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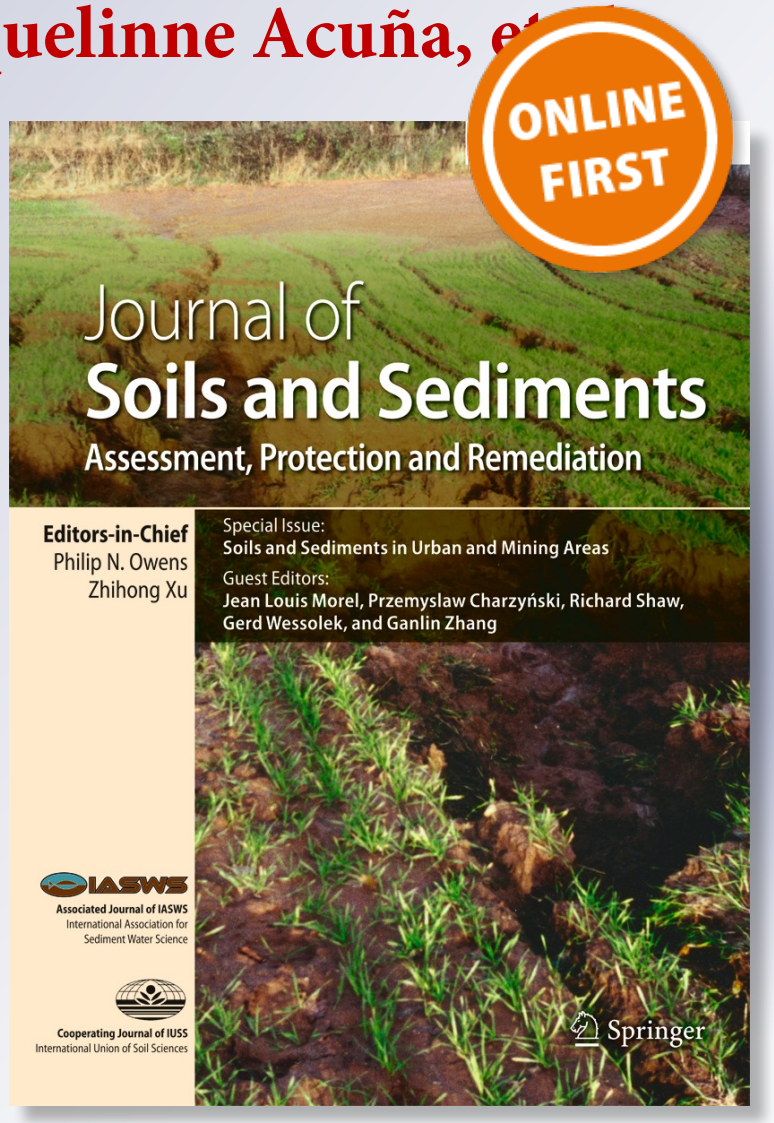

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Effects of biochar on copper immobilization and soil microbial communities in a metal-contaminated soil

Sebastián Meier[1] · Gustavo Curaqueo[1] · Naser Khan[2,3] · Nanthi Bolan[2,3] · Joaquín Rilling[1] · Catalina Vidal[1] · Natalia Fernández[4] · Jacquelinne Acuña[1] · María-Eugenia González[5] · Pablo Cornejo[1] · Fernando Borie[1]

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Abstract
Purpose Copper (Cu) contamination has been increasing in land ecosystems. Biochars (BCs) and arbuscular mycorrhizal fungi (AMF) are known to bind metals, and metallophyte can remove metals from soils. Will BC in combination with AMF contain the Cu uptake by a metallophyte growing in a metal-contaminated soil? The objective of this study was to investigate the effects of BCs on the Cu immobilization and over soil microbial communities in a metal-contaminated soil in the presence of AMF and metallophyte.
Materials and methods Two BCs were produced from chicken manure (CMB) and oat hull (OHB). A Cu-contaminated sandy soil (338 mg kg^{-1}) was incubated with CMB and OHB (0, 1, and 5 % *w*/*w*) for 2 weeks. Metallophyte *Oenothera picensis* was grown in pots (500 mL) containing the incubated soils in a controlled greenhouse for 6 months. A number of analyses were conducted after the harvest. These include plant biomass weight, microbial basal respiration, and dehydrogenase activity (DHA), AMF root colonization, spore number, and glomalin production; changes in fungal and bacterial communities, Cu fractions in soil phases, and Cu uptake in plant tissues.
Results and discussion The BCs increased the soil pH, decreased easily exchangeable fraction of Cu, and increased organic matter and residual fraction of Cu. The BCs provided favorable habitat for microorganisms, thereby increasing basal respiration. The CMB increased DHA by ~62 and ~574 %, respectively, for the low and high doses. Similarly, the OHB increased soil microbial activity by ~68 and ~72 %, respectively, for the low and high doses. AMF root colonization, spore number, and total glomalin-related soil protein (GRSP) production increased by ~3, ~2, and ~3 times, respectively, in soils treated with 1 % OHB. Despite being a metalophyte, *O. picensis* could not uptake Cu efficiently. Root and shoot Cu concentrations decreased or changed insignificantly in most BC treatments.
Conclusions The results show that the BCs decreased bioavailable Cu, decreased Cu uptake by *O. picensis*, improved habitat for microorganisms, and enhanced plant growth in Cu-contaminated soil. This suggests that biochars may be utilized to remediate Cu-contaminated soils.

