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Mechanisms of biochar derived from chicken manure or rice straw that effectively immobilizes cadmium do not apply to sewage-sludge biochar

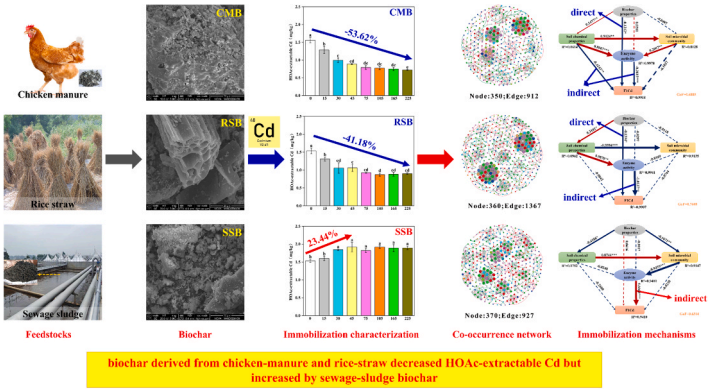
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HIGHLIGHTS

- Available Cd fraction decreased by CMB and RSB but increased by SSB.
- CMB and RSB treatment significantly affected soil chemical properties.
- SSB slightly increased AP and TK content, but decreased urease enzyme activity.
- Microbial diversity was dependent on sampling stage and microbial kingdom.
- CMB and RSB reduced available Cd by direct and indirect effect, whereas indirect effect for SSB.

GRAPHICAL ABSTRACT



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ABSTRACT

In order to explore immobilization characteristic and its possible mechanism of various feedstocks-based biochars, deriving from chicken manure (CMB), rice straw (RSB) and sewage sludge (SSB), the effect of three biochars on speciation transformation of Cd in soil was compared, and soil properties, microbial community diversity were also examined. The treatment of CMB and RSB reduced significantly the content of acid-extractable Cd by 53.62% and 41.18%, respectively, but this available fraction was increased by 23.44% after SSB treatment. Simultaneously, CMB and RSB treatment significantly increased the soil pH, CEC, TOC, AN, AP, TK, microbial biomass carbon, dehydrogenase, phosphatase, catalase and urease enzyme activity, while SSB treatment slightly increased AP and TK content, only decreased urease enzyme activity. Lower bacterial alpha diversity was observed at the initial sampling of day 0, whereas treatment differences in bacterial Shannon diversity were not significant at day 225, it suggested that their effects on microbial alpha diversity were dependent on sampling stage and microbial kingdom. Partial least squares modelling (PLS-PM) speculated that CMB and RSB treatment decreased available Cd through direct effect of biochar and indirect effect of soil, whereas SSB treatment elevated available metal by indirect effect. This increase might be due to the decrease of urease activity indirectly affected by SSB, thus decelerating Cd precipitation and enhancing available Cd. These

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results deepen our understanding on the degree of immobilization and underlying mechanisms for Cd in soil that is critical for evaluating the application of effective biochar treatments.

1. Introduction

Heavy metal contamination in soil, particularly cadmium (Cd), has become a global environmental issue due to its persistence and high mobility in the environment [1]. In China, approximately 19.4% of the agricultural soils are contaminated according to the national survey of soil pollution, among which Cd is ranked the highest in the exceeding percentage of 7% and affected more than 130,000 ha of farmland [2]. Contaminated agricultural soils require the implementation of mitigation strategies, for which the application of biochar has turned out useful, because it immobilizes the heavy metal in what is considered an environmetnally friendly, effective and low-cost remediation technology [3].

Biochar is produced by pyrolysis of waste biomass in an oxygen-limited environment, such as plant residue, livestock manure and sewage sludge. Recently, the majority of previous research has shown that biochar materials had great potential for decreasing the available fraction of heavy metals in acidic soils [4–6]. For example, Zang et al. [7] investigated the immobilization characteristics of Cd by the biochars derived from lignite coal (LCB), rice straw (RSB), silkworm excrement (SEB), and sugar refinery sludge (SSB), respectively, and reported that the acid-extractable fractions of Cd (available speciation) were reduced significantly by 22–35% after biochar treatments. Likewise, Karer et al. [8] studied the immobilization of soil heavy metals by poplar biochar in a 2-year experiment, in which the biochar induced a reduction of available Cd by 87% in the field. Unexpectedly, some other biochars from coconut shell, sugarcane bagasse and sewage sludge did not affect significantly the available fraction of heavy metal in soils, even manure-derived biochar increased available Cd speciation in soil under controlled laboratory settings [9–11]. Thus, application of biochar for heavy metal immobilization in soil needs to be carefully tested before being applied on a commercial scale, and the source of feedstock largely determines the biochar properties and their immobilization efficiencies.

In soil environments, the mechanisms governing the available speciation of heavy metals by biochar could be roughly divided into direct and indirect processes. For the direct process, biochar immobilized heavy metals in soil by chemical adsorption of biochar itself, for which a large specific surface area, presence of certain functional groups, and certain surface minerals are preferred properties. At this point, the surface adsorption mechanisms of biochar mainly included complexation, cation exchange, metal-Cπ coordination, and chemical precipitation, to transform heavy metals from mobile into stable form [12,13]. In comparison with direct effects, indirect effects resulted in the shift of available metal towards residual speciation when biochar addition causes changes in soil pH, organic matter, cation exchange capacity, and microbial activity [14]. For example, the increase of pH could result in chemical precipitation of heavy metals, thus reducing their availability in soils [15]. Another factor contributing to the immobilization of heavy metals in soil by biochar is biological action [16]. Soil microorganisms with a high resistance towards heavy metals could be promoted by biochar, such as certain species of *Pseudomonas*, *Enterobacter* and *Bacillus*, these affected heavy metal availability by multiple mechanisms, including acidification, bioaccumulation and biosurfactant production [17]. These examples highlighted the extreme complexity of heavy metals immobilization by biochars in soil environment, because the relative importance of both immobilization effect (direct and indirect) changed largely depending on biochar feedstock, soil environment, and metal type. Therefore, the relative contribution role of direct and indirect effects on the immobilization for Cd^{2+} in soil remains to be demonstrated.

We selected three commonly used biochar types, produced from chicken manure, rice straw and sewage sludge, to compare immobilization characteristics of Cd in an acidic contaminated soil with the following specific study objectives: (i) to characterize the dynamic changes of Cd speciation, soil chemical properties, and enzyme activity by the presence of different biochars; (ii) to investigate the relationships between soil environmental factors and available Cd after biochar treatments; (iii) to provide insights into the direct and indirect effects of three biochar types on the speciation transformation of Cd. The observations presented here improve our understanding of the controlling factors and mechanisms involved in soil Cd immobilization, which could be critical for evaluating biochar applications.

2. Materials and methods

2.1. Biochar preparation and characterization

Chicken manure (CM) was collected from a local hennery, rice straw (RS) from a farmland and sewage sludge (SS) from a domestic sewage treatment plant in Guangzhou, China. All biochar feedstocks were dried for 48 h at 80°C, followed by grinding and passing through a sieve to obtain < 2.0 mm particles. The powered feedstocks were collected and placed in a stainless steel reaction, and then slowly pyrolyzed increasing from 25 to 700°C at a heating rate of 10 °C/min with the holding temperature of 700°C for 2 h, and under N_2 atmosphere at a flow rate of 50 mL/min. The resulting biochars from CM, RS, and SS were then grinded into powder and passed through a 0.25 mm sieve (60 mesh), which were named CMB, RSB, and SSB, accordingly.

