Environment International 137 (2020) 105576

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Biochar and bacteria inoculated biochar enhanced Cd and Cu immobilization and enzymatic activity in a polluted soil

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ARTICLE INFO

Handling Editor: Da Chen

Keywords:
Biochar
Heavy metal
Microbe loaded biochar
Bioavailability
Stabilization
Soil enzyme activity

ABSTRACT

The application of biochar in the remediation of heavy metal contaminated soil has received increasing global attention during the past decade. Although there has been some review work on the mechanism of heavy metals stabilization by biochar, the effects and mechanisms of interaction between biochar and functional microbes such as heavy metal tolerant, adsorption and transformation microbial strains remains unclear. In this paper, maize biochar and a heavy metal-tolerant strain *Pseudomonas* sp. NT-2 were selected to investigate the dynamic effects and potential mechanisms of biochar and bacteria loaded biochar on the stabilization of Cd and Cu mixed contaminated soil by a 75-day pot experiment. The results showed that, compared to the single biochar amendment, the application of biochar inoculated with NT-2 strain at the rate of 5% significantly increased the soil pH at the initial stage of incubation, and followed by a slight decline to a neutral-alkaline range during the reaction. The addition of NT-2 loaded biochar could also significantly increase the proportion of residual fraction of Cd and Cu, thus reduce the proportion of exchangeable and carbonate bound species in the soil, which lead to the decreasing of plant and human bioavailability of the metal in the soil indicated by DTPA and simulated human gastric solution extraction (UBM), respectively. Finally, the application of bacterial loaded biochar also markedly enhanced soil urease and catalase activities during the later stage of the incubation, and improved soil microbial community at the end of incubation, which indicates a recovery of soil function after the metal stabilization. The research results may provide some new insights into the development of functional materials and technologies for the green and sustainable remediation of heavy metal contaminated soil by the combination of biochar and functional microorganisms.

1. Introduction

Soil pollution, especially heavy metal pollution, is a serious environmental issue of global concern. In the U.S., over 100,000 contaminated sites have been identified (Connell, 2005), while in the E.U. countries, 250,000 polluted sites have been reported needing urgent remediation (Mench et al., 2010). According to the National General Survey Report on Soil contamination, announced by former Chinese Ministry of Environment Protection (MEP) and Ministry of Land and Resources (MLR), up to 16.1% of the total survey sites exceed the soil environment quality standards, with 11.2% register minor pollution. From the perspective of pollution type, the main pollution sources are inorganic pollutants (82.8%), among which Cd, Ni, As and Cu rank the top four of the inorganic contaminants (MEP and MLR, 2014). Considering the large quantity of a wide range of soil contamination in China and worldwide, there is an urgent demand for the developing of green and sustainable remediation strategies and technologies, particularly for the remediation of heavy metal contaminated farmland soil.

One of the principal strategies for the remediation of heavy metal polluted soil is to thoroughly remove the chemical contaminants from the soil, i.e., soil washing and phytoremediation. However, this strategy is either time and cost consuming or less environmentally friendly. Stabilization is another promising strategy to reduce the mobility and bioavailability of chemical pollutants by adding proper amendments

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https://doi.org/10.1016/j.envint.2020.105576
Received 31 October 2019; Received in revised form 9 February 2020; Accepted 11 February 2020
Available online 18 February 2020
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into the polluted soil, thus minimize the health and ecological risks of toxic contaminants in the soil (Li et al., 2017a; Shen et al., 2019). Biochar, a carbon-rich material of thermal conversion of biomass under anaerobic or oxygen-limited condition, has been universally applied in soil remediation due to its porous structure, large surface area, and variable surface compositions (Godlewska et al., 2017). Many published papers have evaluated and demonstrated the ability of biochar to sorb heavy metals, and to reduce the mobility, bioavailability, and consequently toxicity of heavy metals in the soil (Nie et al., 2018; Qi et al., 2018; Wei et al., 2019a).

The main mechanisms under the immobilization of heavy metals in the soil by various biochars have been reviewed by several researchers (Bandara et al., 2019; Hamid et al., 2019; Li et al., 2017b). Firstly, biochar can directly sorb heavy metals via complexation, cation exchange, electrostatic interactions, reduction, and precipitation processes. Secondly, the biochar amendment could affect soil properties such as pH, cation exchange capacity (CEC), and soil organic carbon (SOC), thus leading to an increase in metal retention in the soil indirectly (He et al., 2019a). The stabilization of heavy metals in the soil by the application of biochar is highly dependent on the biochar properties including feedstock type and pyrolysis temperature. For instance, compared to the wood-based biochars, the grass-based biochars (e.g., rice straw or wheat straw biochars) were generally more effective in reducing Cd leaching potential and enrichment in crop tissues (O'Connor et al., 2018). Biochars produced at higher temperatures were performed better for metal immobilization than those produced at lower temperatures (Kavitha et al., 2018; Shu et al., 2016; Wei et al., 2019a). Besides, biochar dosage, mixing depth, soil properties, soil microbial community, and even climate conditions may have complicated effects on the heavy metal stabilization in the soil (O'Connor et al., 2018).

