SE (n = 3)

abundance of *Planctomycetes*, *Bacteroidetes* and *Proteobacteria* were positively correlated with soil DOC, TC, AN, AP and TN, while negatively correlated with soil pH. The relative abundance of *Gemmatimonadetes, Cyanobacteria, Chloroflexi* and *Nitrospirae* were positively correlated with soil pH, while negatively correlated with soil DOC, TC, AN, AP, TN DTPA-Cd and DTPA-Zn.

Discussion

Influences of organic amendments on growth and metal accumulation of S. alfredii

The amount of metal uptake by plants depends on both the shoot metal concentration as well as on the plant biomass (Chen et al. 2017). In this study, application of organic amendments has a positive effect on the extraction of metals, especially the two kinds of SMSs significantly increased the shoot extracted Cd Pb, and Zn. First, this increased metal extraction may have been caused by increasing the shoot biomass under all treatments with organic amendments (Table 2). Plant growth inhibition is a direct and obvious symptom of plants under metal stress due to the toxicity as well as due to the resulting low nutrient conditions (Gong et al. 2019). The enhanced biomass production by organic amendments may be associated with improvement of soil nutrient conditions. In fact, after mushroom harvesting, SMS still has high levels of organic matter, N, P, K and other nutrients (Paula et al. 2017). In this study, the application of organic amendments significantly increased soil available N, P and K (Table 3), which might promote plant growth. Elouear et al. (2016) reported the improved growth of alfalfa in Cd, Pb, and Zn contaminated soil with sheep manure. Similar results were also reported for *Sedum plumbizincicola* X. H. Guo et S. B. Zhou (Lu et al. 2014) and *Boehmeria nivea* (L.) Gaudich (Gong et al. 2019). In addition, plant growth may also be promoted by increased enzyme activities (Table 4) after application of organic amendments. Enzymes such as urease and phosphatase involved in soil N and P cycling

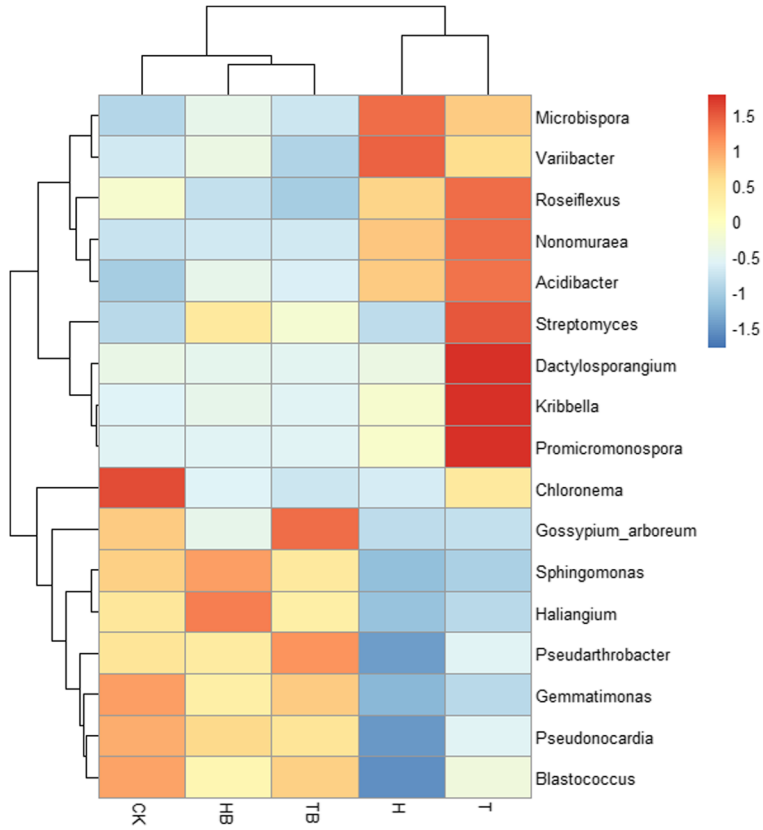

Fig. 4 Heatmap of the relative abundance of the dominant genera (>1%) under different treatments

were shown to be important for soil fertility and plant growth (Bowles et al. 2014).