Biochar properties were determined by the methods described by Huang et al. [4] with the following specific details. The pH of biochar was suspended in water 1:20 (w/v) and measured after stirring for 1 h. The ash content was measured by heating at 800 °C for 4 h in a muffle furnace. The surface area was determined by N_2 adsorption using liquid nitrogen by means of a Gemini 2360 Micromeritics surface area analyzer and calculated with the Brunauer-Emmett-Teller equation. The content of C, H, N, and O was determined by an elemental analyzer (Flash EA1112, Thermo Finnigan, Italy), respectively. The mineral content (K, Ca, Na and Mg) was determined by a flame atomic absorption spectrophotometer (FAAS) (M6, Termo Elemental, USA) after microwave digestion. Water-soluble PO_4^{3-} and CO_3^{2-} concentrations were measured by ionic chromatography (IC1000, Dionex Co., Ltd., USA). The Zeta-potential of biochars was determined at different pH values using a potential analyzer (Malvern, Nano ZS90, UK). The isoelectric point (pH_{IEP}) is the pH at which the Zeta-potential is zero, which was obtained by plotting Zeta-potential versus the initial solution pH. Surface characteristics of the biochars were also analyzed using scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDS, Hitachi, Japan), and Fourier transform infrared spectroscopy (FTIR, PerkinElmer 2000, USA), respectively.

2.2. Soil incubation experiments

Soil sample was collected from a heavily polluted paddy field at a depth of 0–20 cm in Shaoguan city, Guangdong Province, China, which was air-dried for two weeks and then passed through a 2-mm sieve. The soil chemical properties were determined by the methods described by Mei et al. [18] with the following test results: pH 5.97 ± 0.09, cation exchange capacity (CEC) 3.92 ± 0.02 cmol/kg, Total organic carbon (TOC) 34.48 ± 1.96 g/kg, Total nitrogen (TN) 2.09 ± 0.13 g/kg, Available nitrogen (AN) 151.88 ± 2.63 mg/kg, Available phosphorus (AP) 52.32 ± 1.65 mg/kg, Total potassium (TK) 0.26 ± 0.01 g/kg, and the contents of Cu, Zn, As, and Cd were 46.43 ± 2.43, 175.85 ± 6.61,

34.64 ± 3.18, and 3.39 ± 0.32 mg/kg, respectively. Among these heavy metals, only the Cd levels were significantly higher than the permissible limit of 0.3 mg/kg set by national soil environmental quality standards.

Each type of biochar sample (40 g) was separately added to 800 g of soil (5.0%, w/w) and mixed thoroughly, which were performed in polythene pots and cultured at 25 °C for 225 days. Throughout the incubation period, soil moisture levels were basically kept constant at 70% of the field moisture capacity by supplying tap water. Soil samples were taken at 0, 15, 30, 45, 75, 105, 165 and 225 day, in which the fraction of the samples were freeze-dried and sieved to determine of the soil chemical properties, Cd speciation and enzyme activities. The remaining sample was frozen and stored at −80°C for subsequent microbial community analysis. In addition, soil chemical properties including pH, CEC, TOC, AN, AP and TK were determined by the methods described above. Microbial biomass carbon (MBC) and nitrogen (MBN) were measured using a chloroform extraction method [19]. The activities of four informative enzymes including dehydrogenase (DHA), catalase (CA), phosphatase (PA) and urease (UA) were determined by the methods of Mei et al. [18], which were expressed as mg TPF g^{-1} h^{-1}, 0.1 M $KMnO_4$ g^{-1} h^{-1}, and mg p-NP g^{-1} h^{-1}, respectively.

2.3. Determination of Cd content and mobility

The fractional analysis of Cd in soil was determined by the method of European Community Reference Bureau (BCR) as described by Rauret et al. [20]. Following this standard procedure, total soil Cd was separated into four fractions with decreasing chemical mobility: the acid-extractable fraction (F1Cd) was extracted with 0.1 M HOAc, reducible fraction (F2Cd) with 0.5 M NH_2O HCl, oxidizable fraction (F3Cd) with 30% H_2O_2 and 1 M NH_4OAc, and residual fraction (F4Cd) was obtained by the above three fractions subtracted from total Cd content. The Cd content of these four fractions was measured by inductively coupled plasma mass spectrometry (ICP-MS, Perkin Elmer 600X, USA). Additionally, the mobility of Cd was expressed as the mobility factor (MF), which was calculated from the following equation:

$$MF = \frac{F1 + F2}{F1 + F2 + F3 + F4} \quad (1)$$

To compare the relative binding intensity of Cd in soil by three different biochars treatment, the reduced partition index (I_R) was determined by the method described by Zahedifar [11], which was calculated based on the following equation:

$$I_R = \frac{\sum_{i=1}^{K}(F_i \times (i^n))}{K^n} \quad (2)$$

where i is the fraction step number, F_i is the percentage of Cd content in the solid-phase component i out of the total extracted, n is set to 2 and K is the number of extraction stages (K=4). The metal distributes in the soluble and exchangeable speciation when I_R is low rate, while the I_R is close to 1, the metal generally bounds in the residual fraction.

2.4. Microbial diversity analysis

At the beginning (day 0) and end of the experiments (day 225), microbial DNA was isolated from the soil and the V4 region of the bacterial 16S rRNA gene was amplified using the primer pair 338 F/806 R, while primer pair ITS1F/ITS2 served to amplify the ITS1 region of the fungal 18S rRNA gene, using the methodology described elsewhere [18]. The two sampling stages were used to compare initial and final microbial community states, and no intermediate microbial sequencing was performed. The resultant amplicons were externally (338 F: 5′-ACT CCT ACG GGA GGC AGC AG-3′; 806 R: 5′-GGA CTA CHV GGG TWT CTA AT-3′), and Primer pair ITS1F/ITS2 (ITS1F: 5′-CTT GGT CAT TTA GAG GAA GTA A-3′; ITS2: 5′-GCT GCG TTC TTC ATC GAT GC-3′) was used to amplify the ITS1 region of the fungal 18S rRNA gene. Sequenced on an Illumina MiSeq platform (Illumina, San Diego, USA) and analyzed by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). Following operative taxonomic units (OTU) attribution by standard procedures, microbial diversity was expressed by Shannon and ChaoI indices. The sequences were binned into phyla and genera, for which relative abundances were reported.

2.5. Data analysis and statistics

Pearson correlation analysis was used to assess associations between soil factors and the abundance of bacteria and fungi at the phylum level. The correlation between biochar properties and the reduction in Cd fractions of F1 and F4 were also assessed. Microbial community structures were visualized by non-metric multidimensional scaling (NMDS), based on Bray-Curtis dissimilarity matrices as described before [18]. Mantel tests (MT) were used to identify relationships between soil properties and microbial community structure, and their interaction patterns were also determined. Co-occurrence network analysis was used as an exploratory approach to characterize statistical association among microbial taxa, for which the WGCNA package was used [21]. OTUs whose relative abundance exceeded 0.1% were retained (Spearman $r > 0.70$ with FDR-corrected p-values < 0.05). The resulting networks were visualized using Gephi 0.10.1. Partial least squares path modeling (PLS-PM) analysis was used as an exploratory multivariate framework to summarize hypothesized direct and indirect statistical associations among biochar properties, soil chemical properties and enzyme activities, and microbial communities with exchange metal fraction F1, using Smart-PLS software v. 3.0 [22].

Path coefficients were interpreted as standardized associations within the specified model rather than as evidence of experimentally demonstrated causal mechanisms. Model evaluation included R values for endogenous constructs and the goodness-of-fit (GoF) index. The retained model output included standardized path coefficients, R^2 values for endogenous constructs, and the goodness-of-fit (GoF) index. Moderate multicollinearity exists among the indicators of the PLS-PM model (variance-inflation, VIF< 10), which falls within the acceptable range and satisfies the model assumptions. GoF and R were used as descriptive measures of the specified models and were not interpreted as evidence of causality.

