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ORIGINAL PAPER

Effects of biochar on Cd and Pb mobility and microbial community composition in a calcareous soil planted with tobacco

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Abstract

An experiment was conducted with tobacco (*Nicotiana tabacum L.*) grown in a Cd- and Pb-contaminated calcareous soil amended with 0.0, 1.0, 2.5, and 5.0% (*w*/*w*) tobacco stalk biochar (BC). The BC amendment significantly increased organic matter, total C, N, P, and K contents of soil, and the C/N ratio. Bioavailable metal concentrations (DTPA extraction) decreased by increasing BC application rate. The 5.0% BC amendment significantly decreased the DTPA-extractable Cd and Pb by 10.4 and 13.6%, respectively. Correspondingly, the bioaccumulation and translocation factors of Cd and Pb also decreased by increasing the BC addition rates and this indicated that BC inhibited the uptake and transfer of both Cd and Pb by tobacco plants. Moreover, high-throughput sequencing revealed that BC increased Chao1 richness, Shannon's diversity and Simpson's diversity of bacterial communities of soil. The relative abundance and genera composition of *Adhaeribacter*, *Rhodoplanes*, *Pseudoxanthomonas*, and *Candidatus Xiphinematobacter* increased under BC treatments, while those of *Kaistobacter*, *Lacibacter*, and *Pirellula* decreased. Overall, BC increased soil nutrients (C, N, P, and K contents), enhanced bacterial diversity indexes and richness, and changed the bacterial community composition, which may all have contributed to reduce the mobility and bioavailability of both Cd and Pb in a calcareous soil.

Keywords Biochar · Heavy metal · Bioavailability · Translocation · Bacterial community

Introduction

In recent years, heavy metals (HMs) introduced into soil have moved into agricultural crops and through soil–plant–food chain transfer, have caused elevated levels of toxic metals in human organs (Chaney et al. 2004). To decrease HMs pollution in soil is necessary to reduce their accumulation in food or crops (Puga et al. 2015). The potential toxicity of HMs depends on various factors, such as chemical form, concentration, reactivity, mobility, and availability. In order to ensure food security and health, it is needed to reduce bioavailability of metals in croplands (Mulligan et al. 2001). A variety of conventional remediation techniques have been developed to minimize the bioavailability and mobility of HMs in agricultural soils and involve replacement, solidification, electrokinetic extraction, and washing strategies (Mulligan et al. 2001; Rutigliano et al. 2008). However, most of these techniques are unfeasible on large scale since they are time-consuming, expensive, and not environmentally friendly (Houben et al. 2013).

Biochar (BC) is an organic C rich material derived from the pyrolysis of waste biomass, such as agricultural and forestry residues under an oxygen-limited environment (Lehmann 2007). It is used to mitigate CO_2 emissions due to its long-term persistence in soil (Lehmann 2007; Budai et al. 2016) and to immobilize soil HMs, thus decreasing the risk of HMs entering the human food chain (Beesley and Marmiroli 2011) due to its large surface area, highly microporous structure, presence of active organic functional groups, and generally high CEC and pH (Xie et al. 2015).

Soil pH affects soil physicochemical reactions and processes, and governs the surface charges of soil particles. At high pH values, HMs can be precipitated or adsorbed by soil particles (including electrostatic and specific adsorption) (Inyang et al. 2016), thus decreasing their bioavailability. To our

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knowledge, little information is available about the effects of BC on HMs-contaminated calcareous soils. Generally, the application of BC can change pH, exchangeable acidity, point of zero net charge, CEC, and specific surface area (Chintala et al. 2014), and changes in these soil properties may determine HMs forms and availability. However, the effects of BC on the mobility and availability of metals in a calcareous soil are poorly known.

Moreover, HMs can also affect soil microbial communities, which play important roles in soil biogeochemical processes, such as nutrient cycling and decomposition of organic materials and pollutants (Acosta-Martinez et al. 2010). Generally, HMs have a negative impact on soil microbial communities, but bacterial community compositions in the BC-amended soil might show different responses depending on the dose and type of metals. The abundance of *Actinobacteria* substantially increased whereas those of *Acidobacteria* and *Chloroflexi* decreased in the BC-treated soils polluted with Pb and As (Ahmad et al. 2016). However, BC amendments decreased the abundance of *Actinobacteria* and increased those of the *Bacteroidetes* and *Planctomycetes* in soils contaminated with As and organochlorine (Gregory et al. 2015). Therefore, it is worthwhile examining the effects of BC amendments on the diversity and composition of soil bacterial communities in a calcareous soil, polluted by HMs.

The total annual crop straw output in China is more than 7.22×10^8 t, of which 5.21×10^6 t are produced annually by tobacco (*Nicotiana tabacum L.*) stalk, which are generally burned during harvest season after removing the marketable leaves; this management may result in severe air quality and risk to human health (Ai et al. 2015). Tobacco stalks can be converted to BC, which can be used as to mitigate soil pollution (Lu et al. 2014; Puga et al. 2015). Tobacco tends to accumulate high levels of HMs, especially Cd and Pb, with risk for humans due to the tobacco consumption. Indeed, smoking is the major contributor to Cd intake. Cd is known to cause kidney and bone damages whereas Pb can cause a variety of neurological disorders (Akesson et al. 2006; Bellinger 2008).

The main objectives of the present work were to investigate (1) the changes in soil chemical properties in Cd- and Pb-contaminated soils amended with BC produced from tobacco stalks, (2) the immobilization of Cd and Pb in calcareous soils after BC addition, and (3) the effects of BC on microbial community diversity and composition in Cd- and Pb-contaminated soils.

Materials and methods

BC and soil characteristics

The BC was made from tobacco stalks taken from Longgang Tobacco Experiment Station (26° 52′ 24.8″ N, 107° 06′ 40.8″ E), Guizhou Academy of Tobacco Science, China. Air-dried tobacco stalks were pyrolyzed under oxygen-limited conditions in a combined carbonization furnace, with a cylindrical furnace body (1.2-m height and 1.2-m diameter). The main stalk BC properties were 760.09 g kg^{-1} C, 23.10 g kg^{-1} N, 52.20 g kg^{-1} K; 5.78 g kg^{-1} P, pH (H_2O) of 9.77, and a C/N ratio of 32.95. The Cd and Pb contents of BC were 2.74 and 12.0 mg kg^{-1}, respectively. The BC was ground to pass through a 2-mm sieve and homogenized before being used.