The combination of biochar and bioremediation with functional bacterial or fungal strains is considered as an effective and emerging strategy for the sustainable remediation of polluted soil (Chen et al., 2019; Wu et al., 2019). For the heavy metals in the soil, many microbial strains with strong metal tolerant or adsorption capability were isolated and applied in to the soil as microbial agents either by direct inoculation of free-living cells, or by immobilization of cells with a particular carrier substance. The carrier materials used for the microbial cell immobilization generally consist of natural materials (e.g., activated sludge, zeolite, and diatomite), synthetic macromolecular materials (e.g., polyvinyl alcohol, polyurethane, and acrylamide), artificial inorganic materials (e.g., porous ceramics and activated carbon), and composite materials (e.g., alginate-chitosan-carbon microcapsules) (Bouabidi et al., 2019; Partovinia and Rasekh, 2018). Recently, biochar has been used as an ideal carrier material for the plant growth-promoting microbes, and the increased the biomass of hyperaccumulators (*Chrysopogon zizanioides* L.) in the Cd polluted soil (Wu et al., 2019). The potential interaction mechanisms between biochar and soil indigenous microbes have been proposed by some reviews, including (1) biochar can act as a microbial shelter; (2) biochar can provide nutrients to microbes; (3) biochar can change soil property, soil enzyme activity and microbial community; (4) biochar can enhance the transformation and degradation of pollutants via sorption, electron transfer and free radicals (Bandara et al., 2019; Zhu et al., 2017). However, the combination mechanism between biochar and exogenous functional microbes, especially heavy metal resistant and immobilizing microbes, is not well understood. The respective contribution of biochar and functional microbes to the retention of heavy metals in the soil remains unclear. Furthermore, the evaluation of stabilization effects of heavy metals in soil mainly relies on the analysis of metal speciation based on single or sequential chemical extraction, which lacks a systematic assessment of metal bioavailability based on different receptors at multiple scales. There is also a research gap in the dynamic variation of soil properties and soil function recovery during the biochar-microbe combined remediation of heavy metal polluted soil.

In this study, a maize straw biochar and a heavy metal tolerant bacteria (*Pseudomonas* sp. NT-2) loaded biochar were tentatively applied into the Cd-Cu mixed polluted soil at different rate, with the aims to illustrate the effects of microbial inoculation on the stabilization of Cd-Cu in the soil, and to investigate the dynamic variations of metal bioavailability to multiple receptors including plants and human beings, as well as the dynamic impacts on soil property and function during the whole remediation period. The results of this study may bring some new insights into the development of functional materials and related technologies for the green and sustainable remediation of heavy metal polluted soil.

2. Materials and methods

2.1. Preparation of biochar and Pseudomonas NT-2 loaded biochar inoculant

The maize straw (MS) biochar was selected as the test amendment due to its high yield and excellent performance in metal immobilization according to the previous studies (Li et al., 2016). It was produced anaerobically using a slow-pyrolysis machine at 400 °C with a retention time of 8 h (Liu et al., 2017). The basic properties of the biochar are listed in Table 1. Before used, the biochar was pulverized and passed through a 0.25 mm sieve.

The bacterial strain NT-2 was initially isolated from a Cu contaminated orchard soil with a cultivation history for more than 20 years in Shandong Peninsula of China. Based on previous morphological and molecular characterization, NT-2 was identified as the genus *Pseudomonas frederiksbergensis*, which exhibited strong plant growth-promoting capacity and high tolerance to Cu contamination in soil (Tu et al., 2015). Recently, our unpublished data showed that strain NT-2 also showed remarkable tolerance to Cd (up to 100 mg L^{-1}) in the Cd-containing medium.

For the preparation of NT-2 loaded biochar inoculant, the strain NT-2 was firstly inoculated in beef extract peptone medium at a rate of 5% and cultivated in a thermostatic oscillator at 200 rpm, until the OD_{600} value reached 1.0. Subsequently, the biochar was blended with the strain culture at a ratio of 1:3 (w/v) and incubated for 8 h before application. Before amended into the soil, a subsample of as-prepared NT-2 loaded biochar was separated and freeze-dried for the characterization of strain NT-2 on the biochar surface using a scanning electron microscope (S-4800 SEM, Hitachi, Japan). For the non-inoculated biochar, neither the bacterium nor the bacterium-free medium was incubated with the biochar before the observation by SEM.

2.2. Soil incubation experiment

The soil sample used in this study was collected from the topsoil (0–20 cm) of an uncontaminated farmland in the Shandong Peninsula. The methods described by Lu (1999) were applied to measure soil pH, CEC, organic matter, total N, P, and K content of the soil. The basic properties of the soil are presented in Table 1. The soil was air-dried

Table 1
Basic property of the biochar and soil.

Property	Biochar*	Soil
pH (H_2O)	9.60	7.85
Texture	–	sandy loam
Organic matter (g kg^{-1})	–	8.04
Total C (g kg^{-1})	597.7	–
Total N (g kg^{-1})	13.4	0.56
Total P (g kg^{-1})	2.47	1.09
Total K (g kg^{-1})	29.8	15.36
CEC (cmol kg^{-1})	17.0	5.40

* From Liu et al. (2017).

and sieved to remove impurities such as stones and organic residues, then spiked with $Cd(NO_3)_2$ and $Cu(NO_3)_2$ solutions, mixed thoroughly, and aged at room temperature for 2 weeks. The final Cd and Cu concentration of soil achieved 56 and 247 mg kg^{-1}, respectively.

The incubation experiment was conducted in a thermostatic chamber at 25 °C for 75 days. The contaminated soils were amended with biochar and NT-2 loaded biochar inoculant at ratios of 1% and 5% (dry weight of biochar in soil), namely, B1, B5, BN1, and BN5, respectively. During the incubation period, the soil moisture was kept at 40% of water holding capacity to facilitate equilibrium. All treatments and control (CK, without amendment) were performed in triplicate. Soil samples were air-dried and sieved again for further analyses.

2.3. Tessier sequential extraction of heavy metals in the soil

Changes in the speciation and redistribution of Cd and Cu in soil were analyzed using the Tessier sequential extraction method (Tessier et al., 1979). Five different fractions designated by this method included: exchangeable (EX), carbonate bound (CB), Fe-Mn oxide bound (OX), organic matter bound (OM), and residual fraction (RS), of which the first four were extracted by 1.0 mol L^{-1} $MgCl_2$ (pH 7), 1.0 mol L^{-1} NaOAc (pH 5), 0.04 mol L^{-1} $NH_2OH{\cdot}HCl$ (in 25% HO-Ac), and 0.04 mol L^{-1} HNO_3 (in 30% H_2O_2, pH 2), respectively, while the last was obtained by digesting the residual with HNO_3-HF-$HClO_4$ mixture.