The obtained data are reported as the mean ± standard deviation where appropriate. Statistical evaluations were performed using SPSS 26.0 software (SPSS Co., Ltd., USA). Owing to temporal dependence of repeatedly sampled data, linear mixed-effects models (LMMs) and repeated-measures ANOVA were applied to quantify the effects of amendment treatment, sampling time and their interaction on measured parameters, with replicate unit as random effect. Tukey's HSD test was used for pairwise comparisons ($p < 0.05$). Origin 2022 (Origin Lab, USA) and R (version 4.3.1) were also employed for data handling and figure generation.

3. Results and discussion

3.1. Biochar properties

The chemical properties of three biochar types were summarized in Table 1. The pH of CMB and RSB was alkaline but that of SSB was weakly acidic, this is probably because the former contained much more alkaline mineral elements including K, Ca, Na and Mg than the latter, in which case these alkaline elements may begin to separate from the organic matrix at high temperature (700 ℃), causing the increase of pH to almost 10.0 [23]. While the pH of SSB was 6.75, this might be resulted from the abundance of Fe demonstrated by EDS spectra (Fig. S1), since it was easy to hydrolysis producing H^+ into the solution [24]. For the ash content, SSB had the largest amount (90.67%) among the three biochars ranging from 42.33% to 90.67%, as partly revealed by SEM observations (Fig. S1). Such combination of high ash and low pH was observed for

Table 1
Properties of the three biochar types.

Index	CMB	RSB	SSB
pH	11.81	10.69	6.75
Ash (%)	54.78	42.33	90.67
pH_{IEP}	2.28	2.15	3.78
S_{BET} (m^2/g)	10.89	41.42	34.8
C (%)	33.77	43.29	8.1
H (%)	0.48	1.45	0.08
O (%)	16.22	14.01	4.11
N (%)	2.84	0.87	0.65
H/C	0.17	0.4	0.12
O/C	0.36	0.24	0.38
K^+ (mg/g)	6.39	6.34	0.06
Ca^{2+} (mg/g)	0.15	0.16	0.14
Na^+ (mg/g)	2.46	0.27	0.04
Mg^{2+} (mg/g)	0.21	0.07	0.03
PO_4^{3-} (mg/g)	1.74	2.25	0.01
CO_3^{2-} (mg/g)	12.17	6.52	N.D

SSB, this was also reported by Xu et al. [24], who compared the biochars produced from sewage sludge and wheat straw. The BET surface area (S_{BET}) was the highest for RSB (41.42 m^2/g), as also supported by SEM results (Fig. S1). In addition, the pH_{IEP} was calculated to be 2.28, 2.15, and 3.78 for CMB, RSB, and SSB, respectively, which might be of benefit to the surface adsorption between heavy metal cations and the biochars by reducing the electrostatic repulsion at pH > pH_{IEP} [4].

Elemental analysis showed that CMB and RSB had higher contents of C, H, O and N than SSB, this was likely due to the strong enrichment rather than volatilization from chicken manure and rice straw feedstocks during the pyrolysis process [25]. Among these biochars, SSB exhibited lower H/C ratio, this suggested that it had more intensive aromatic structure and decreased hydroxyl [26]. The values of O/C were 0.36, 0.24, and 0.38 for CMB, RSB, and SSB, respectively, suggesting the oxygen-containing functional groups was rich in these biochars, as also supported by FTIR observations (Fig. S2).

Compared to SSB, the other two biochars had higher contents of alkaline metal ions including K^+, Ca^{2+}, Na^+, and Mg^{2+}, which was in line with their higher pH. These alkaline metal ions could be replaced by Cd^{2+} during the surface adsorption owing to electrostatic cation exchange [27]. In addition, CMB and RSB contained higher fractions of phosphate and carbonate compared with SSB, these anions played key roles in chemical precipitation of heavy metals [28]. Note that the content of inherent heavy metals in these biochars including Cd, Pb, As, Cu, Zn, Cr, and Ni, were much lower than the maximum allowable thresholds for theses metals (Table S1) according to the International Biochar Initiative (IBI). In combination, significant differences in chemical properties among three biochar types were observed, which might have a profound effect on the transformation characteristics of cadmium speciation in soils after biochar treatment.

3.2. Cd speciation

Total Cd content of actual contaminated soil was relatively high (3.39 ± 0.32 mg/kg), which was divided into the four fractions of acid-extractable (F1Cd, account for 46.23%), reducible (F2Cd, 22.72%), oxidizable (F3Cd, 3.79%), and residual speciation (F4Cd, 27.26%). After biochar treatment, the F1Cd fraction decreased considerably by 53.62% and 41.18% for CMB and RSB, respectively, but it increased by 23.44% in presence of SSB (Fig. 1A). Compared with CK, both the F2Cd and F3Cd

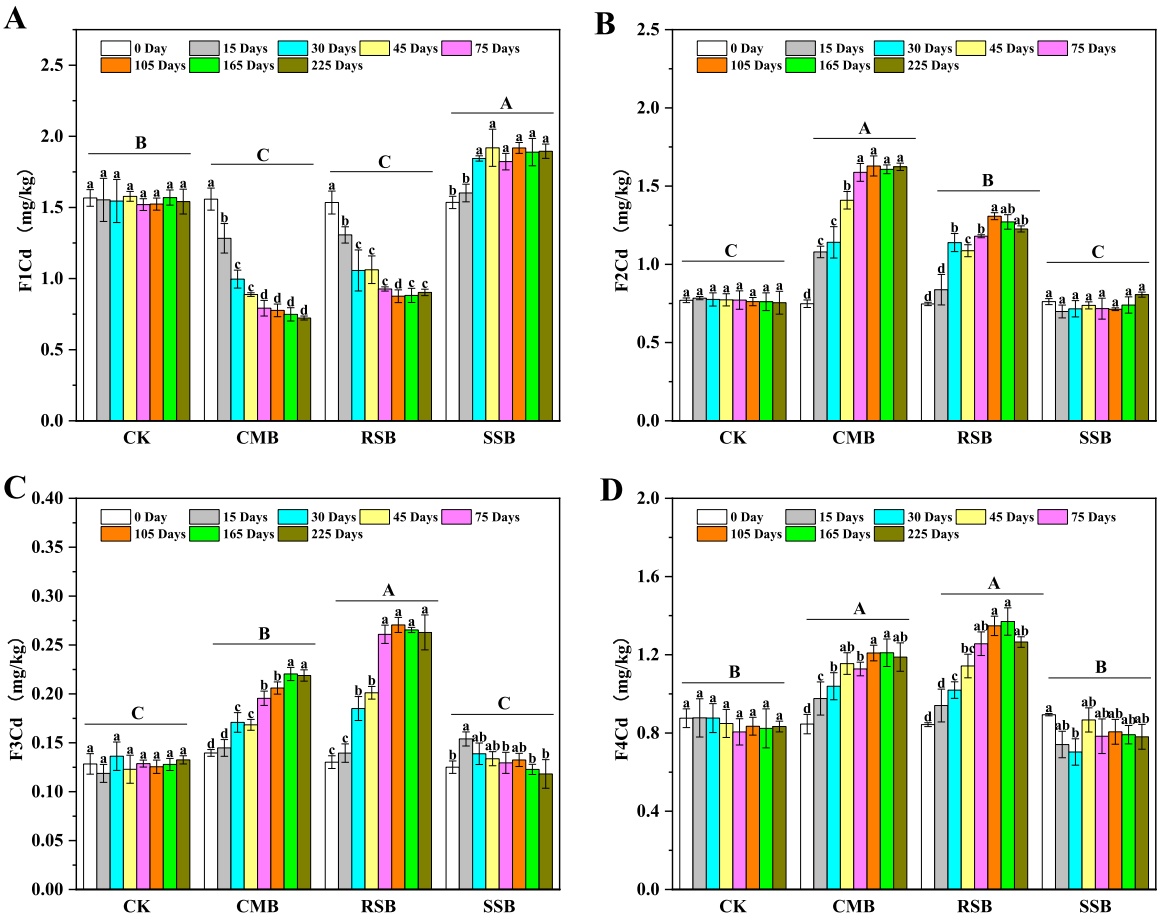


Fig. 1. Changes of Cd speciation during the incubation under CK, CMB, RSB and SSB treatment, respectively. Uppercase letters represent differences among treatments (Duncan's test, $p < 0.05$), and lowercase letters above error bars show significant differences among time points.