The soil was collected from the plow-layer (0 to 20-cm depth) in a tobacco field at the Longgang Tobacco Experiment Station. The region has a subtropical humid monsoon climate with a mean annual temperature of 13.5–14.6 °C, and an average annual rainfall of 1130–1206 mm over four distinct seasons. The soil type is classified as a calcareous soil (Chinese Soil Taxonomy), equivalent to a calcaric cambisol in the FAO/UNESCO Taxonomy and had the following main properties: pH of 7.85; the average organic matter, total C, N, K, and P contents of 43.67, 35.42, 1.92, 8.90, and 1.06 g kg^{-1}, respectively; and Cd and Pb contents of 0.46 and 57.8 mg kg^{-1}, respectively. Soil samples were air-dried, homogenized, and sieved (< 2 mm) before the experimental use.

Pot experiment

Plastic pots were filled with 15 kg of soil, and amended with 0.0, 1.0, 2.5, and 5.0% (*w*/*w*) of tobacco stalk BC. Each pot was artificially mixed with a dose of Cd (in the form of $CdCl_2$) and Pb (in the form of $Pb(NO_3)_2$) at 5 and 100 mg kg^{-1}, respectively. These metal concentrations were chosen to simulate a moderately contaminated soil and represent the average pollution levels of Cd and Pb in mining areas of Guizhou province; we also assumed that BC addition rates would be appropriate for mine soil remediation. Each treatment was replicated three times. After application of the BC to soil, pots received deionized water to bring soil moisture to 50% of the field water-holding capacity (WHC); then pots were pre-incubation for 1 week.

The K326 tobacco, a hybrid from the Northup King Seed Company, is a disease-resistant and heat-tolerant variety, widely cultivated in China. After 1 week of pre-incubation, uniform size tobacco seedlings (0.2-cm diameter, 10-cm height) were transplanted directly from the seedbeds into pots. The tobacco plants were watered every 2–3 days to prevent drought stress and grown in a greenhouse under natural sunlight with an average temperature of 26–34 °C and a constant relative humidity of 70%.

After 3 months, the aboveground parts (leaves and stems) of plants were harvested by cutting the stems at the soil surface. The roots were carefully removed from soil, washed with tap water and deionized water, and then air-dried for 24 h. The fresh weight of roots, stems, and leaves were measured for

each pot and the respective were taken to determine moisture content and Cd and Pb concentrations. Rhizosphere soil samples were collected by removing the tobacco plants from the pots and obtaining the soil tightly attached to roots at five different locations; then rhizosphere subsamples of each plot were thoroughly mixed to get a composite sample, which was divided into two parts. One subsample was used for DNA extraction and microbial community analysis. The other subsample was used for soil chemical analysis.

Chemical analysis

The pH of BC in water solution was measured at 1:20 (*w*/*v*) ratio after occasionally stirring over 1 h. Total C (TC) and total N (TN) contents of BC were determined with an Elemental Analyzer (EA) (Vario Macro Cube, Germany). Total P, K, Cd, and Pb contents of BC were determined using inductively coupled plasma optical emission spectroscopy (ICP-OES, Vista-MPX, Agilent) after a HNO_3-H_2O_2-HF-$HClO_4$ digestion (Bird et al. 2012).

Soil pH was measured in a soil:water slurry at a 1:2.5 (*w*/*v*) ratio using a pH-meter. Soil organic matter (SOM) was determined by oxidation method with potassium dichromate (Allison 1965). Soil TC and TN concentrations were measured with the EA. Soil total P (TP) content was analyzed by H_2SO_4-$HClO_4$ digestion, available P (AP) by the $NaHCO_3$ extraction and Mo-Sb colourimetric method (Wang et al. 2016). Total K (TK) and available K (AK) contents were measured by the NaOH melt and CH_3COONH_4 extraction, respectively (Wang et al. 2016). The total Cd and Pb contents in the different treatments were analyzed using a digestion mixture of HNO_3-H_2SO_4-$HClO_4$ followed by atomic absorption spectrometry (AAS) (PinAAcle 900F, PE) (Allen et al. 1986). Available Cd and Pb were extracted by DTPA solutions (pH 7.3) (0.005M DTPA, 0.01M $CaCl_2$, and 0.1M TEA) and analyzed by AAS (Lindsay and Norvell 1978). The Cd and Pb concentrations of plant tissues were determined by HNO_3-$HClO_4$ digestion followed by AAS (Burzynski and Klobus 2004).

Soil DNA extraction and 16S rRNA MiSeq sequencing

Total genomic DNA was extracted from a soil (0.25 g) sampled from each pot using the PowerSoil® DNA Isolation Kit (MOBIO Laboratories, Carlsbad, CA, USA) according to manufacturer protocol. DNA was diluted to 10 ng μl^{-1} with sterile ultrapure water and stored at − 80 °C until downstream applications. To amplify the V4 hypervariable regions of 16S rRNA genes, the universal primer 515F (5′-GTGC CAGCMGCCGCGGTAA-3′) and 909R (5′-CCCC GYCAATTCMTTTRAGT-3′) with the 12-nt unique barcode were used (Tamaki et al. 2011). The PCR was carried out in 25 μl final volume containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.4 μM each deoxynucleoside triphosphate (dNTPs), 1.0 μM each primer, 0.5 U of Ex Taq polymerase (TaKaRa, Japan) and 10 ng DNA template. The PCR program consisted of an initial heating step at 95 °C for 3 min, 30 cycles at 94 °C for 40 s, at 56 °C for 60 s, at 72 °C for 60 s, and a final extension step at 72 °C for 10 min. Amplicon quality was checked by gel electrophoresis, and then amplicons were pooled and purified using a SanPrep DNA Gel Extraction Kit and quantified with Nanodrop. All samples were pooled together at equal molar amounts. The sequencing libraries were constructed using a TruSeq DNA sample preparation kit following the manufacturer's instructions. The purified library was diluted, denatured, re-diluted, and mixed with PhiX (equal to 30% of final DNA amount) according to the Illumina library preparation protocols, and then the samples were sequenced by the Illumina Miseq system.