2.4. Phytoavailability and bioaccessibility of heavy metals in the soil

Heavy metals in soil extracted by diethylenetriamine pentaacetic acid (DTPA) are considered to be the most available fraction to plant uptake (Fellet et al., 2014). Therefore, the effects of biochar and NT-2 loaded biochar treatments on the phytoavailability of Cd and Cu were determined by the DTPA extraction method. Soil samples were added into the extract solution of 0.005 mol L^{-1} DTPA, 0.01 mol L^{-1} $CaCl_2$, and 0.1 mol L^{-1} triethanolamine (pH 7.3) at a solid to water ratio of 1:2 (m/v). The suspensions were agitated on a thermostat shaker at 180 r min^{-1} and 25 °C for 2 h, centrifuged, and passed through a 0.45 μm polyethersulfone filter for Cd and Cu analyses.

A modified Unified Bioaccessibility Method (UBM) test was applied to evaluate the fraction of Cd and Cu taken up by the human body via oral ingestion (Wragg et al., 2011). Here the gastric digestion was adopted because the bioaccessible concentration of metals in the stomach is commonly higher than that in the intestine (Zhong and Jiang, 2017). Briefly, each treated soil sample (2 g) was mixed with the simulated gastric extract (45 mL) prepared according to the modified UBM protocol (Wragg et al., 2011). The pH of the suspension was adjusted to 1.1 ± 0.05. And then the suspension was rotated end-over-end slowly at 37 °C for 1 h. After rotation, all suspensions were centrifuged, filtered, and stored at 4 °C before Cd and Cu analysis.

The Cd and Cu concentration in the filtrates of the aforementioned sequential extraction, DTPA extraction, and UBM test were measured by an atomic absorption spectrophotometer (TAS-990 AAS, Beijing Persee, China). The bioaccessibility (BAF) was expressed as a ratio of the Cd and Cu concentration in the gastric extract to their total concentrations in the soil.

2.5. Soil enzymatic activity analysis

Soil enzymatic activities, including urease and catalase, were determined following the procedures detailed in Tu et al. (2018a). The obtained soil urease and catalase activities were expressed as mg NH_4^+-N g^{-1} 24 h^{-1} and as mL (0.1 mol L^{-1} $KMnO_4$) h^{-1} g^{-1}, respectively.

2.6. Microbial diversity analysis

Soil DNA was extracted using a FastDNA SPIN Kit (MPbio, USA) according to manufacturer's protocols. The DNA samples which met the quality standards were sent to Shanghai Majorbio Bio-pharm Technology Co., Ltd (Shanghai, China) for 16S rRNA gene high-throughput sequencing using the Illumina MiSeq PE300 platform. Bacterial relative abundance, alpha-diversity, and PCoA analysis were performed using the free online platform of Majorbio Cloud Platform (www.majorbio.com).

2.7. Data analysis

All the experiments were conducted in triplicate. Data were presented as means with standard deviations. Statistical analyses were performed by SPSS Statistics 19.0 software. One-way analysis of variance (ANOVA) was applied to evaluate the effects of different amendment treatment on the analyzed parameters, and the differences between the treatments means were determined using the Duncan test at $p < 0.05$.

3. Results and discussion

3.1. Characterization of NT-2 loaded biochar

The SEM images of the parent biochar (Fig. 1a) show the coarse surface and porous structures, which might facilitate the attachment and proliferation of NT-2. As expected, the strain NT-2 (Fig. 1b) adhered well to the biochar. Most of the cells scattered or aggregated on the biochar surface (Fig. 1c); however, it is reasonable that some cells entered the pore structures given their small size (< 2 μm). The colonization of microorganisms on surfaces and in the pores of biochar depends on its physiological features and the biochar properties (Zhu et al., 2017). In a field experiment conducted by Quilliam et al. (2013), only sparse concentrations microorganisms were observed adhering to the aged biochar buried for 3 years. They considered that the high mineral salts content and possible presence of polycyclic aromatic hydrocarbons (PAHs) might potentially inhibit microbial colonization. On contrast, Chen et al. (2014) found that *Geobacter metallireducens* and *Geobacter sulfurreducens* were able to colonize synergistically on the biochar surface in a short period (10 days). In the present study, the strain NT-2 colonized well on the biochar surface (Fig. 1c), and showed a quick colonizing capacity (8 h). Interestingly, extracellular polymeric substances (EPS) were observed around NT-2 cells (Fig. 1c), which was

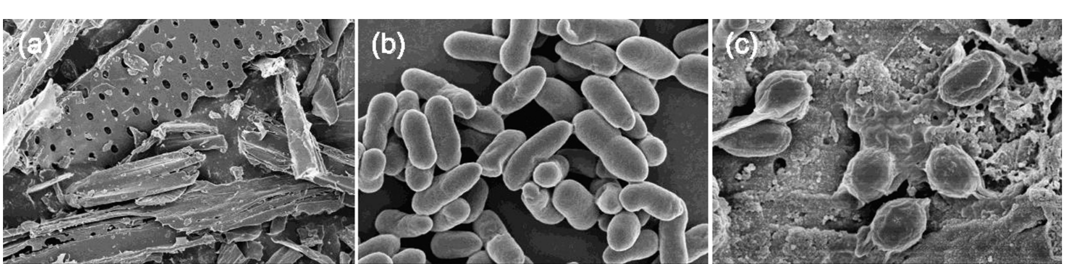

Fig. 1. Scanning electron micrographs of biochar (a), NT-2 strain (b), and NT-2 loaded biochar (c).