fractions were increased significantly by CMB and RSB treatment, whereas these did not change much for SSB treatment (Fig. 1B and C). Similar increase was observed for the F4Cd fraction under CMB and RSB treatment, and no significant changes were found for SSB, remaining constant throughout the incubation period (Fig. 1D). These indicated that CMB and RSB treatment had resulted in the strong shift from F1Cd towards the other speciation, indicative of immobilization degree, while SSB had only produced slight effects that favored the F1Cd fraction, resulting in unwanted mobilization. This was also confirmed by the results of metal mobility evaluation after biochar treatments (Fig. S3), in which both CMB and RSB treatments reduced the MF values and increased the I_R values, suggesting Cd mobility was decreased and stability was improved. While the opposite phenomenon was observed for SSB, it suggested that SSB treatment enhanced the mobility of Cd in soil. In addition, no statistically significant differences were detected between day 165 and day 225 under the applied comparison for the measured Cd fractions. However, the absence of a significant difference between two late sampling points is not sufficient to demonstrate a formal plateau, thus, day 225 is referred to as the final sampling point rather than a stabilized state.

High ash content of SSB should not be equated with high alkalinity or metal adsorption capacity, because the effect of SSB depended strongly on its ash composition. In fact, the reviews of heavy metal immobilization by SSB had likewise emphasized that its behavior in soil was highly feedstock- and process-dependent [29–31]. In the present study, SSB had the highest ash content (90.67%) but much lower pH (6.75) than CMB and RSB, only 8.1% C, very low contents of K^+, Na^+, Mg^{2+} and phosphate, and no detectable carbonate (Table 1). In other words, the highest ash content of SSB did not correspond to strongly alkaline or carbonate/phosphate-rich matrix. Weaker liming capacity and lower abundance of alkaline and precipitating components for SSB, these could limit pH-driven adsorption and precipitation when compared with CMB and RSB. At the same time, soluble mineral constituents and feedstock-derived metals in SSB may contribute to the mobile metal pool under some conditions. Because the detailed mineral phases and SSB-borne Cd were not independently characterized in this study, these possibilities are discussed as plausible explanations rather than demonstrated mechanisms. Despite these, these immobilization characteristics of Cd by three biochar types depended largely on internal biochar properties and exterior soil environmental changes.

3.3. Soil chemical properties

As expected, the strong alkaline biochars of CMB and RSB had increased the soil pH significantly from acid to alkali, with 1.84 and 1.27 pH units at day 225, respectively, compared to the control (Fig. 2A). Meanwhile, there were no significant changes in soil pH under SSB treatment, it had a pH of 5.89 at the end point, comparable to that of soil itself (5.97). In the case of CMB and RSB, the initial increase of pH was instantaneous attributed to dissolution of alkaline substances present in both biochars, after that the pH slightly decreased when these readily available alkaline substances were depleted [32]. Compared with SSB, much stronger pH increase of both biochars was thus beneficial for the Cd immobilization in soil. Similar phenomenon was also observed for soil CEC changes, in which the CEC increased by 42.50% and 49.49% at peak values for CMB and RSB addition, respectively, while this increase by SSB was much weaker (8.93%) compared to CK (Fig. 2B). Such improvement of soil CEC, particularly with CMB and RSB, would suggest that the increase of available adsorption sites capable of adsorbing heavy metal ions, resulting in the enhancement of metal immobilization by soil particles [33]. In combination, the different changes of soil pH and CEC resulted from biochar addition seemed to further confirm the phenomenon that the decrease of available Cd fraction by CMB and RSB treatments, whereas increase by SSB treatment (Fig. 1).

The TOC content by three biochars increased significantly compared to CK (Fig. 2C), in which the average content increased from 34.34 g/kg to 46.96 g/kg for RSB (36.75% increase) throughout the incubation period, followed by CMB (19.34%). This descending order was consistent with that of C content in the bicohars (Table 1), and the increase of TOC might be benefit for the transformation of acid-extractable speciation into less available speciation [34]. Similarly, the content of AP increased significantly under the three biochars treatment, among which CMB had the highest increase, followed by RSB and SSB (Fig. 2E and F). Total potassium (TK) was much higher in soil treated by CMB and RSB compared to CK (with 50.82% and 43.89% increase at day 30,

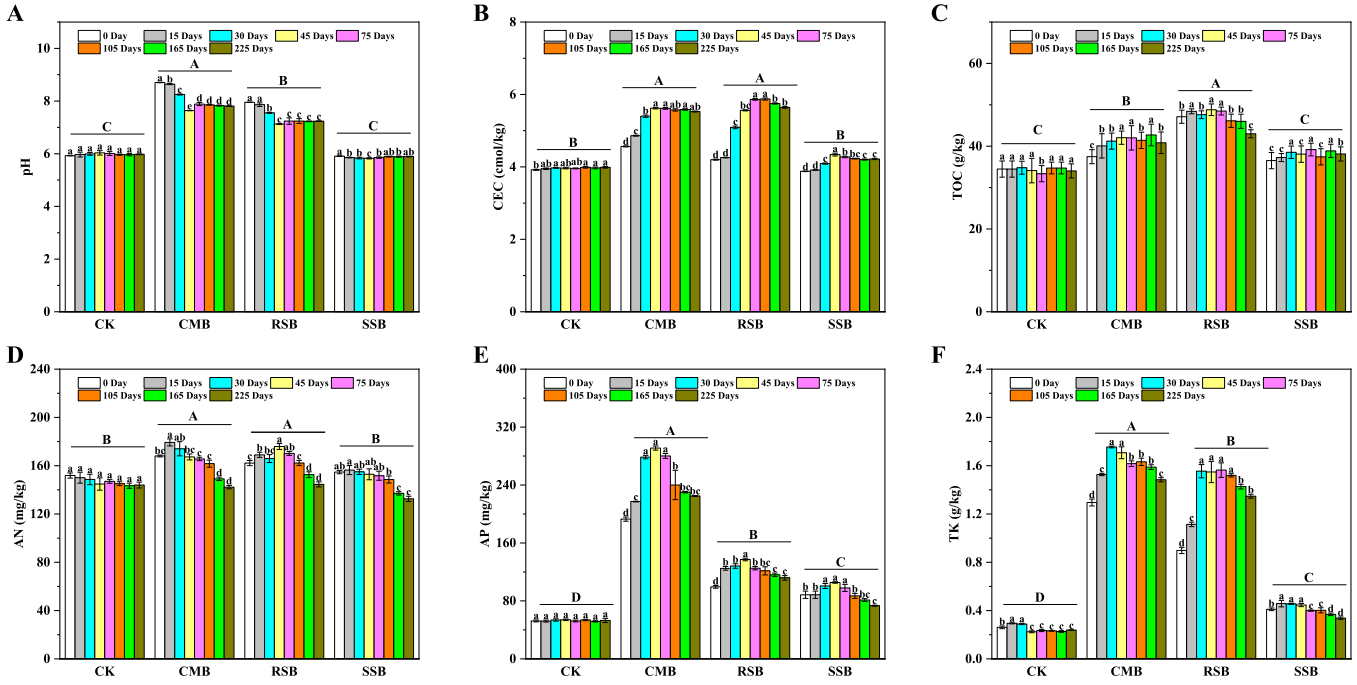


Fig. 2. Changes of soil properties during the incubation under CK, CMB, RSB and SSB treatment, respectively.

respectively), and it was slightly higher 11.8% following SSB addition. In comparison, the content of AN by CMB and RSB exhibited significantly higher than that of CK, with increases of 10.66% and 6.14%, respectively, whereas no significant difference was observed between SSB and CK (Fig. 2D). These increases of nutrient elements at varying degrees, particularly in CMB and RSB, were largely due to the biochar addition, with different levels of these elements inside biochars (Table 1). This situation seemed fairly comparable to the findings of Wu et al. [35], who reported that biochar treatment could increase soil available N and P content to reduce metal availability, applicable for explaining the observations of CMB and RSB.