Analysis for bacteria community compositions

Raw sequence data were processed using the QIIME Pipeline-Version 1.7.0 toolkit (http://qiime.org/tutorials/tutorial.html). All sequence reads were trimmed and assigned to each sample based on their barcodes. The sequence libraries were split and denoised to avoid an overestimation of diversity caused by sequencing errors. The sequence reads were processed by removing the incorrect primer, only accepting reads with an average quality score ≥ 30 and read lengths ≥ 250 bp. All chimeric sequences were identified and removed by the UCHIME algorithm (Edgar et al. 2011). Then sequences were clustered into operational taxonomic units (OTUs) at the ≥ 97% identity threshold. Singleton OTUs were filtered out. These OTUs were then used as a basis for calculating alpha diversity (Shannon's and Simpson's diversity index, and Chao1 estimator of richness) and the taxonomy was assigned using the Ribosomal Database Project (RDP) classifier (Wang et al. 2007). The original sequence data have been deposited in the European Nucleotide Archive (ENA) under accession number PRJEB23281 (http://www.ebi.ac.uk/ena/data/view/PRJEB23281).

Bioaccumulation factor and translocation factor

The bioaccumulation factor (BAF) refers to the accumulation capability of HMs of plants. The translocation factor (TF) reflects the transference of HMs in plant organs.

$$\mathrm{BAF} = C_{plant}/C_{soil}$$
$$\mathrm{TF} = C_{aboveground}/C_{belowground}$$

where, C_{plant} is the metal concentration in plant tissue (mg kg^{-1}), C_{soil} is the metal concentration in soil (mg kg^{-1}), $C_{\mathrm{aboveground}}$ is the metal concentration in plant leaf and stem (mg kg^{-1}), and $C_{\mathrm{belowground}}$ is the metal concentration in plant root (mg kg^{-1}).

Statistical analysis

The differences in soil chemical properties, bacterial diversity and abundance, and availability and plant uptake of Cd and Pb among different treatments were compared using one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test using SPSS 13.0 software package (SPSS Inc., Chicago, IL, USA). The composition of microbial communities were compared by principal coordinates analysis (PCoA) in Fast UniFrac. All significance levels were set at $p < 0.05$.

Results

Effect of BC on soil properties and plant biomass

Table 1 shows the changes in chemical properties of soils amended with tobacco stalk BC at four application rates. After incubating for 90 days, the addition of BC significantly increased SOM content from 46.47 ± 0.78 g kg^{-1} of the control soil (0.0% BC amendment) to 94.21 ± 10.22 g kg^{-1} in the 5.0% BC-amended soil. The TC, TN, TP, and TK contents and the C/N ratio also significantly increased by increasing BC application rates. However, there were no significant differences in pH between BC-unamended and amended calcareous soils at the end of the incubation. In addition, no significant difference in AP concentration was detected between 0.0, 1.0, and 2.5% BC-treated soils, which were significantly lower ($p < 0.05$) than the value of the 5.0% BC-amended soil. The AK content significantly increased by increasing BC application rates. In general, the nutrient status of Cd- and Pb-polluted calcareous soils increased after BC application (Table 1).

The biomass (dry weight) of the tobacco cultivated in the BC-amended and not amended soils did not differ ($p < 0.05$) (Fig. 1). The average of total plant biomass (including root, stem, and leaf) were 217.96, 217.15, 220.63, and 233.16 g plant^{-1} in the 0.0, 1.0, 2.5, and 5.0% BC-amended soils, respectively.

Effect of BC on soil Cd and Pb availability and plant uptake

The concentrations of available Cd and Pb of soils were affected by BC application rates (Fig. 2). Compared with the control, the 5.0% BC amendment decreased the available Cd and Pb concentrations by 10.4 and 13.6%, respectively. There were significant differences in DTPA-extractable Cd between the control and the 5.0% BC amendments, and in DTPA-extractable Pb between the control and the 2.5, and 5.0% BC amendments (Fig. 2). Therefore, the immobilization of Cd and Pb in the calcareous soil was only significantly affected at the high BC addition rate.

Table 1 Soil chemical properties under different BC treatments

Treatments (%)	pH	TC (g kg^{-1})	TN (g kg^{-1})	TP(g kg^{-1})	TK(g kg^{-1})	C/N	SOM(g kg^{-1})	AP(mg kg^{-1})	AK(mg kg^{-1})
0.0	7.85 ± 0.05^{a}	35.88 ± 0.74^{d}	1.89 ± 0.06^{c}	0.94 ± 0.01^{c}	5.35 ± 0.03^{a}	19.08 ± 1.03^{b}	46.47 ± 0.78^{b}	45.09 ± 0.96^{b}	350.82 ± 58.13^{c}
1.0	7.90 ± 0.05^{a}	40.13 ± 0.35^{c}	1.97 ± 0.06^{bc}	0.95 ± 0.01^{c}	5.33 ± 0.55^{a}	20.37 ± 0.36^{b}	47.56 ± 10.36^{b}	43.10 ± 1.87^{b}	624.89 ± 216.56^{bc}
2.5	7.92 ± 0.13^{a}	47.89 ± 0.20^{b}	2.13 ± 0.10^{b}	0.99 ± 0.02^{b}	5.48 ± 0.37^{a}	22.50 ± 0.89^{a}	54.24 ± 0.63^{b}	45.21 ± 2.83^{b}	995.37 ± 149.26^{b}
5.0	8.01 ± 0.20^{a}	56.75 ± 1.21^{a}	2.41 ± 0.04^{a}	1.13 ± 0.01^{a}	5.83 ± 0.35^{a}	23.54 ± 0.09^{a}	94.21 ± 10.22^{a}	55.08 ± 1.32^{a}	1959.50 ± 7.79^{a}

Values are presented as mean ± standard deviation. *TC*, total C; *TN*, total N; *TP*, total P; *TK*, total K; *SOM*, soil organic matter content; *AP*, available P content; *AK*, available K content. The different letters in columns represent a significant difference ($p < 0.05$) (one-way ANOVA and LSD test)