In addition to the enhancement of plant biomass, the SMS (T) treatment also increased the Cd and Zn

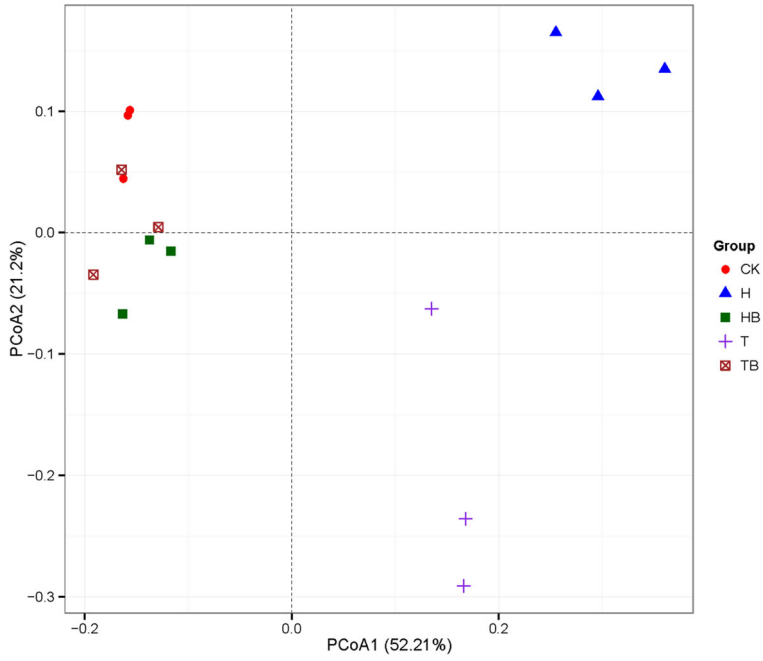

Fig. 5 PCoA of bacteria OTU at 97%level based on Bray-Curtis distance under different treatments