3.4. Enzyme activities

For the levels of microbial biomass carbon (MBC), CMB and RSB treatment significantly increased the content of MBC compared to CK, with increases of 55% and 114% at day 225, respectively, while SSB addition did not have a significant effect on MBC levels in soil (Fig. 3A). In contrast, the levels of microbial biomass nitrogen (MBN) were considerably higher under RSB treatment than the other treatments, and no significant difference was observed between the others and CK (Fig. 3B). Similar phenomenon was described by Sánchez et al. [36], who considered it was likely that the occurrence of mineral ions and negatively charged surfaces on the alkaline biochars, together with the elevation of soil pH they had caused, could stimulate the increase of soil microbial biomass.

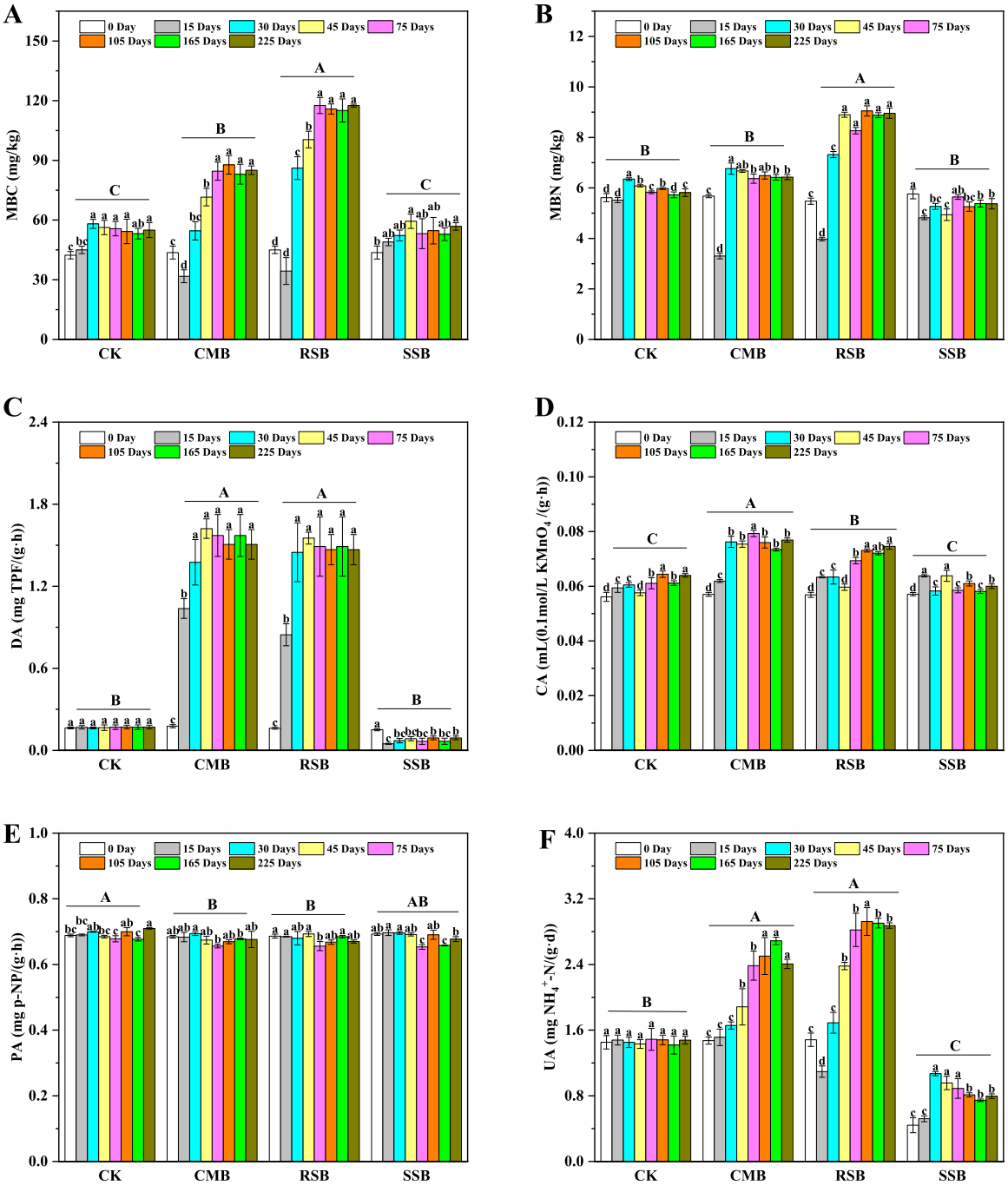


Fig. 3. Changes of soil MBC, MBN, and enzyme activity during the incubation period under CK, CMB, RSB and SSB treatment, respectively.

In the cases of four soil enzymes, the activity of dehydrogenase (DHA) was low in CK and remained low (and even decreased) following SSB addition, it strongly increased in presence of CMB and RSB, resulting in the levels at day 225 that were 7.5-fold and 8.0-fold higher compared to CK, respectively (Fig. 3C). The catalase (CA) activity was less dramatic, this also increased in CMB and RSB but not in SSB (Fig. 3D), this enzyme was oxidoreductase that mediated direct changes in the chemical speciation of heavy metals, thereby affecting available metal content [37]. While the levels of phosphatase (PA) hardly changed under all three treatments with slightly higher levels after each biochar treatment (Fig. 3E). This situation was completely different from urease activity (UA), in which UA activity increased significantly after CMB and RSB treatments, but decreased obviously by SSB treatment (Fig. 3F). The average percentage of increase reached by 41.33% and 55.58% for CMB and RSB, respectively, while this percentage decreased significantly by 46.68% for SSB after the incubation period. At this regard, cadmium was the most toxic metal to inhibit UA activity, according to the report of Chaperon and Sauvé [38], who investigated the UA response in soil exposed to single metals and metal mixtures, such as Cd, Cu, and Pb. Furthermore, the increase of UA activity could accelerate cadmium carbonate precipitation in soil, and vice versa [39], thus, the decrease in UA activity by SSB treatment compared to CK, might cause an increase of available Cd fraction. It should be emphasized that this was only one possible explanation, the other possibilities existed in SSB, such as specific microorganism activation, even Cd release from the SSB into the soil under long-time incubation. Taken together, the response of the investigated soil enzyme activities to these treatments was heterogeneous, resulting from the differences in responses to biochar types in terms of soil chemical properties and microbial activities.

3.5. Microbial community

3.5.1. Alpha diversity

At day 0 and day 225, high-throughput sequencing of informative amplicons derived from soil microbial DNA was performed and microbial richness was assessed by Chao1 indices, while Shannon's index served to evaluate microbial diversity [40]. The microbial data were accordingly interpreted as an initial-to-final comparison rather than as a continuous temporal trajectory (Table 2). At the initial sampling (day 0), bacterial Shannon diversities under CMB and RSB treatment were 11.6% and 4.7% lower than those of CK, respectively, and bacterial Chao1 richness were also 15.6% and 7.8% lower than in CK, while SSB did not differ significantly from CK for both bacterial index. At the final sampling (day 225), bacterial Shannon values were similar among these treatments (CK 6.43, CMB 6.37, RSB 6.32, SSB 6.43). Relative to their initial values, bacterial Shannon diversity increased by 9.8% for CMB and 1.1% for RSB. In contrast, fungal Shannon diversity declined from 4.36 to 2.87 in RSB (34.2%) and from 4.24 to 3.21 in SSB (24.3%), whereas fungal Chao1 richness did not differ significantly among the treatment-stage combinations. These results suggested that alpha-diversity responses depended on microbial kingdom, biochar type, and sampling stage rather than following a uniform decline. Because only two sequencing time points were available, these changes are described as initial-to-final differences rather than continuous temporal dynamics.