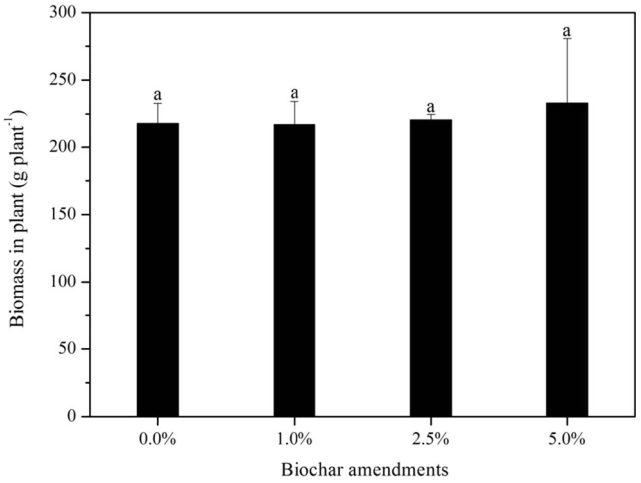

Fig. 1 Biomass (dry weight) of tobacco plants cultivated in a Cd- and Pb-contaminated soil amended with different BC application rates. Error bars indicate standard deviation. Same letters indicate no significant difference in mean values ($p > 0.05$)

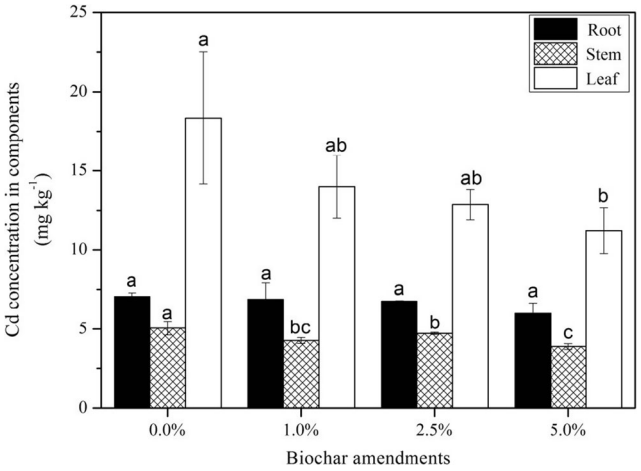

Fig. 3 Concentration of Cd in tissues (root, stem, and leaf) of tobacco under different BC application rates. Error bars indicate standard deviation. Different letters indicate significant difference ($p < 0.05$)

Compared with the control (18.35 ± 4.17 mg kg^{-1}), the Cd concentration in tobacco leaves decreased to 14.00 ± 1.98, 12.88 ± 0.95, and 11.23 ± 1.45 mg kg^{-1}, in the 1.0, 2.5, and 5.0% BC amendments, respectively (Fig. 3). The Cd concentration in tobacco stem significantly decreased from 5.06 ± 0.44 mg kg^{-1} of the control to 3.89 ± 0.18 mg kg^{-1} of the 5.0% BC-amended soil. However, there were no significant differences in Cd concentrations in tobacco roots between BC-unamended and -amended soils. The Pb concentration in tobacco roots significantly decreased from 75.45 ± 1.20 mg kg^{-1} in the control soil to 51.45 ± 1.27 mg kg^{-1} in the 5.0% BC-amended soil (Fig. 4). The Pb concentrations in tobacco leaves also significantly decreased from 6.50 ± 1.56 mg kg^{-1} in the control to 2.58 ± 0.21 mg kg^{-1} in the 5.0% BC amendment, respectively. The BC amendment had no significant influence on Pb concentration in the tobacco stem, but there were significant differences in Pb concentrations of roots and leaves between the control and 1.0, 2.5, and 5.0% BC amendments, and between the control and 2.5%, and 5.0% BC amendments, respectively (Fig. 4).

Bioaccumulation and translocation factors of Cd and Pb

The BAF_{Cd} and BAF_{Pb} of root, stem, and leaf decreased by increasing BC application rates (Table 2). The lowest leaf BAF_{Cd} (1.94) occurred with the 5.0% BC amendment being 63.0% lower ($p < 0.05$) than the control value. The lowest root and stem BAF_{Cd} were also observed with the 5.0% BC amendment being 87.4 and 78.8% ($p < 0.05$) of the highest value observed in the control, respectively. Likewise, there were significant differences in the BAF_{Pb} of tobacco leaf

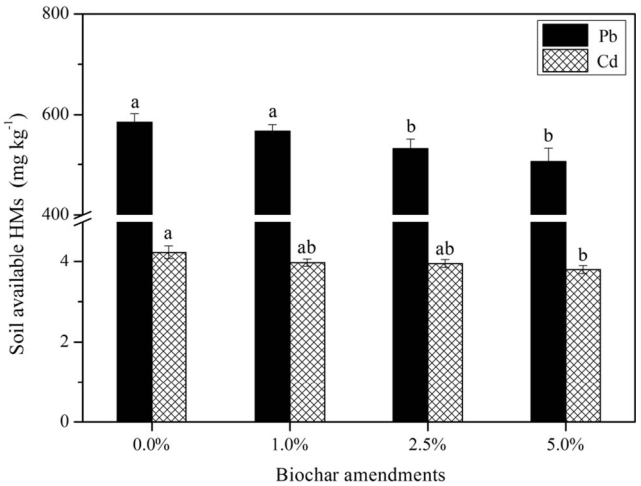

Fig. 2 Concentrations of DTPA-extractable Cd and Pb in soil amended with different levels of BC. Different letters indicate significant difference ($p < 0.05$)