Keywords Arbuscular mycorrhizal fungi · Biochar · Contamination · Copper · Immobilization · Microorganism

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1 Introduction

Anthropogenic activities such as metal mining, smelting, and refining have been contaminating soils with heavy metals (Meier et al. 2012b). The conventional practices to remediate these hazardous soils include physicochemical approaches (Mulligan and Galvez-Cloutier 2003), which are costly and intrusive in nature causing soil degradation (Khan 2006). Over the last decade, plants are emerging as a cheaper method for phytoremediation, which refers to use of plants (and associated microorganisms) to remove, transfer, stabilize, decrease, and/or decompose contaminants in the ecosystems (McGrath and Zhao 2003). Phytoremediation has been widely used by public agencies and industries because of its low implementation cost and the appreciation by those who prefer "green technologies" over chemicals or heavy machinery (Schwitzguébel 2001).

Nevertheless, the success of the phytoremediation depends on the ability of metallophytes to produce biomass, which is in fact difficult in a soil with metals present at phytotoxic levels, and poor health (Ginocchio et al. 2004). However, soil amendments may be used to remediate a polluted soil and make it suitable for plant establishment (Meier et al. 2011).

Biochar (BC) is the solid product from pyrolysis of waste biomass residues from agricultural or forestry production (Wei et al. 2014). Application of BC to soil has been considered as to having great potential to enhance long-term carbon stabilization because most carbon in BC has an aromatic structure and is very recalcitrant in the environment (Khan et al. 2015). Typically BC has a high pH value and cation exchange capacity (CEC) (Belyaeva and Haynes 2012). A number of studies have demonstrated that BC can adsorb metals from soils (Park et al. 2011; Lu et al. 2012), thereby reducing their availability. At the same time, BC can enhance plant growth by improving soil health (Rutigliano et al. 2014).

Most of previous studies that investigated the interactions between biochar and soil metal contaminants are mainly focused on metal availability and retention (Park et al. 2011; Beesley et al. 2013). However, these studies paid less attention to simultaneous effects of BC on plant growth, soil microbial activity, and changes in microbe communities.

Microbes (bacteria, and fungi) are ubiquitous and integral parts of soils; they play significant roles in the recycling of soilborne C, N, P, S, and metallic elements, making them available to plants (Lehmann et al. 2011). Heavy metals in bioavailable forms adversely affect soil microbes by reducing their populations and changing the community structure and diversity (Carrasco et al. 2006). The adverse impacts are reflected in terms of decreased soil enzyme activities (Karami et al. 2011) and interferences in plant-soil-metals association (Meier et al. 2012b). Carrasco et al. (2006) noted that heavy metals reduce functional diversity of the affected soil microbial communities.

Nevertheless, one prominent microorganism with a potential use under metal stressful conditions is the arbuscular mycorrhizal fungi (AMF) mainly due to its ubiquity in soil environments (even in metal-contaminated soils), and also because these fungi can employ several strategies that allow the plant to tolerate high metal concentration in the soil (Meier et al. 2012b). AMF are microscopic fungi present in the soil, which form symbiotic associations with the roots of terrestrial plants in almost all ecosystems, even in those disturbed by high metal concentrations. The AMF contributes to the plant growth establishment especially under stringent conditions of water, fertility, and metal toxicity (Meier et al. 2012c) improving plant nutrient uptake and allowing metallophyte survival under high metal concentrations (Meier et al. 2011). In turn, the AMF receive carbonaceous compounds from the photosynthesis, which are indispensable for their metabolic processes because they are obliged symbionts (Smith and Read 2008).

If biochar is able to reduce metal uptake of plants, will the biochar amendments protect the microbial communities in Cu-polluted soils from harm? We hypothesized that the biochar amendments in soil would enhance the activity of bacterial and fungal (including AMF) communities in Cu-contaminated soils using a metallophyte. The objective of this study was to investigate the effects of BCs on the Cu immobilization and over soil microbial communities in a metal-contaminated soil, in the presence of AMF and using the metallophyte *Oenothera picensis*.