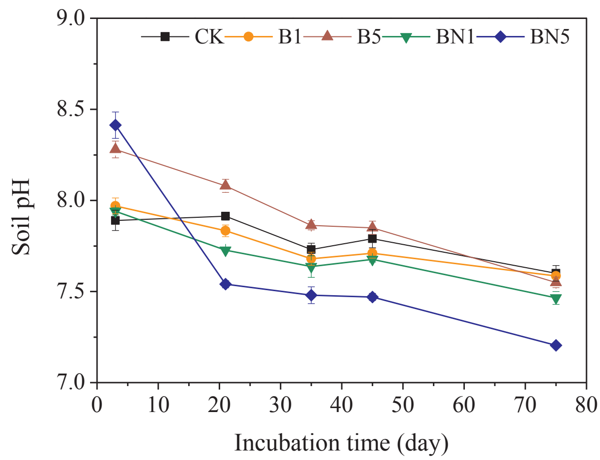

Fig. 2. Effects of biochar and NT-2 bacteria loaded biochar on soil pH during incubation. CK denotes the soil samples without amendments; B1 and B5 denote the soil samples from 1% and 5% biochar treatment; BN1 and BN5 denote the soil samples from 1% and 5% NT-2 loaded treatment, respectively.

different from the colonization status exhibited in the works of Quilliam et al. (2013) and Chen et al. (2014). This suggests that the EPS could alleviate the harmful effects caused by toxic substances, as well as reinforce the adhesion between the NT-2 cells and biochar.

3.2. Changes in soil pH during the incubation

The application of biochar and NT-2 loaded biochar both elevated the soil pH at the preliminary stages of incubation (day 3), especially with the application rate of 5%. As shown in Fig. 2, the soil pH of all the treatments presented a declining trend with the increasing incubation time. Yuan and Xu (2011) reported a similar fluctuation in soil pH within the incubation with biochar from crop residues. They suggested the quick increase of soil pH was due to the dissolution of alkaline substances (such as inorganic carbonate) in the biochar, and then the pH was slightly changed after these readily released alkaline substances were depleted. The NT-2 loaded biochar treatment exhibited a more pronounced pH decline effect compared with its counterpart of single biochar treatment. In particular, the BN5 treatment significantly reduced the pH ($p < 0.05$) in the soil after the 20-day incubation. These observed results suggested that a high dose of NT-2 loaded biochar can effectively mediate soil pH during the whole incubation period. The substances secreted by *Pseudomonas* strain NT-2 might contain some low molecular weight organic acids, which would cause a decrease in soil pH of the BN treatment. However, in this study, the soil pH remained in the neutral-alkaline range after NT-2 loaded biochar amendment. Regardless, the pH buffering ability of NT-2 loaded biochar is an interesting phenomenon, which is worthy of further investigation of the influences on the speciation and bioavailability of Cd and Cu in the soil, especially in the acidic soil.

3.3. Fractionation of Cd and Cu in soil

The fractions of Cd and Cu in soil were clearly affected by the addition of biochar amendments (Fig. 3). For the initial, untreated soil indicated by CK at 0 day, the predominant Cd fraction was exchangeable Cd (56.80%), followed by the carbonate bound Cd fraction (26.5%), while the residual Cd and Fe-Mn oxide bound Cd accounted less (10.13% and 6.35%, respectively), and the organic matter bound Cd fraction was lower than 0.5%. After 75 days of incubation, the proportion of exchangeable Cd in soil shrank to varying degrees, suggesting the exchangeable Cd fraction transformed into other fractions. Compared with the CK treatment, the exchangeable Cd fraction in B1 and BN1 treatment decreased by 1.18% and 1.82%, whereas Fe-Mn oxide bound Cd fraction increased by 1.29% and 1.95%. The B5 and BN5 treatment showed a significant decrease in the exchangeable Cd fraction by 5.74% and 12.82%, meanwhile increased in the Fe-Mn oxide bound Cd fraction by 4.28% and 8.17% and in the residual Cd fraction by 1.72% and 5.49%, respectively. Unlike Cd, Cu in the initial soil (CK, 0 day) was mainly in the form of carbonate bound Cu (49.4%), followed by residual Cu (28.36%), Fe-Mn oxide bound Cu (17.97%), while organic matter bound Cu and exchangeable Cu accounted for a lower proportion (2.90% and 1.42%). On the 75th day, the exchangeable Cu fraction of BN5 reduced to an even lower level ($< 1\%$), as well as other treatments. Compared with the CK treatment, the carbonate bound Cu fraction of B5 and BN5 treatments was significantly reduced by 22.59% and 26.55%, respectively, while residual Cu was increased by 8.39% and 11.54%. It is worth noting that in CK treatment, the carbonate bound and Fe-Mn oxide bound Cu fraction transferred to the organic matter bound Cu fraction during the incubation. Some researchers indicated that Cu had a strong affinity to soil organic matter, and the proportion of organic bound Cu could be notably elevated after biochar application (Park et al., 2011; Rinklebe and Shaheen, 2015). Park et al. (2011) pointed out that the application of 15% chicken manure-derived biochar increased the proportion of organic matter bound Cu from 46.2% to 64.2%. However, our results show that although the application of biochar amendments increased the soil organic matter content, the proportion of organic matter bound Cu did not increase as proportionately expected. The carbonate bound Cu in this study was more inclined to convert to the most recalcitrant fractions, i.e. Fe-Mn oxide bound Cu and residual Cu fractions.