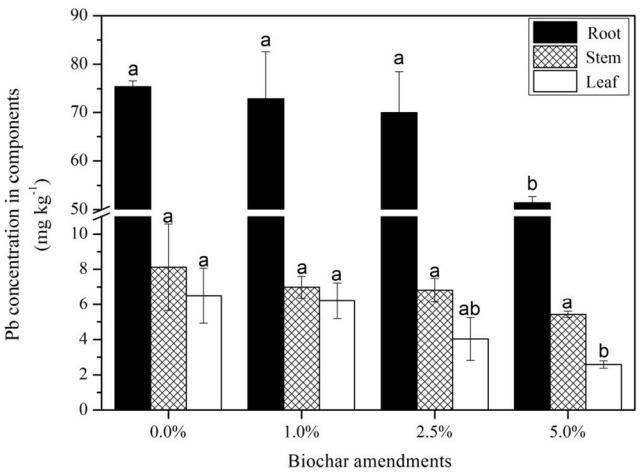

Fig. 4 Concentration of Pb in tissues (root, stem, and leaf) of tobacco under different BC application rates. Error bars indicate standard deviation. Different letters indicate significant difference ($p < 0.05$)

Table 2 Bioaccumulation factor (BAF) and translocation factor (TF) of Cd and Pb in tobacco tissues under different BC amendments

Treatments (%)	BAF_{Cd}			TF_{Cd}		BAF_{Pb} ($\times 10^{-2}$)			TF_{Pb} ($\times 10^{-2}$)	
	Leaf	Stem	Root	Leaf	Stem	Leaf	Stem	Root	Leaf	Stem
0.0	3.08 ± 0.66^{a}	0.85 ± 0.06^{a}	1.19 ± 0.05^{a}	2.61 ± 0.67^{a}	0.72 ± 0.09^{a}	0.62 ± 0.15^{a}	0.77 ± 0.23^{a}	7.19 ± 0.11^{a}	8.59 ± 1.93^{a}	10.74 ± 3.09^{a}
1.0	2.33 ± 0.28^{ab}	0.71 ± 0.05^{ab}	1.15 ± 0.20^{a}	2.08 ± 0.60^{a}	0.63 ± 0.07^{a}	0.59 ± 0.09^{a}	0.66 ± 0.05^{a}	6.78 ± 1.16^{ab}	8.93 ± 2.85^{a}	9.99 ± 2.47^{a}
2.5	2.18 ± 0.06^{ab}	0.80 ± 0.05^{ab}	1.15 ± 0.06^{a}	1.90 ± 0.15^{a}	0.70 ± 0.01^{a}	0.40 ± 0.13^{ab}	0.66 ± 0.09^{a}	6.70 ± 0.73^{ab}	6.04 ± 2.67^{a}	10.03 ± 2.47^{a}
5.0	1.94 ± 0.25^{b}	0.67 ± 0.03^{b}	1.04 ± 0.11^{a}	1.88 ± 0.43^{a}	0.65 ± 0.04^{a}	0.26 ± 0.01^{b}	0.55 ± 0.00^{a}	5.20 ± 0.28^{b}	5.02 ± 0.54^{a}	10.55 ± 0.60^{a}

Values are presented as mean ± standard deviation. BAF_{Cd} *and* BAF_{Pb}, BAF of Cd and Pb, respectively, TF_{Cd} *and* TF_{Pb}, TF of Cd and Pb, respectively. The different letters in columns represent a significant difference ($p < 0.05$) (one-way ANOVA and LSD test)

and root between the control and the 5.0% BC amendment; in addition, no significant differences of BAF_{Pb} of tobacco stem were observed among the different treatments. Both leaf TF_{Cd} and TF_{Pb} decreased by increasing the BC rate. The lowest leaf TF_{Cd} and TF_{Pb} were observed in the 5.0% BC-amended soil being 72.0 and 58.4% of the control value, respectively, and suggesting that BC could affect the transfer of Cd and Pb from roots to leaves. However, there were no significant changes in the TF_{Cd} or TF_{Pb} of leaf and stem in response to BC amendment.

Effect of BC on soil bacterial community diversity

To compare the alpha diversity of bacterial communities among all BC treatments, the same number of sequences per sample (7910) was randomly selected. The coverage values of all samples were more than 84.0%, decreasing by the increasing BC application rates. After BC amendment, the average number of observed OTUs increased from 1971 in the control soil to 2278 in the 5.0% BC-amended soil. Similarly, the 5.0% BC-amended soil had higher values for Chao1 richness, Shannon's diversity and Simpson's diversity than the other BC amendment rates. Therefore, the alpha diversity of bacterial communities in the calcareous soil increased by increasing BC addition rates (Table 3).

The effect of different BC addition rates on the composition of bacterial communities were analyzed by two dimensional Principal coordinates analysis (PCoA) of weighted UniFrac distance (Fig. 5), which explained 36.5% (PCoA1) and 15.6% (PCoA2) of the total variance and confirmed a separation between 2.5% BC amendment and both the 0.0 and 1.0% BC-amended soils, while the 5.0% BC amendment was more distant from other groups.

Effect of BC on soil bacterial community composition

A total of 35 phyla were shared by all soil samples. The main phyla were *Proteobacteria*, *Bacteroidetes*, *Acidobacteria*, *Chloroflexi*, *Crenarchaeota*, *Actinobacteria*, *Gemmatimonadetes*, *Verrucomicrobia*, *Planctomycetes*, and *Nitrospirae* (Fig. 6). Regardless of BC application rates, *Proteobacteria* was the most dominant of the 35 phyla, accounting for approximately 24.8–29.1% of the total relative abundance. *Bacteroidetes* was the second largest phylum in all samples, accounting for 16.5–26.9% of the total. The relative abundances of *Chloroflexi* and *Gemmatimonadetes* significantly decreased by increasing the BC application rates. For example, the relative abundances of *Chloroflexi* decreased from 12.0% in the control soil to 6.7% in the 5.0% BC-amended soil, while the corresponding values of *Gemmatimonadetes* were from 3.3 and 1.5% in the 0.0 and 5.0% BC amendments, respectively. The relative abundances of *Verrucomicrobia* increased after BC

Table 3 Bacterial community richness and diversity of Cd- and Pb-contaminated soils with different BC amendments

Treatments (%)	Coverage	Observed OTUs	Shannon	Simpson	Chao1
0.0	0.8636 ± 0.0105	1971 ± 103	9.27 ± 0.11	0.9942 ± 0.0004	3615 ± 324
1.0	0.8617 ± 0.0100	1985 ± 136	9.23 ± 0.30	0.9929 ± 0.0030	3746 ± 312
2.5	0.8515 ± 0.0117	2077 ± 111	9.40 ± 0.16	0.9948 ± 0.0009	4032 ± 315
5.0	0.8415 ± 0.0240	2278 ± 306	9.71 ± 0.42	0.9956 ± 0.0016	4145 ± 582

Values are presented as mean ± standard deviation. *Coverage*, good's nonparametric coverage estimator; *Shannon*, nonparametric estimation of Shannon diversity index; *Simpson* nonparametric estimation of Simpson diversity index; *Chao1*, species richness estimators

addition, from 2.4% in the control to 2.6 and 4.5% in the 2.5 and 5.0% BC amendments, respectively. Moreover, the addition of BC had no significant impact on the relative abundances of *Planctomycetes* and *Nitrospirae* in calcareous soils.