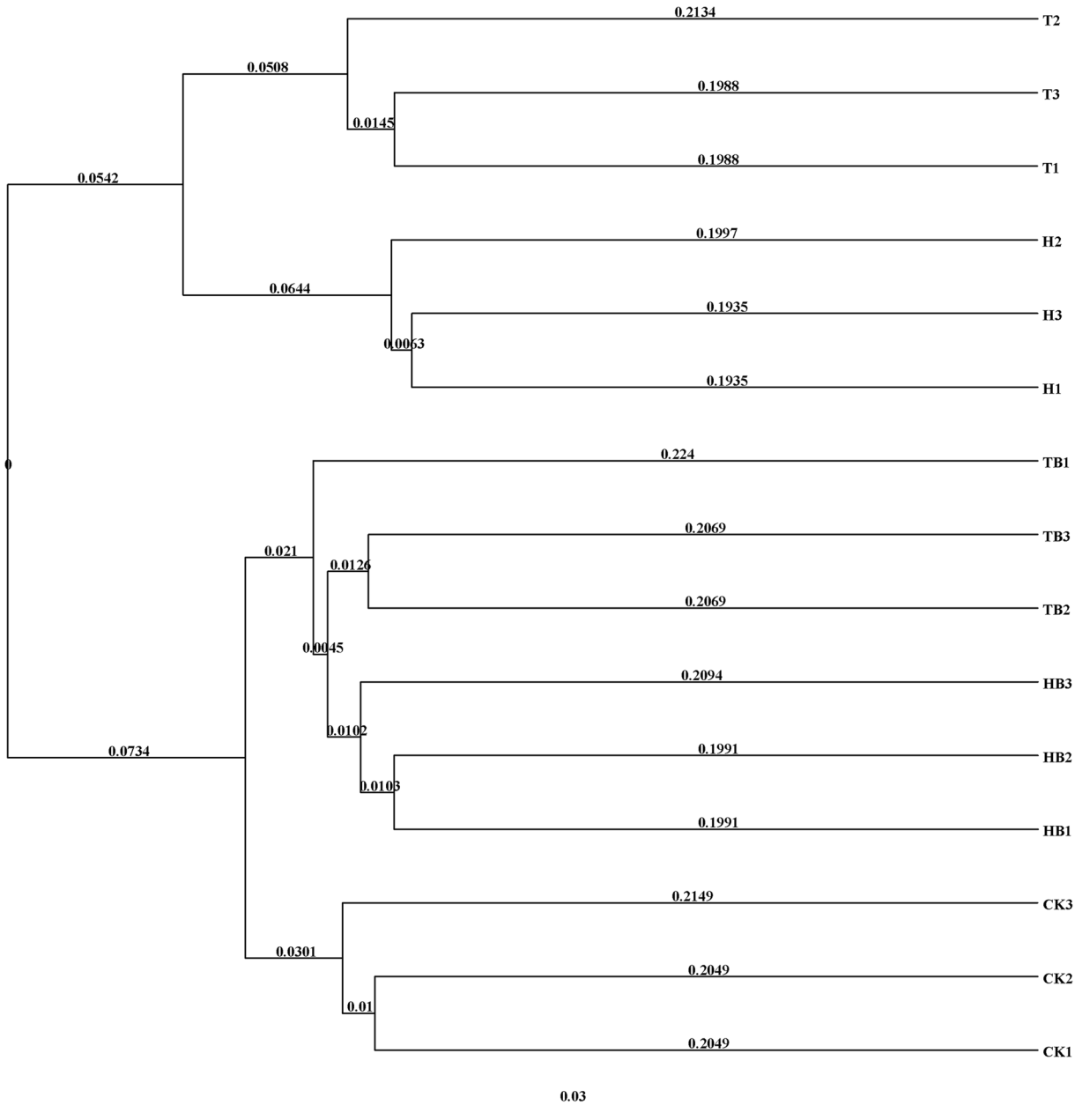

Fig. 6 Hierarchical cluster dendrogram of the bacterial community based on Bray-Curtis distance under different treatments

concentrations of *S. alfredii* (Fig. 1). The increased shoot metal concentrations may also contribute to the increased amount of total metal extraction. The metal concentrations of the hyperaccumulators were reported to correlate with bioavailable metals in soil during phytoextraction (Li et al. 2014). In this study, the T treatment significantly increased soil DTPA-extractable Cd and Zn (Table 3) which facilities the uptake by plants. Positive relationships were found between soil DTPA-extractable Cd and Zn and shoot Cd and Zn concentrations (data not shown). The changes of available metals might correlate to the variations of DOC and pH, which were regarded as key factors controlling metal bioavailability (Alfonso et al. 2016; Rodríguez-Vila et al. 2016). It was found that soil available Cd and Zn were negatively correlated with pH while positively correlated with DOC (data not shown). Thus, the lower soil pH and the higher DOC in the T treatments might

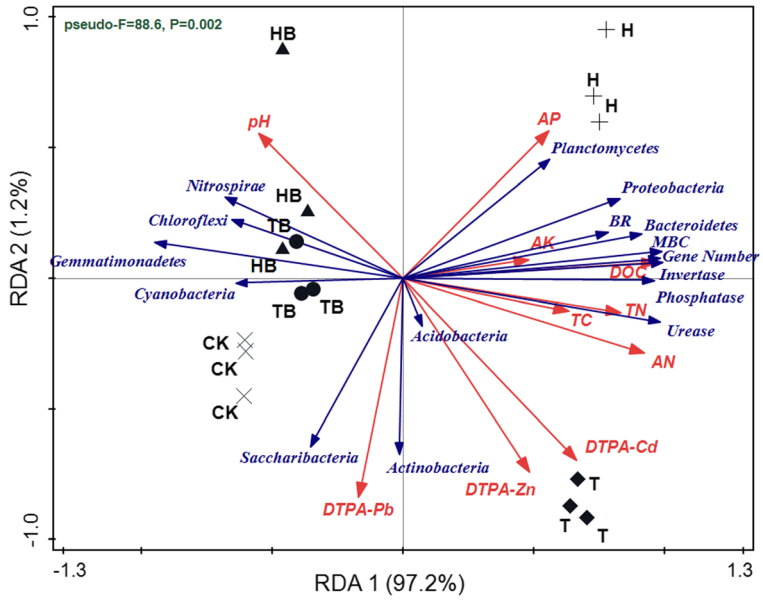

Fig. 7 Biplot of redundancy analysis (RDA) of the relationships between soil microbial biomass, activity and phylum abundance and soil physico-chemical properties. AP, available phosphorus; AK, available potassium; TC, total carbon; TN, total nitrogen; AN, available nitrogen; DOC, dissolved organic carbon; MBC, microbial biomass carbon; BR, basal respiration