The exchangeable and carbonate bound fractions represent the easily labile fractions, while the Fe-Mn oxide, organic matter bound, and residual fractions can be ascribed to the recalcitrant (less bioavailable) fractions. Both the labile and recalcitrant fractions can be used to evaluate the effectiveness of in-situ immobilization (He et al., 2019b; Jiang et al., 2012; Rinklebe et al., 2016). Recently, Nie et al. (2018) found that the labile fractions of Cd and Cu in soil decreased with the increasing biochar addition, while their recalcitrant fractions increased. By using synchrotron-based X-ray absorption spectroscopy, Cui et al. (2019) and Ippolito et al. (2012) further demonstrated that biochar application promoted both Cd and Cu to form (oxy)hydroxide, carbonate, and organically bound species. In the present work, similar trends were observed that both labile Cd and Cu concentrations of B5 treatment were significantly lower than those of B1 treatment (Fig. 3a and b). Moreover, compared to the single biochar treatments, the NT-2 loaded biochar treatments could effectively reduce the labile Cd and Cu in the soil; thus increase the recalcitrant Cd and Cu, especially at the addition rate of 5%. Both biochar and NT-2 loaded biochar could induce transformation of Cd and Cu from labile fractions to recalcitrant fractions, especially the formation of Fe-Mn oxide bound and residential fractions. However, the fraction distributions of Cd and Cu in the treated soils varied. Specifically, the exchangeable fraction of Cu in the soil is obviously lower than that of Cd, while the organic matter bond fraction of Cu is markedly higher than that of Cd. This may due to Cu having a higher affinity sequence and larger sorption capacity to organic matters (e.g., humic acid and biochar) and minerals in soil (Ding et al., 2019; Sellaoui et al., 2018; Sipos et al., 2019).

3.4. Phytoavailability and bioaccessibility of Cd and Cu

The contents of DTPA extractable Cd and Cu (i.e., DTPA-Cd, DTPA-Cu) in the treated soil progressively declined with increasing incubation time (Fig. 4). After 75 days, the concentration of DTPA-Cd decreased from 29.5 mg kg^{-1} in CK to 24.7 mg kg^{-1} in B1, and to 20.8 mg kg^{-1} in B5. The reduction in DTPA-Cu was more pronounced, from 127.3 mg kg^{-1} in CK to 108.2 mg kg^{-1} in B1, and to 73.4 mg kg^{-1} in B5. Similar to our results, Lu et al. (2017) found that the application of bamboo biochar at the dose of 5% was able to reduce the contents of DTPA-Cd and DTPA-Cu by 0.16 and 87.5 mg kg^{-1} in contaminated soil

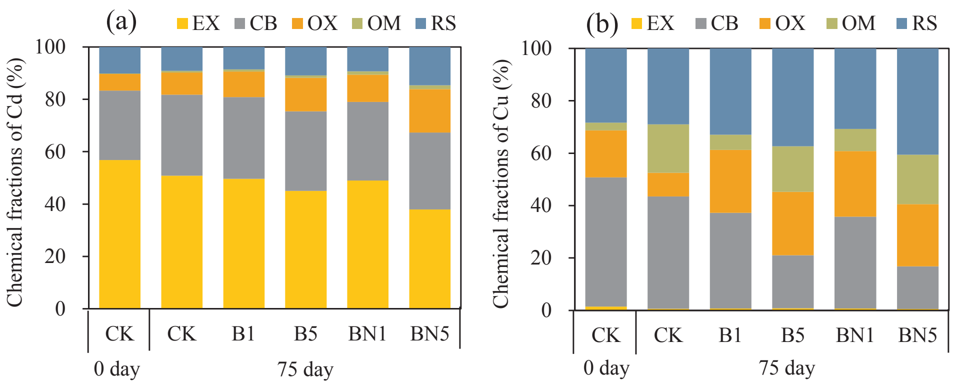

Fig. 3. Cd (a) and Cu (b) speciation in the polluted soil (EX, exchangeable; CB, carbonate bound; OX, Fe-Mn oxide bound; OM, organic matter bound; RS, residual).

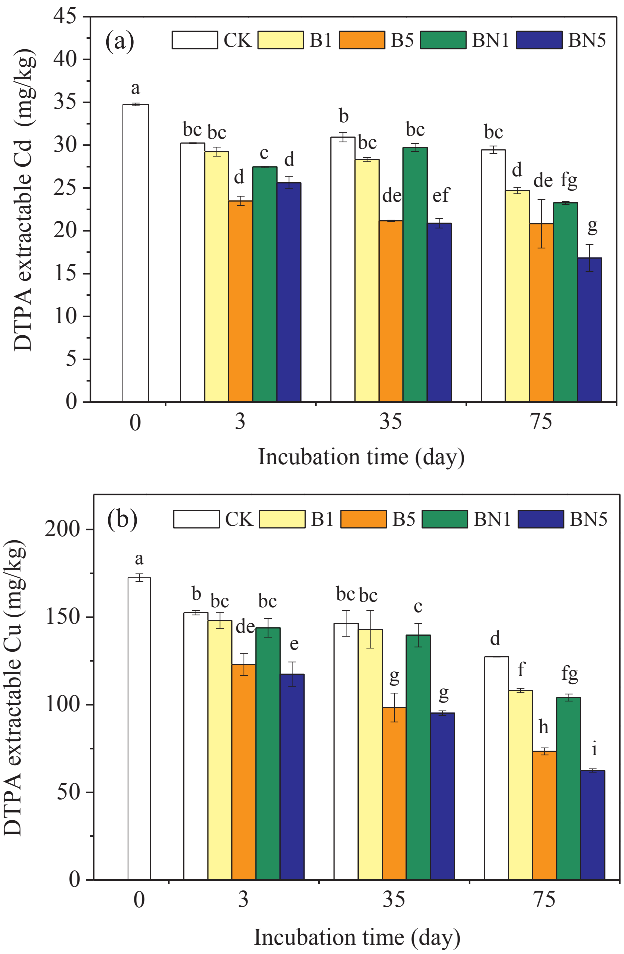

Fig. 4. Changes in DTPA-Cd (a) and DTPA-Cu (b) in soil during incubation. Lowercase letters above the error bars indicate significant differences among different treatments analyzed by Duncan test ($p < 0.05$).