Saprospirales was the most dominant order in all soil samples, accounting for approximately 9.4–17.3% of the total abundance (Fig. 7). The relative abundance of *Rhizobiales* increased by increasing application rates of BC. Compared to the soil amended with 0.0, 1.0, and 2.5% BC, *Rhizobiales* were significantly ($p < 0.05$) more abundant in the 5.0% BC-amended soil. However, the relative abundance of *envOPS12* and *iiil-15* decreased by increasing the BC application rates. *Sphingomonadales*, *envOPS12*, and *iiil-15* were significantly ($p < 0.05$) the least abundant in the 5.0% BC-amended soil (Fig. 7).

Sixteen bacterial genera generally had percentages greater than 1.5% and significant differences at least between two treatments; they were *Kaistobacter*, *Candidatus Nitrososphaera*, *Chryseobacterium*, *Flavobacterium*, *DA101*, *Flavisolibacter*, *Rhodoplanes*, *Bradyrhizobium*, *Nitrospira*, *Adhaeribacter*, *Lacibacter*, *Pirellula*, *Lysobacter*, *Pseudoxanthomonas*, *Thermomonas*, and *Candidatus Xiphinematobacter*. As shown in Table 4, the relative abundances of *Candidatus*

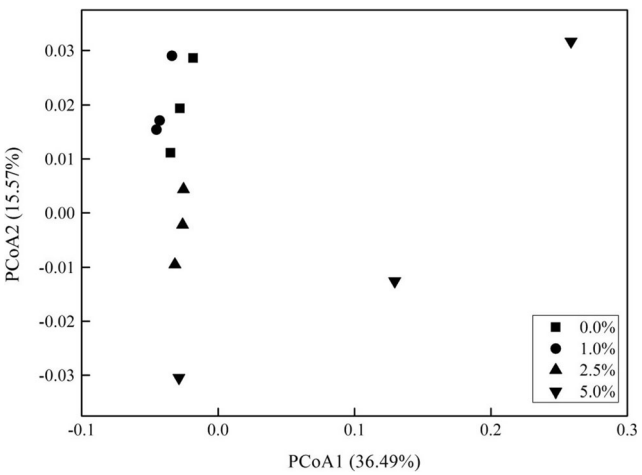

Fig. 5 PCoA plot based on weighted UniFrac distance generated for the bacterial communities of the Cd- and Pb-polluted soil amended with different BC application rates

Nitrososphaera and *Kaistobacter* were higher than 4.0% in all treatments. The relative abundances of *Adhaeribacter*, *Flavobacterium*, *Rhodoplanes*, *Pseudoxanthomonas*, and *Candidatus Xiphinematobacter* increased after BC amendments in Cd- and Pb-polluted calcareous soils, from 0.05–0.8% in the control up to 0.1–1.1% and 0.3–3.4% in the 2.5 and 5.0% BC amendments, respectively. However, the relative abundances of *Chryseobacterium*, *Lacibacter*, *Pirellula*, and *Kaistobacter* decreased as the BC amendment rate increased. The corresponding values of the 0.0% BC treatment were 0.1–8.0%, while those of amendments with 2.5 and 5.0% BC amendments were 0.1–7.7%, and 0.02–4.3%, respectively. The relative abundances of *Kaistobacter*, *Flavobacterium*, *Rhodoplanes*, *Pirellula*, *Pseudoxanthomonas*, and *Candidatus Xiphinematobacter* were significantly ($p < 0.05$) higher in the 5.0% BC-amended soil than in the control.

Discussion

Effect of BC on calcareous soil properties and plant biomass

All BC treatments did not significantly increase the calcareous soil pH (Table 1), thus confirming what already reported for alkaline soils (Lentz and Ippolito 2012; Nabavinia et al. 2015). On the contrary, the BC treatment can increase pH values in acidic soils (Lu et al. 2014; Teutscherova et al. 2017; Zhang et al. 2017). Calcareous soils have alkaline pH values and a high buffering capacity toward acidification (Zhang et al. 2016). The application of tobacco stalk BC significantly increased the SOM concentration in calcareous soils, thus confirming what found by Laird et al. (2010), and the available P and K, particularly at the application rate of 5.0%, also confirming previous studies (Tammeorg et al. 2014; Madiba et al. 2016). The BC is also a net source of these nutrients, contained in the ash (Laird et al. 2010). It is important to underline that the total N, K, and P contents of the calcareous soil were only 1.92, 8.80, and 1.04 g kg^{-1}, respectively (data not shown), whereas, the corresponding values of the tobacco stalk BC were 23.10, 52.20, and 5.78 g kg^{-1}, respectively. Therefore, the BC produced from the incomplete combustion