Table 2
Alpha diversity of microbial community after biochar treatment.

Treatment	bacteria		fungi	
	Shannon	Chao1	Shannon	Chao1
CK (0 day)	6.56 ± 0.01a	3845.62 ± 27.02a	4.25 ± 0.31a	708.92 ± 173.92a
CK (225 day)	6.43 ± 0.11abc	3323.59 ± 149.93bc	3.64 ± 0.61ab	671.90 ± 34.80a
CMB (0 day)	5.80 ± 0.10d	3243.81 ± 122.98c	4.36 ± 0.12a	668.47 ± 171.33a
CMB (225 day)	6.37 ± 0.03bc	3160.34 ± 108.67c	4.34 ± 0.11a	699.09 ± 21.50a
RSB (0 day)	6.25 ± 0.05c	3545.09 ± 117.45c	4.36 ± 0.29a	737.69 ± 63.01a
RSB (225 day)	6.32 ± 0.09bc	3072.03 ± 66.53c	2.87 ± 0.30b	576.04 ± 42.61a
SSB (0 day)	6.44 ± 0.10ab	3842.53 ± 194.94a	4.24 ± 0.16a	811.25 ± 26.87a
SSB (225 day)	6.43 ± 0.07abc	3285.35 ± 60.50bc	3.21 ± 0.67b	573.08 ± 18.13a

3.5.2. Microbial community structure

Under all the treatments, ten bacterial phyla with relative abundances above 1% were detected in the soil, including *Acidobacteria*, *Actinobacteria*, *Bacteroidota*, *Chloroflexi*, *Desulfobacterota*, *Firmicutes, Gemmatimonadota, Myxococcota, Verrucomicrobiota,* and *Proteobacteria* (Fig. 4A), while the phyla with abundances below 1% were collectively grouped as "Others". Among the detected phyla, *Proteobacteria, Actinobacteria, Chloroflexi,* and *Acidobacteria* were particularly abundant, accounting for approximately 60% of all identified phyla, these suggested the characteristic microbial community of Cd-contaminated soil [41]. It should be noted that the relative abundance of *Bacteroidota, Firmicutes* and *Gemmatimonadota* were higher under CMB and RSB treatments, but lower after SSB treatment when compared with the CK. Among the three bacterial phyla, *Firmicutes* have been shown to be an abundant phylum in Cd-contaminated soil [3], in which members of this phylum could survive under potentially harsh conditions, such as heavy metal pollution [42]. In addition, both CMB and RSB treatments reduced the relative abundance of *Acidobacteria*, which is beneficial for reducing available Cd fraction, since members of this phylum were known to activate Cd by releasing acidic compounds [43].What's more, the relative high abundance of bacterial genus after biochar treatment were recorded for *Gaiella* (2.05%–6.05%), *Sphingomonas* (0.95%–10.70%), *Pseudolabrys* (0.82%–2.12%) and *Bacillus* (0.57%–3.95%) (Fig. S2A). Among the most abundant genera, *Bacillus* had been extensively studied and it was confirmed that these could co-immobilize heavy metals in soil with biochar [18,44].

For the detected fungal phyla, *Ascomycota* constituted 65–93% of the fungal community, *Basidiomycota* accounted for 3–14% and *Mortierellomycota* for 2–13%, whereas the remaining fungal phyla comprised only negligible portions (Fig. 4B). Under each treatment, the fungal community was dominated by *Ascomycota* by a large margin, with *Basidiomycota* and *Mortierellomycota* as the subsequent most abundant phyla. Compared with the initial soil, *Basidiomycota* and *Mortierellomycota* decreased under biochar treatments, but increased *Ascomycota*. These fungal taxa could be potentially the major microorganisms responsible for Cd stabilization in soils, among which *Ascomycota* constituted the dominant fungal phylum in an arid loess region by multimetal contamination [45]. What's more, at the fungal genus level, the highest relative abundances were recorded for *Fusarium* (7.00%-14.96%), *Talaromyces* (3.22%-10.79%), *Clonostachys* (2.71%-7.02%), *Saitozyma* (0.81%-6.13%), *Westerdykella* (2.01%-4.39%), *Mortierella* (0.95%-3.23%), *Neurospora* (0.71%-3.50%) and *Psilocybe* (0.28%-3.24%) (Fig. S2B). Some of the fungal genera including *Fusarium*, *Talaromyces*, and *Mortierella* had previously been described as dominant taxa in heavy metal-contaminated environments [46,47]. Microorganisms that persist and thrive in heavy metal-contaminated environments frequently develop tolerance to heavy metals, involving primarily cellular-level structural changes that lead to metal resistance [48].

3.5.3. Co-occurrence network

Network analysis was used to compare exploratory microbial co-occurrence patterns between the initial and final sampling stages (Fig. 5 and Table S2). Compared to CK (the node and edge of network increased by 73 and 236 after incubation), CMB and RSB treatments significantly increased by the node of 173 and 89, and the edge of 699

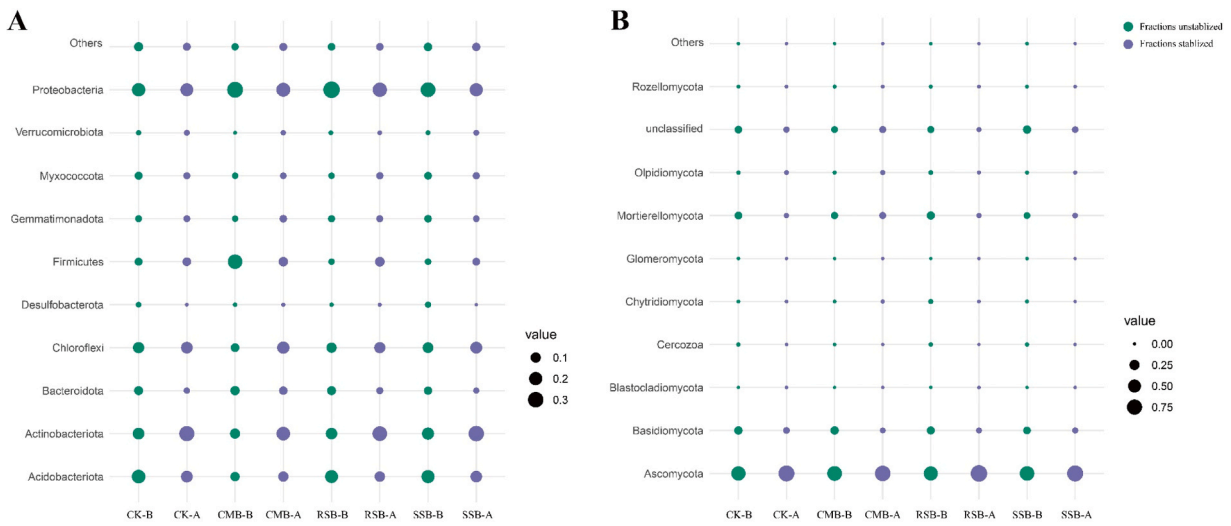


Fig. 4. Relative abundance of microbial phyla (A: bacterial phyla reaching >1% relative abundance, B: all detected fungal phyla).