near a copper smelter. However, contrasting results were observed in other studies. Fellet et al. (2014) reported that with the application of biochars from manure and prune residues, the DTPA-Cd in the mine tailing soils decreased from 5.23 mg kg^{-1} in CK to 3.77 and 1.62 mg kg^{-1} at 1.5% and 3% dose, respectively, whereas the DTPA-Cu increased from 6.24 mg kg^{-1} to 7.38 and 6.89 mg kg^{-1}. An increase in the phytoavailability of Cu as indicated by DTPA-Cu was also observed by Mendez et al. (2012). This phenomenon may be due to an increased concentration of dissolved organic matter (DOM) as a consequence of the biochar application. DOM released from biochar, which is rich in functional groups such as carboxylate and hydroxyl, can chelate Cu cations and further increase its bioavailability and mobility (Wei et al., 2019b). Therefore, bench testing to identify biochars that release the least amount of DOM before large-scale field application would curb unintended mobility of contaminants. In our study, the maize straw biochar contained a low concentration of DOM (3.40 g kg^{-1}) (Liu et al., 2017) and would limit adverse effects of DOM on the bioavailability of Cd and Cu in the soil. As shown in Fig. 4, compared to the single biochar treatment, the NT-2 loaded biochar treatment with the same input rate generally had lower contents of DTPA-Cd and DTPA-Cu. In particular, on the 75th day, the DTPA-Cd concentration was 23.3 and 16.8 mg kg^{-1} in BN1 and BN5; while the DTPA-Cu concentration was 104.1 and 62.4 mg kg^{-1} in BN1 and BN5, respectively. These indicated that the inoculation of NT-2 to the biochar made a significant contribution in reducing the phytoavailability of Cd and Cu in soil, especially at the 5% amendment dose.

Fig. 5 shows the bioaccessible fraction of Cd and Cu in the soil extracted by the UBM test. Similar to the DTPA-phytoavailability, the Cd and Cu bioaccessibility of the treated soil decreased to various levels with the addition of two amendments at different ratios. For the CK treatment, the percentage of bioaccessible Cd and Cu was 83.7% and 80.2%, indicating a high release risk under the harsh gastric conditions. After 75 days of incubation, the Cd bioaccessibility slightly declined for the B1 and BN1 treatment, while significantly reduced to 75.5% and 74.8% for B5 and BN5, respectively. In contrast, an evident decrease in Cu bioaccessibility was observed for all the four treatments, in particular to 69.5% and 62.6% for B5 and BN5. Many studies have

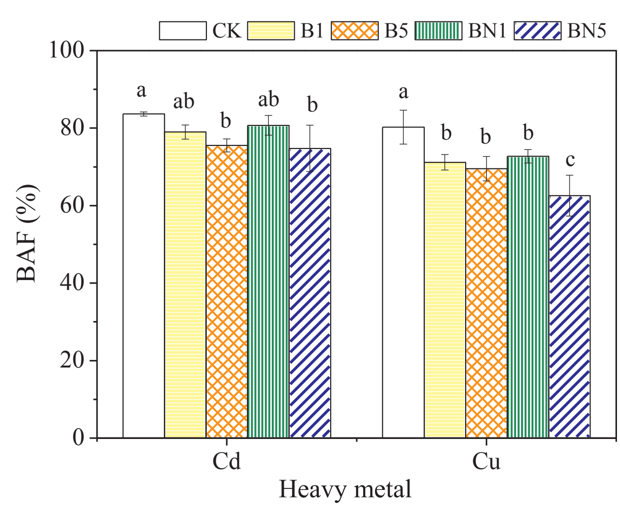

Fig. 5. The oral bioaccessibility of Cd and Cu in gastric phase after 75 days incubation. Lowercase letters above the error bars indicate significant differences among different treatments analyzed by Duncan test ($p < 0.05$).

demonstrated the ability of biochar to reduce the metal bioaccessibility, even at low amendment levels (Bashir et al., 2018; Lin et al., 2017; Uchimiya et al., 2012; Yoo et al., 2018). However, most of these researches were using acidic soils. Recently, Janus et al. (2018) evaluated the influence of three biochars (pH 8.41–10.24) on the oral bioaccessibility of Cd, Pb, and Zn in slightly alkaline soil (pH 7.55). In their study, with amendment rates up to 2%, the biochar application did not necessarily decreased the bioaccessible metal content in the soil. In the study by Qi et al. (2018), the application of an acid biochar (pH 3.25) also showed no effect on the bioaccessible Cd in two alkaline soils (pH 7.87 and 8.47), however, the neutral biochar (pH 7.00) was capable of decreasing the bioaccessibility of Cd by 29.7% and 18.0% after 140 days incubation. Hence, based on the above observation, our results confirmed the feasibility of the application of NT-2 loaded biochar inoculant to reduce the human health risks of Cd and Cu in contaminated alkaline soil.

3.5. Soil enzyme activity

Heavy metal contamination can have seriously negative influences on the biological activities of soil microbes (Jiang et al., 2010; Pan and Yu, 2011). Soil catalase and urease activity are sensitive biomarkers to heavy metals; hence, they are widely used to assess the effects of soil amendments application on soil biological functions (He et al., 2019b; Shi and Ma, 2017; Tu et al., 2018a). It has been observed that the effects of biochar on enzyme activity in the contaminated soil are highly variable, which may likely be associated with the biochar property and soil environmental conditions (Bashir et al., 2017; Liu et al., 2018; Nie et al., 2018; Zhu et al., 2017). The dynamic changes of soil catalase and urease activities during the 75 days incubation are presented in Fig. 6. With the increase of the incubation time, both catalase and urease activities gradually decreased up to 45 days in all treatments, and then rebounded at the end of the incubation. The reduction in the activities of the enzymes at an early stage could be the result of inhibitory effect by the high concentrations of Cd (56 mg kg^{-1}) and Cu (247 mg kg^{-1}) in the soil. Gong et al. (2019) found a similar trend for the urease activity in Cd contaminated sediment after the addition of tea waste biochar.