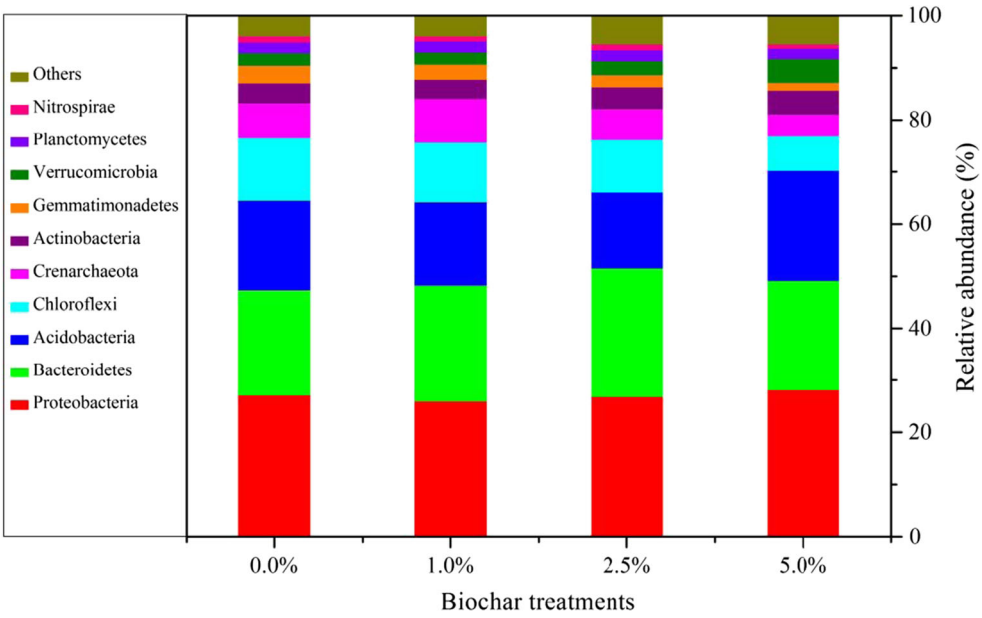

Fig. 6 Relative abundance of dominant phyla in Cd- and Pb-contaminated soils amended with different application rates of BC

of tobacco stalks increased these nutrients in Cd- and Pb-polluted soils (Table 1).

In contrast to previous results (Jeffery et al. 2011; Nguyen et al. 2016), total plant biomass (dry weight) was not significantly enhanced by the BC addition (Fig. 1), which is consistent with the results with plants grown in HMs polluted soil (Namgay et al. 2010; Zhang et al. 2013). Probably any positive effect of BC on crop yield was masked by the negative effects of HMs of the polluted calcareous soil. Furthermore, the positive effect of BC amendment on crop yield may be more pronounced in acidic than in the calcareous soil.

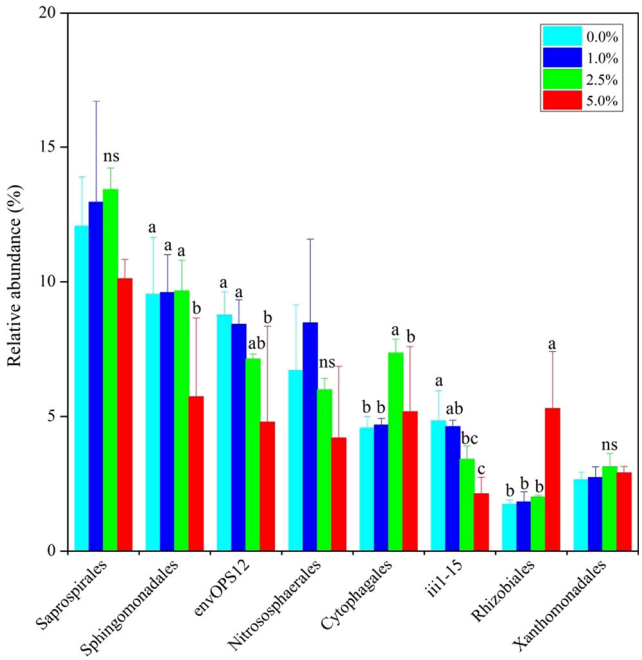

Fig. 7 The dominant distribution of soil bacteria at the order level amended with different BC application rates. The different letters indicate significant difference at $p < 0.05$

Effect of BC on soil Cd and Pb bioavailability

Previous studies have reported that BC can reduce the bioavailability of HMs in soil (Karami et al. 2011; Zhang et al. 2013; Lu et al. 2014), due to the three main mechanisms: (a) increasing adsorption of HMs to organic matter; (b) increasing formation of stable metal-organic complexes (Kumpiene et al. 2008); and (c) stimulating the adsorption and uptake by microbial cells (Javanbakht et al. 2014). Soil Cd and Pb extracted by the DTPA solution are considered to be plant available, especially in calcareous soil (Al-Farraj et al. 2010), and they decreased by increasing BC application rates (Fig. 2). The 2.5% BC amendment reduced DTPA-extractable Cd and Pb by only 6.84 and 9.20%, respectively (Fig. 2), whereas a significant reduction in DTPA-extractable Cd and Pb was observed (7.7–24.2% and 18.1–27.3%, respectively) by the same BC amendment rate in a non-calcareous soils (Bian et al. 2014; Hu et al. 2014), suggesting that BC addition had a low influence on the immobilization of both Cd and Pb in calcareous soils. Probably this depended on the fact that the BC amendments did not significantly change the pH values since pH is considered the dominant factor controlling the availability of HMs in soil (Rieuwerts et al. 2006). The BC amendment decreased extractable HMs concentrations because promoted precipitation or co-precipitation of HMs as oxides, hydroxides, carbonates, and phosphates, due to the increase in soil pH (Beesley and Marmiroli 2011; Houben et al. 2013). This did not occur in the calcareous soil due to its alkaline pH and its buffering capacity, as already mentioned.