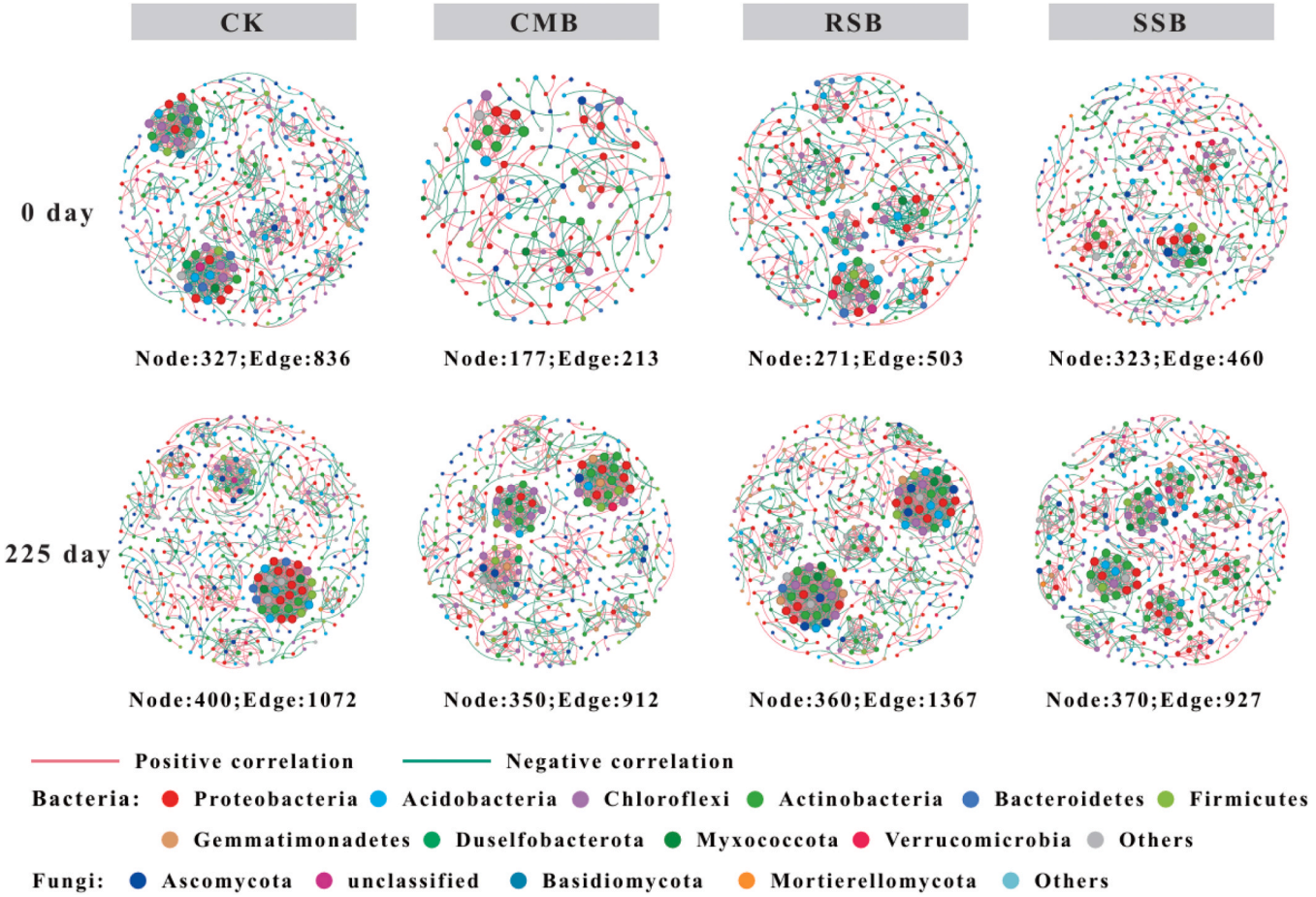


Fig. 5. Co-occurrence networks of bacterial and fungal communities combined under different treatments. Nodes are colored according to the dominant microbial phyla with edges showing positive (red) and negative (green) correlations. The node size is proportional to the abundance of taxa.

and 864, respectively, while in SSB, the node and edge of network slightly increased by 47 and 467, respectively. This suggested that complex interactions in soil microbial community by CMB and RSB might determine their responses to the Cd-contaminated environment [49]. In addition, biochar treatment initially reduced the total nodes and edges compared to CK, particularly in CMB and RSB, after 225 days of incubation, the microbial communities in the CMB and RSB interacted more strongly compared to the initial community. The prolonged incubation time probably facilitated the adaptation of certain microorganisms to the shifts in the Cd speciation [50]. These were also revealed by larger average degrees of connectivity and higher node and edge counts (Table S2), which implied that the complex co-occurrence network within the microbiome had formed, particularly in the treatments of CMB and RSB. It should be pointed out that the patterns were

interpreted as differences in statistical co-occurrence architecture between the two sampling stages, rather than as complete evidence of stronger biological interactions, continuous network maturation, or a causal contribution of particular taxa to the immobilization of Cd speciation.

3.5.4. Relationships between microbial community and soil properties

Microbial community structure exhibited high sensitivity to heavy metal stress and is simultaneously strongly affected by the alterations in soil properties (Liu et al., [51]). Despite Cd content being a dominant factor in structuring soil bacterial communities, other soil variables, such as pH, TOC, and available NP, were frequently more decisive and capable of reducing Cd mobility [52]. Therefore, we explored the associations between microbial taxa, the most relevant fractions F1Cd (most mobile) and F4Cd (most immobile), and soil properties, to which we added MBC, MBA and the activities of soil enzymes (Fig. 6).

At the initial sampling, bacterial community structure was significantly associated with pH, CEC, and available NP, whereas no significant association was detected for the fungal community (Fig. 6A). At day 225, bacterial community structure was significantly associated with F1Cd, F4Cd, pH, CEC, AP, TK, CA, and DHA, whereas the fungal community showed a significant association only with available P (Fig. 6B). Phylum-level correlation patterns are further shown in Fig. S4, where several bacterial phyla were significantly associated with pH, CEC, and available NPK, whereas *Chytridiomycota* was associated with TOC at the initial sampling. At day 225, *Proteobacteria, Actinobacteria, Firmicutes*, and *Bacteroidota* were associated with multiple soil variables, and *Proteobacteria, Firmicutes, Bacteroidota,* and *Gemmatimonadota* were negatively correlated with F1Cd. These results suggested that variation in bacterial community composition covaried with Cd fraction and soil properties, whereas the corresponding associations for fungi were weaker. For the fungal community, no phyla significantly associated with the investigated factors, this indicated that F1Cd was a key factor in the variation of microbial communities during the incubation period. Furthermore, the correlation of fungal communities with the investigated variables was markedly weaker than that of bacterial communities.

3.6. Relative role of direct and indirect effects

Partial Least Squares Path Modeling (PLS-PM) was used as an exploratory multivariate framework to summarize hypothesized statistical associations among biochar properties, soil chemical properties, microbial communities, enzyme activities, and F1Cd (Fig. 7). The resulting paths were interpreted as covariance patterns within the specified models rather than as experimentally demonstrated causal mechanisms. In the CMB model, negative path coefficients linking biochar properties, soil chemical properties, and enzyme activity with F1Cd were observed ($\beta = -0.1312$, -0.4243, and -0.3615, respectively). In the RSB model, negative path coefficients linking biochar properties and enzyme activity with F1Cd were observed ($\beta = -0.1394$ and -1.0137, respectively). While in SSB model, a positive path coefficient linking enzyme activity with F1Cd was observed ($\beta = 0.6663$), while a negative association was observed between SSB properties and the soil-chemical-property block. These suggested that CMB and RSB reduced the content of F1Cd by the direct effect of biochar and by the indirect effect of soil chemical properties, while F1Cd content increased by SSB treatment was largely related to the indirect effect of biochar on the soil enzyme activity, such as urease activity (Fig. 3). Additionally, the heat map further showed that most measured soil properties were negatively correlated with F1Cd (Fig. S5). Overall, the biochars of CMB and RSB exhibited distinct multivariate association patterns linking material properties, soil chemistry, microbial variables, enzyme activity, and F1Cd. It should be noted that these paths represent covariance within the hypothesized models and should not be interpreted as experimentally demonstrated immobilization or mobilization mechanisms. In particular, the positive enzyme-to-F1Cd path in the SSB model is treated as a statistical association rather than proof that enzyme activity caused the increase in F1Cd, despite urease-mediation could be one explanation for the increase of available Cd fraction by SSB.