Notably, in the early 3 days, soil amended with 5% NT-2 loaded biochar had the highest level of catalase activity among all treatments (Fig. 6a), which is likely due to the protection of porous biochar structures (Quilliam et al., 2013). During the range of the 21st day to the 45th day, there was no significant difference among all treatments. However, on the 75th day, the catalase activity of the biochar and NT-2 loaded biochar treatments all became higher than that of the untreated soil. Compared to the CK, the urease activity of soil amended with biochar declined more pronounced at first 35 days, but sharply increased after the 45th day (Fig. 6b). Moreover, biochar treatment at the low level (1%) exhibited a better promotion effect for urease activity. Previous studies also demonstrated that high doses of biochar application could lead to a decrease in soil urease activity, possibly because of the detrimental effects of biochar to soil microbes (Bhaduri et al., 2016; Gong et al., 2019; Huang et al., 2017). In the case of NT-2 loaded biochar treatment, the urease activity also decreased at the earlier period but started to exceed the CK and biochar treatment after the 35th day of incubation. These observations indicated a faster and higher self-restoring capacity of soil function in contaminated soil amended with NT-2 loaded biochar.

3.6. Relative abundance of *Pseudomonas* genus and overall microbial community

The relative abundance of bacteria from *Pseudomonas* genus in different treatments at the end of pot experiments (75 days) was quantified from high-throughput DNA sequencing results. Fig. 7a shows that the abundance percentage of *Pseudomonas* sp. in BN5 treatment was

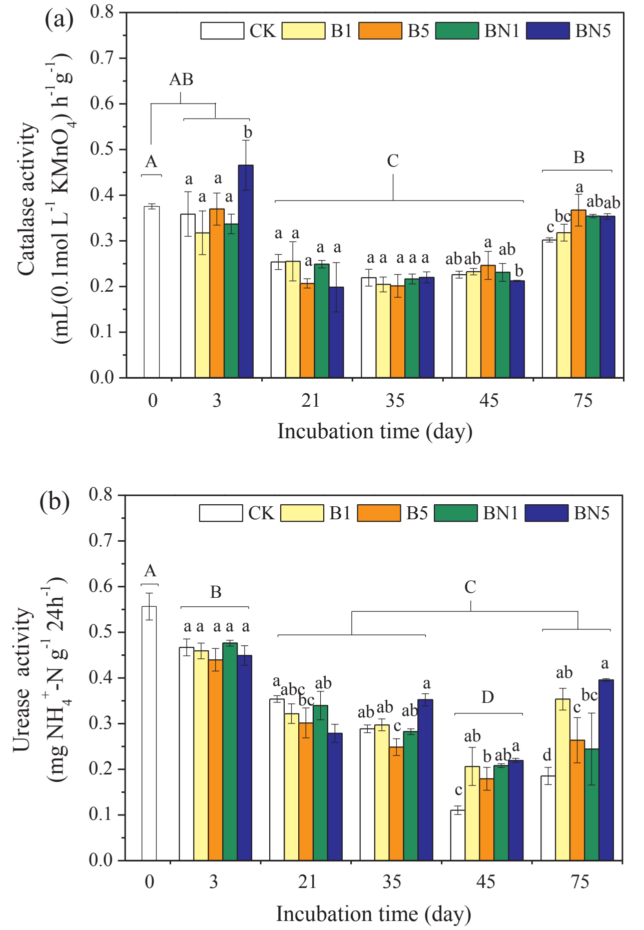

Fig. 6. Impact of biochar and NT-2 loaded biochar on the activities of urease (a) and catalase (b) in the soil. Lowercase letters indicate significant differences among different treatments ($p < 0.05$), while capital letters indicate significant differences among different incubation time ($p < 0.05$).

1.8-fold higher than in CK, and was 1.6-fold higher than in B5 treatment, which indicated that the strain NT-2 inoculated to the biochar successfully survived in the soil. However, the microbial alpha diversity in terms of Shannon index showed slight decrease in the BN5 treatment compared with other treatments (Fig. S1). This could be due to the competition of the niche in the soil between the inoculated strain and the indigenous microbes (Wu et al., 2019). Furthermore, PCoA and ANOSIM tests showed significant differences of the microbial community among different treatments ($p = 0.001$) (Fig. 7b). This indicates that the amendment of biochar and NT-2 loaded biochar at different dosages contributed distinctly in the variation of microbial community structure. Furthermore, based on the results from heavy metal speciation, enzyme activity and 16S rRNA gene sequencing analyses, NT-2 played a dominant role in the microbe-biochar complex inoculants during the resistance and stabilization of Cd and Cu in the soil.