Table 4 Relative abundances (%) of dominant genera in Cd- and Pb-contaminated soils with different application rates of BC

Genera	0.0%	1.0%	2.5%	5.0%
Kaistobacter	7.98 ± 1.85^{a}	7.98 ± 1.24^{a}	7.74 ± 0.84^{a}	4.27 ± 2.20^{b}
Candidatus Nitrososphaera	6.66 ± 2.41^{a}	8.43 ± 3.09^{a}	5.95 ± 0.42^{a}	4.18 ± 2.66^{a}
Chryseobacterium	2.41 ± 1.31^{a}	3.88 ± 6.37^{a}	2.19 ± 2.11^{a}	1.68 ± 2.53^{a}
Flavobacterium	0.77 ± 0.42^{b}	0.41 ± 0.26^{b}	1.08 ± 0.44^{b}	3.38 ± 2.11^{a}
DA101	0.37 ± 0.09^{b}	0.30 ± 0.15^{b}	0.36 ± 0.07^{b}	2.27 ± 1.94^{a}
Flavisolibacter	1.90 ± 0.27^{a}	2.18 ± 0.77^{a}	2.26 ± 0.25^{a}	0.96 ± 0.49^{b}
Rhodoplanes	0.51 ± 0.12^{b}	0.52 ± 0.03^{b}	0.52 ± 0.07^{b}	1.91 ± 1.37^{a}
Bradyrhizobium	0.09 ± 0.10^{b}	0.11 ± 0.03^{b}	0.07 ± 0.02^{b}	1.15 ± 1.02^{a}
Nitrospira	0.79 ± 0.10^{a}	0.63 ± 0.19^{ab}	0.80 ± 0.10^{a}	0.50 ± 0.14^{b}
Adhaeribacter	0.09 ± 0.07^{a}	0.15 ± 0.06^{a}	0.14 ± 0.05^{a}	0.25 ± 0.24^{a}
Lacibacter	0.12 ± 0.08^{ab}	0.12 ± 0.03^{a}	0.08 ± 0.05^{ab}	0.02 ± 0.02^{b}
Pirellula	0.27 ± 0.07^{a}	0.22 ± 0.03^{ab}	0.18 ± 0.07^{ab}	0.14 ± 0.05^{b}
Lysobacter	0.47 ± 0.10^{ab}	0.63 ± 0.21^{a}	0.60 ± 0.14^{ab}	0.34 ± 0.07^{b}
Pseudoxanthomonas	0.05 ± 0.07^{b}	0.07 ± 0.03^{b}	0.18 ± 0.11^{ab}	0.40 ± 0.24^{a}
Thermomonas	0.16 ± 0.04^{bc}	0.27 ± 0.02^{a}	0.23 ± 0.05^{ab}	0.12 ± 0.05^{c}
Candidatus Xiphinematobacter	0.14 ± 0.06^{b}	0.15 ± 0.04^{b}	0.19 ± 0.07^{b}	0.31 ± 0.08^{a}

Values are presented as mean ± standard deviation. The different letters after the values of the same line between treatments represent significant difference ($p < 0.05$) (one-way ANOVA and LSD test)

Effect of BC on Cd and Pb accumulation and transfer in plants

Zhang et al. (2013) reported that wheat chaff-derived BC significantly reduced the Cd concentration in *Juncus subsecundus*, and the BC additions decreased Cu and Pb concentrations in rice and ryegrass (Karami et al. 2011; Zheng et al. 2012). The application of 1.0, 2.5, and 5.0% BC significantly decreased the Cd and Pb concentrations in roots, stems, and leaves of tobacco (Figs. 3 and 4), probably due to their adsorption by soil organic matter (Cao and Ma 2004), whose content was increased by BC amendments (Table 1). For the same BC-treatment, the Cd concentration in leaves was higher than that in roots, indicating that Cd was easily transferred in tobacco plants, thus confirming what observed by Angelova et al. (2004). Conversely, Pb was more highly concentrated in roots than in leaves and stems of tobacco, which is consistent with del Piano et al. (2008), due to the accumulation of Pb in the insoluble fraction of cell walls and nuclei of roots (Pahlsson 1989). Moreover, the capacity of root cell walls to bind Pb mainly depends on the amount of polysaccharides, with have carboxyl groups (Inoue et al. 2013). Our results showed that tobacco organs have different abilities to bind Cd and Pb, and BC addition can effectively reduce their accumulation and transfer between different tobacco organs.

Effect of BC on bacterial community composition in the calcareous soil

Changes in physicochemical properties (soil pH, bulk density, soil water content, and soil aeration) and the availability of C, N, and other nutrients in soil can change microbial abundance and composition (Lehmann et al. 2011). The BC addition increased bacterial richness and diversity (Chao1, Shannon, and Simpson indices and observed OTUs). The relative abundance of the *Verrucomicrobia* phylum was increased in soils treated with BC, whereas the relative abundance of the phyla *Chloroflexi* and *Gemmatimonadetes* was reduced. *Acidobacteria*, generally prefer soil acid environments with low resource availability (Rousk et al. 2010), and therefore, the relative abundance of *Acidobacteria* decreased in response to the BC-related shift to a more copiotrophic and neutral environments (Jaiswal et al. 2017). Probably, the relative abundance of the *Acidobacteria* phylum did not decrease in the calcareous soil after BC addition, because the initial pH of calcareous soil was alkaline and not significantly changed by the BC amendments. Similar changes in bacterial diversity were observed in BC-amended soils by others (Xu et al. 2016; Jaiswal et al. 2017). The BC improves nutrient retention in soil micropores by significantly increasing SOM content (Table 1), which influences the availability of nutrient elements to microorganisms (Lehmann et al. 2011). Probably changes soil microbial community composition may increase the tolerance of microbes to HMs.

Conclusion

This study has demonstrated that the application of tobacco stalk BC to a calcareous soil has the potential to reduce the availability of Cd and Pb to plants. However, the influence of BC on Cd and Pb availability and uptake varied depending on the type of HMs and application rate of BC. Generally, the BC amendments significantly increased SOM, total C, N, P, and

K, but not pH of the calcareous soil. The DTPA-extractable Cd and Pb decreased by increasing BC application rate, thus reducing their uptake by plants and probably this occurred due to the increased SOM content. Moreover, the BC addition increased the diversity and richness of bacteria and influenced soil bacterial abundances. The relative abundance of *Adhaeribacter*, *Rhodoplanes*, *Pseudoxanthomonas*, and *Candidatus Xiphinematobacter* increased in the BC-treated soil whereas those of *Lacibacter*, *Pirellula*, and *Kaistobacter* were lower than those of the control. Therefore, the BC amendment improved soil macronutrients, changed bacterial community composition and immobilized Cd and Pb, with the consequent reduction in the mobility and bioavailability of HMs. It is important to underline that greenhouse conditions are different from field conditions and thus caution should be used in extrapolating the results of this study to the field conditions, as well as to other soils and crops (Tuberosa 2012).

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