The GoF values were 0.6885, 0.7608, and 0.6314 for the CMB, RSB, and SSB models, respectively. The corresponding R values for F1Cd were 0.9918, 0.9907, and 0.9410. R values for the soil-chemical-property block were much lower (0.0614, 0.0962, and 0.1752 for CMB, RSB, and SSB, respectively), whereas the microbial-community block showed R values of 0.8128, 0.9135, and 0.9147 and the enzyme-activity block showed R values of 0.9978, 0.9941, and 0.9410. These values are reported as descriptive properties of the specified models. Because path-level bootstrap significance and VIF diagnostics were not available, the work does not label individual paths as statistically significant and does not claim that multicollinearity was absent. These diagnostics were used to evaluate the internal statistical performance of the hypothesized models, not to infer causal mechanisms.

4. Conclusions

Treatment of heavy-metal contaminated soil by CMB and RSB demonstrated effective reductions of available Cd content, while SSB increased the most mobile Cd fraction. CMB and RSB treatments increased soil pH, CEC, TOC, AN, AP, TK, MBC, DA, CA, PA and UA

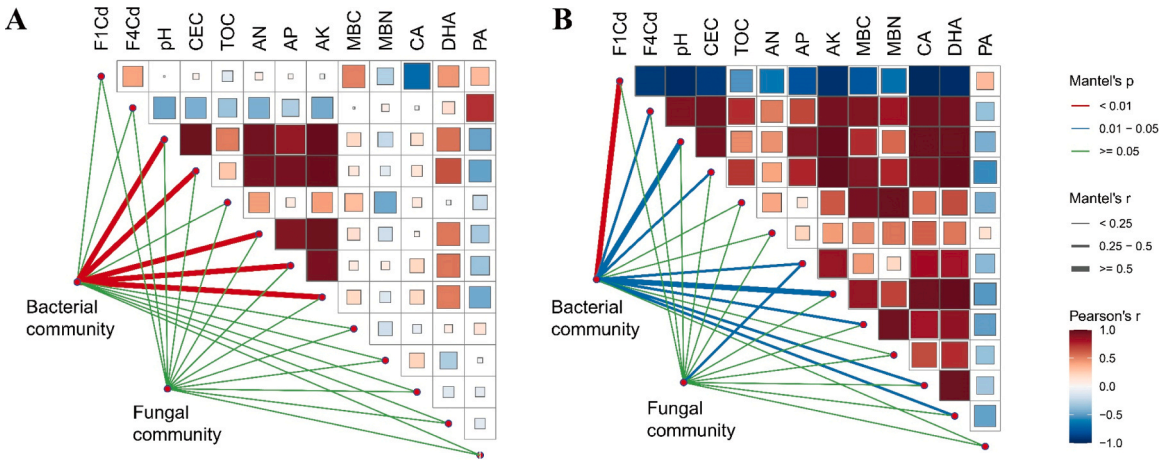


Fig. 6. Relationships between F1Cd and F4Cd, soil variables and microbial community structures. Pairwise comparisons of the analyzed factors are displayed with a color gradient denoting spearman's correlation coefficient. Phyla of fungi and bacteria were related to each soil variable by mantel test.

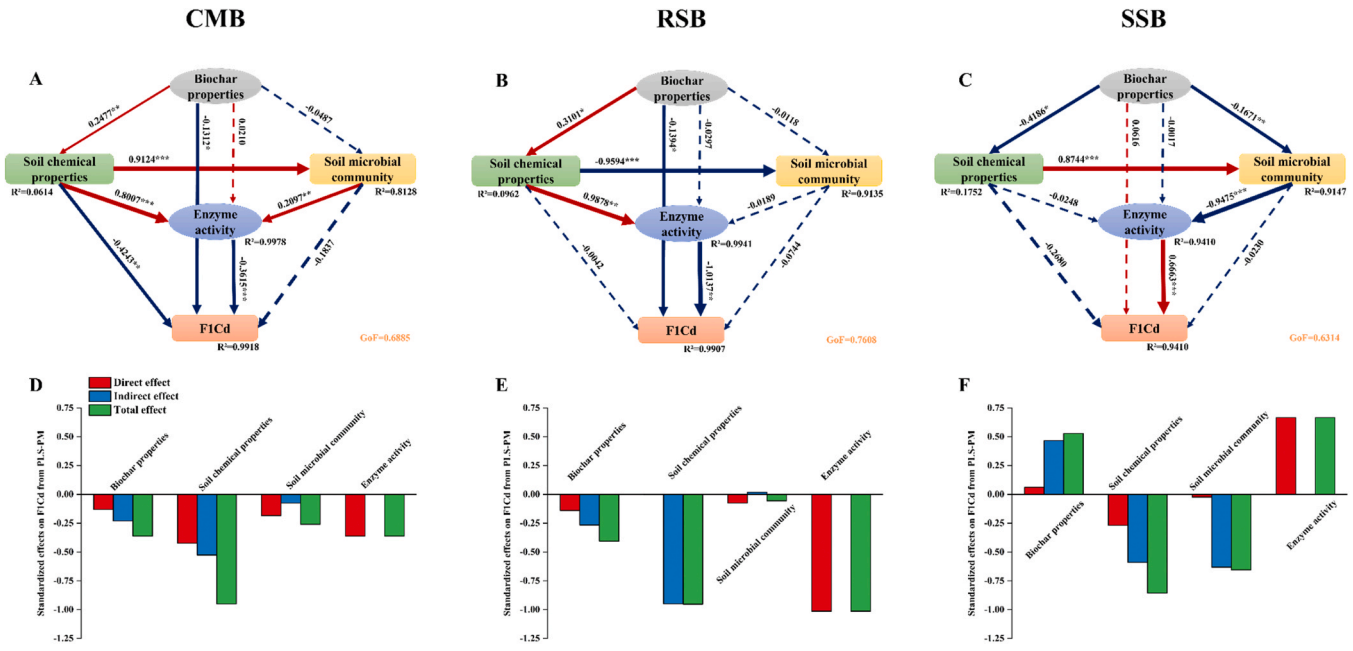


Fig. 7. PLS-PM shows the direct and indirect effects of various factors on F1Cd fraction under different biochar treatments (A–C: Relationship among biochar properties, soil chemical properties, microbial communities, enzyme activity and F1Cd content. Red and blue arrows represent positive and negative effects, respectively. Arrow thickness corresponds to path coefficient values. D–F: Standardized direct, indirect and total mean effects derived from PLS-PM).

enzyme activities, SSB treatment slightly increased AP, TK, and only decreased UA enzyme activity. Mantel test combined with correlation analysis suggested that the F1Cd, pH, CEC, available NP, DA and CA activity served as the key determinants of bacterial and fungal community structure. PLS-PM deduced that CMB and RSB treatment reduced F1Cd content by direct effects of the respective biochar properties, as well as indirect effects regulated by soil chemical properties, while SSB treatment increased F1Cd content indirect effect of biochar on soil enzyme activities, such as UA activity. These demonstrated that CMB and RSB were suitable biochars for the remediation of Cd-contaminated soil, but the increased risk of available Cd and its underlying mechanisms by SSB treatment require further attention.

CRediT authorship contribution statement

Kai-Ming Liang: Writing – original draft, Formal analysis. **Fei Huang:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Yi-Rong Deng:** Visualization, Supervision. **Long-Yong Lin:** Methodology, Data curation. **Zhi-Wen Hu:** Methodology, Investigation, Formal analysis. **Jun-Chun Li:** Software, Investigation.

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Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2026.143892.

Data availability

Data will be made available on request.

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