3.7. Mechanisms and implications

According to our observation, the labile and bioavailable fraction of Cd and Cu evidently decreased in both biochar and NT-2 loaded biochar amended soils, hence alleviated the negative impacts to microbes and consequently simulated the soil enzyme activity. Among these different treatments, the addition of NT-2 loaded biochar at 5% rate exhibited the optimal effects on heavy metal stabilization and soil restoration, suggesting two possible mechanisms. First, biochar is well known to stabilize heavy metals in contaminated soil (Hamid et al., 2019; Igalavithana et al., 2019; Kuppusamy et al., 2016). In this study, the

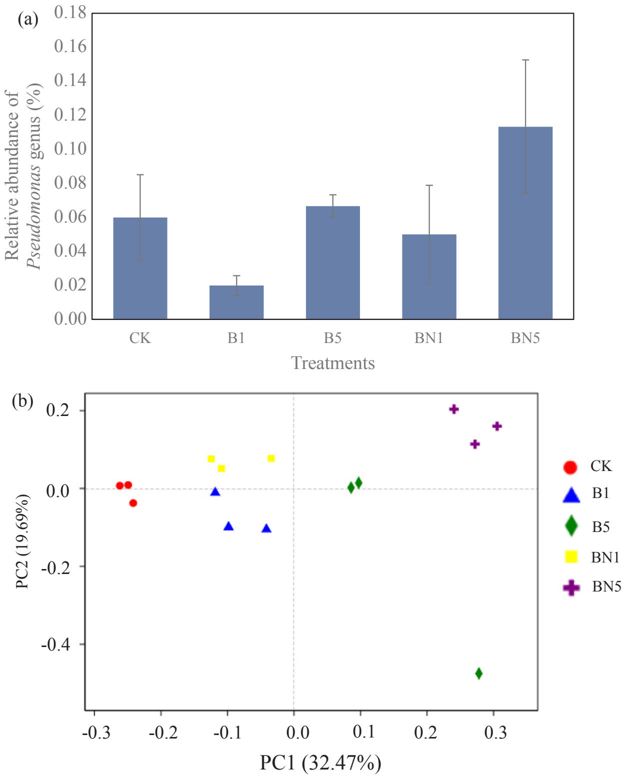

Fig. 7. Relative abundance of *Pseudomonas* genus (a) and the principal co-ordinates analysis (PCoA) of microbial community structure (b) at different treatments.

maize straw biochar could capture the soluble Cd and Cu by the abundant organic functional groups, i.e., via complexation with hydroxyl, carboxyl, and phenolic groups, as well as π electron-rich domain on the aromatic structures (Wang et al., 2018; Wei et al., 2019a). Moreover, OH^-, CO_3^{2-}, and PO_4^{3-} released from the bulk biochar could precipitate with Cd and Cu (Chen et al., 2015; Inyang et al., 2012). Cation exchange and surface complexation (i.e., ionic, covalent or hydrogen bonding) with biochar mineral components might also contribute to the Cd and Cu adsorption (Bian et al., 2014; Harvey et al., 2011; Qian and Chen, 2013). Secondly, the biochar immobilized NT-2 cells were also responsible for the Cd and Cu stabilization. Fig. S2 indicates that the functional groups (i.e., −NH−, −COOH, −OH, and PO_4^{3-}) on the NT-2 cell surface might be involved in biosorption and biomineralization of Cd and Cu, which were similar to the observations of Tu et al. (2018b) and Chen et al. (2019). Besides, the biochar could provide shelter for NT-2 to resist contaminant toxicity, meanwhile, supply nutrients to NT-2 and indigenous soil microbes for their growth (El-Naggar et al., 2019; Zhu et al., 2017). Therefore, our results demonstrated that the application of NT-2 loaded biochar could effectively improve the biochemical properties of heavy metal contaminated soil.

When planning sustainable soil remediation, requirements from environmental, agricultural, economic, and social aspects should be comprehensively considered (Hou et al., 2018). Basically, regarding the effects of biochar application on soil productivity, numerous studies have proved that the soil pH increases after the biochar application (Huang et al., 2017; Laird et al., 2010; Ramtahal et al., 2019; Sohi et al., 2010). Although an increase in soil pH can favor the stabilization of heavy metals, a high pH may seriously interfere and inhibit both the growth and development of plants (Fellet et al., 2014). In our study, the soil pH in the NT-2 loaded biochar treatments were ranged from 7.0 to 8.0, within the satisfactory ranges for agricultural use. As incubation time increased, the bulk biochar material experienced an ageing process in the soil. Ageing leads to the formation of oxygen-containing functional groups and hydrophilic surfaces, which could facilitate heavy metal adsorption (Bian et al., 2014; Rechberger et al., 2019). Furthermore, given the loaded NT-2 cells have a high tolerance to Cd and Cu contamination in the soil, and could proliferate during the incubation, NT-2 loaded biochar is expected to have a long-term effect of for the Cd and Cu stabilization.

4. Conclusions

NT-2 loaded biochar application effectively decreased the lability and bioavailability of both Cd and Cu, especially at the higher application rates. Moreover, NT-2 loaded biochar remarkably enhanced soil enzymatic activities in the contaminated soil. Compared to the treatment of biochar alone, even at a lower rate of biochar substrate, the NT-2 loaded biochar inoculant could achieve superior performances on heavy metal stabilization and soil environmental quality improvement, supporting its cost-effective benefit. This study indicated that the combination of functional microbes and a biochar amendment could be a promising technology for the green and sustainable remediation of polluted soil in the near future.

CRediT authorship contribution statement

Chen Tu: Conceptualization, Methodology, Writing - review & editing, Funding acquisition. **Jing Wei:** Data curation, Formal analysis, Writing - original draft, Funding acquisition. **Feng Guan:** Investigation, Visualization. **Ying Liu:** Software. **Yuhuan Sun:** Resources. **Yongming Luo:** Conceptualization, Supervision, Writing - review & editing, Funding acquisition.

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.

Acknowledgements

The biochar was kindly provided by Prof. Zubin Xie from the Institute of Soil Science, Chinese Academy of Sciences. The authors would like to thank Mr. Shiyao He and Mr. Jianhua Zhao from Shanghai Majorbio Bio-pharm Technology Co., Ltd for their helps in high throughput DNA sequencing data analysis. The authors would also like to thank Dr. Kirk G. Scheckel from US EPA for the language polishing. This research was funded by the National Key R&D Program of China (Grant No. 2016YFE0106400, 2018YFC1801001), the National Natural Science Foundation of China (Grant No. 41977139, 41571310), and the Yantai Key Research and Development Program (Grant No. 2019XDHZ104).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.105576.

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