lead to the increased soil DTPA metals than the CK and biochar (TB) treatment. Similarly, the increase of total metal extraction by plants after organic amendments was previously reported (Rees et al. 2015; Álvarez-López et al. 2016; Liang et al. 2017). In this study, the increased extraction of Pb under the TB treatments was attributed to an increase in the shoot biomass of *S. alfredii* as well as the increased Pb concentration in the shoots. The significantly higher shoot Pb concentrations under the TB treatment was probably correlated to the increase in DTPA-extractable Pb concentrations (Table 3). Previous studies also showed that the effects of biochar on plant metal extraction varied with biochar types and metal types. Houben and Sonnet (2015) reported that biochar derived from miscanthus straw did not significantly reduce the concentration of Cd, Zn and Pb in both roots and shoots of plants and even increased the Pb concentration in the shoots.

Microbial biomass and activity changed by organic amendments

Soil microbial parameters have great value as biological indicators of soil health. Microbial biomass carbon (MBC) is the most commonly used property to determine microbial abundance and effective indicator of soil fertility (Gregorich et al. 1994). While basal respiration and enzyme activities related to the cycling of C, N and P are most frequently used as indicators of soil microbial activity. Microbial biomass and activity could be negatively affected by the chemical pollutants, such as toxic metals (Burges et al. 2015; Ciarkowska 2018). In the present study, the organic amendment application resulted in significant increases in soil microbial biomass, bacterial 16S rRNA gene numbers, basal respiration and enzyme activities (urease, phosphatase and invertase) relative to the control. The higher microbial biomass and activity due to organic amendments might be due to the following two reasons. The first possible reason was that the high surface area of SMS provided habitats for soil microbes. Moreover, the organic amendment itself can bring exogenous microbes into the soil, which might also contribute to the shift in the soil microbial community. The second reason might be the improvement of soil nutrient levels, such as available C, N and P contents (Table 3). The nutrient conditions were reported to be an important factor restricting the growth of microorganisms in mining soils (Juan et al. 2015; Zhao et al. 2019). Thus, organic amendments increase the supply of organic C (as a source of microbial energy) in addition to increasing available nutrients, which stimulates the growth of the microbial community. The positive correlation between soil microbial biomass, basal respiration and enzyme activities and available C and N, P, K found in this study support this hypothesis (Fig. 7). These findings were in accordance with previous studies. Burges et al. (2016) demonstrated that the organic amendments could increase the microbial biomass and activities of a mine soil. Gregory et al. (2014) also reported that, in an arsenic-

contaminated soil, soil microbial activity was significantly increased under biochar treatments. In this study, these promotions were higher under H and T treatment than the TB and HB, which may be related to the higher soil labile fraction of C and N in the H and T treatments (Table 3). In fact, fresh amendments have more available N than their biochars (Table 1). A previous study showed that differences in organic matter composition and thus substrate availability were the reasons for the differences in microbial properties (Juan et al. 2015).

Responses of microbial community composition to organic amendments

The high-throughput sequencing technology has been shown to be effective analyzing diversity and composition of the microbial community in metal-contaminated and phytoremediated soils (Burges et al. 2017; Fan et al. 2018). In this study, the significant shifts in bacterial community composition (Fig. 3) were consistent with our hypothesis that organic amendments could alter the bacterial community during the phytoextraction process. Especially, the fresh amendments (T and H) showed more obvious effects on bacterial community composition than the biochars (TB and HB). This could be explained by the higher available C and N introduced by T and H. At the phyla level, the predominant phyla in the rhizosphere soils of *S. alfredii* were *Proteobacteria, Actinobacteria, Gemmatimonadetes* and *Chloroflexi*, similar to the bacterial community composition in the rhizosphere of *Sedum alfredii* Hance as previously reported (Hou et al. 2017a, b). *Proteobacteria, Actinobacteria* and *Chloroflexi* were often reported to display high resistance to metals (Gremion et al. 2003; Zhao et al. 2019). In this study, the phyla of *Proteobacteria*, *Bacteroidetes* and *Planctomycetes* displayed greater abundance in the organic amended rhizosphere soils. This greater abundance is probably due to the more nutrient-rich soil conditions after addition of these amendments. Especially under the SMS treatments (T and H), which have relatively large amounts of organic C and available mineral nutrients (N, P and K) (Table 3). These phyla were classified as 'copiotrophic' bacteria, which display higher growth rates under nutrient-rich conditions and are thus enriched in soils with high nutrient availability (Trivedi et al. 2013; Dai et al. 2016; Zheng et al. 2016). The positive relationships between the relative abundances of *Proteobacteria, Bacteroidetes* and *Planctomycetes* and available C and N supported this (Fig. 7). The study by Wu et al. (2019) also showed that the biochar addition increased the relative abundances of *Proteobacteria* and *Bacteroidetes* in a Cd contaminated soil. *Actinobacteria* has been reported as an important group of microbes that colonizes plant rhizosphere, which plays an important role in decomposition of organic matters (Kramer and Gleixner 2008). Due to their often high metal resistance, *Actinobacteria* was identified as one of the most dominant phyla in metal-contaminated soils (Har-Peled et al. 2015). In this study, an increase in the relative abundance of *Actinobacteria* was found in the T, TB and HB amended soil. The increase of *Actinobacteria* due to biochar application have been well documented (Mitchell et al. 2015; Xu et al. 2017). The relative abundances of *Chloroflexi* and *Gemmatimonadetes* were decreased in the T and H amended soils. These lower abundances may be due to a lower dependence of *Chloroflexi* and *Gemmatimonadetes* on the easily available substrates provided by T and H. In contrast, *Chloroflexi* and *Gemmatimonadetes* exhibited relative high abundance in conditions of low nutrient status, such as TB and HB treatments, which due to their higher substrate affinities (Fierer et al. 2007). This could also account for the decreased pH level following the addition of the T and H. Soil pH is reported to have strong effects on the composition of the microbial community. Positive relationships between the relative abundances of *Chloroflexi* and *Gemmatimonadetes* and pH were found in this study. This indicated that *Chloroflexi* and *Gemmatimonadetes* may prefer alkaline environments.

At the genus level, the dominant bacteria were found to be different between treatments. Some beneficial genera were enhanced under the SMSs (H and T) treatments soils (Fig. 4). Among them, *Roseiflexus* have been shown to be involved in organic substances degradation in soil, such as cellulose (Schellenberger et al. 2010). The genus such as *Nonomuraea* has been reported to produce enzymes which are beneficial for the plants (Pudjiraharti et al. 2011). *Streptomyces* had previously been reported to stimulate metal solubility by secretion of siderophores and enhanced the phytoremediation potential of plants (Dimkpa et al. 2009). In addition, *Streptomyces* was reported to participate in the mineralization of various complex compounds and enhance plant disease resistance and also promote plant growth by producing IAA (Golinska and Dahm 2011; Huang et al. 2014). The

genus *Dactylosporangium* was reported to be a rich source of novel secondary metabolites notably antibiotics (Kim et al. 2011). It has been reported that *Variibacter* has the capacity of encoding functional enzymes such as nitrate reduction, gibberellin biosynthesis and inactivation which have interactions with plants (Lee et al. 2016). The genus *Kribbella* belongs to the family *Nocardioidaceae*, which possess metal resistance and produced IAA to promote plant growth (Huang et al. 2014). The species belong to the genus *Promicromonospora* reported to produce plant hormones which were beneficial to plant growth and development (Kang et al. 2012). Thus, the organic amendments recruit certain bacteria with the capacity to break down organic matter, promote nutrients cycling and play a positive role in promoting plant growth. In addition, the increase of these bacteria appears helpful for the better performance of phytoextraction by *S. alfredii*.

Conclusions

In this study, spent mushroom substrate (SMS) and biochar derived from them (SMSB) were used to facilitate the phytoextraction efficiency of *S. alfredii.* The results showed that, the addition of SMS and SMSB to Cd-Zn-Pb-contaminated soil increased the biomass of *S. alfredii*. The Cd, Zn and Pb uptake by *S. alfredii* was enhanced under the SMS amendments. In addition to enhancing metal extraction, rhizosphere soil microbial biomass, activity and diversity (indicators of soil health and functioning) were improved in the organic amendment (SMS) assisted phytoextraction. Positive effects on microbial properties indicated that the combination of *S. alfredii* and SMS could be a suitable strategy for the recovery of soil function in metal-contaminated soils. Our results highlight the potential of SMS in facilitating phytoextraction of metal-contaminated soils. However, long-term field experiments will be needed to assess the feasibility of this assisted phytoextraction.

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