2 Materials and methods

2.1 Soil

This soil was obtained from the vicinity of Ventanas Cu smelter, a division of National Copper Corporation of Chile (CODELCO), situated in Puchuncaví Valley of Central Chile (32° 46′ 30″ S, 71° 28′ 17″ W). It was a sedimentary Alfisol (Achreptic haploxeral) from Chilicauquén soil series and had fine sandy loan in texture. The soil properties are available in Meier et al. (2015); however, some are as follows: pH 5.76, OM 3 %, CEC 4.63 cmol(+)kg^{-1}, N 6 mg kg^{-1}, P 11 mg kg^{-1}, K 21 mg kg^{-1}, and Zn 17 mg kg^{-1}.

Arbuscular mycorrhizal fungi are naturally present in this soil. A preliminary morphological analysis of AMF revealed that almost all the spores present in the soil belonged to *Glomus* genus, being *Glomus* aff. *intraradices* the dominant ecotype.

2.2 Biochar production and analysis

Chicken manure biochar (CMB) and oat hull biochar (OHB) were produced at 500 and 300 °C, respectively, at the Center

of Waste Management and Bioenergy, Universidad de La Frontera. The pyrolyzer was fed at full load (5 kg) and then purged with nitrogen gas to displace air before starting the process. The temperature during the pyrolysis process was increased at a rate of 3.6 min^{-1} until that the specific temperature of 500 and 300 °C was reached. This temperature was maintained for 2 h.

A number of physicochemical properties of both BCs were determined (Table 1). The pH (1:5) of BCs was measured electrochemically with a pH meter (Thermo Orion 3 Star pH Benchtop). Total N, C, and H were measured using a CHN Elemental Analyzer (CE instruments EA 1108). Total P, K, Ca, Mg, and Al were measured by the methods described in Sadzawka et al. (2006). Total acidity ($Ba(OH)_2$ method) and carboxylic acidity ($Ca(C_2H_3O_2)_2$ method) were determined according to Tan (2005). Specific surface area (Brunauer-Emmett-Teller), and pore size volume were determined using a Nov. 1000e porosimeter (QUANTACHROME) by adsorbing and desorbing nitrogen at 77 K on samples previously dried and outgassed at 160 °C for 16 h.

The surface charge of the samples was determined by measuring the zeta potential (ζ) of colloidal BC according to the procedure of Johnson et al. (1996). About 1 g was added to 100 mL of deionized water, and the solution was shaken at 250 rpm for 30 min using a mechanical shaker. The shaken solution was then placed in a sonic bath to break the particles into colloids, and then the solution was filtered. The ζ of each supernatant solution obtained was determined using a Zetasizer Nano ZS (Malvern Co, UK). Smoluchowski's formula was used to convert the electric mobility into zeta potential.

The surface functional groups of BCs were characterized by Fourier transform infrared (FTIR) spectroscopy using SPOTLIGHT 400 FTIR Microscopy and Imaging System (PerkinElmer). FTIR spectrum was recorded at 64 scans per point with resolution of 8 cm^{-1} from wave numbers 4000 to 500 cm^{-1}. The BC morphology was analyzed using a scanning electron microscope (SEM, JEOL JSM 6380LV) equipped with energy-dispersive spectrometer (Oxford Instruments INCAx Sight) at 80 kV.

2.3 Plant growth experiment

The soil was mixed by hand with CMB or OHB (0, 1, and 5 % *w*/*w*) until reaching homogeneity. Accordingly, the BC treatments are termed as CMB1, CMB5, OHB1, and OHB5. The natural soil without biochar addition was the control (NS). The mixture was left for 2 weeks for equilibration at field capacity and then placed into 500-mL pots (triplicate). Seeds of *O. picensis* were surface-sterilized with 2 % chloramin-T solution for 5 min and rinsed thoroughly with distilled water. Seeds were germinated and plantlets were grown before transplanting in greenhouse (25±3/15±3 °C day/night temperatures; 16/8-h light/dark photo period; 80–90 % relative humidity). Afterward, the plants were transplanted to 500-mL pots. Since the plant growth rate was very low, they were allowed to grow for 6 months. At harvest, shoots and roots were separated, washed with Milli-Q water, oven-dried at 70 °C for 2 days, and weighed. Then, the samples were ground, ashed at 550 °C, and digested using H_2O/HCl/HNO_3 mixture (8:1:1, *v*/*v*/*v*). Copper in the extracts was analyzed by AA spectrometry (UNICAM Solar 969).

2.4 Arbuscular mycorrhizal fungi analysis

Arbuscular mycorrhizal fungal root colonization was quantified using a dissection microscope (×20–×40) after clearing a portion of the roots in 10 % KOH (*w*/*v*) and staining in 0.05 % trypan blue in lactic acid (*v*/*v*). The gridline intersection method (Giovannetti and Mosse 1980) was used to determine the proportion of AM root colonization.

The AMF spores were separated from the soil by wet sieving and decanting in a 70 %*w*/*v* sucrose solution (Gerdemann and Nicholson 1963) and quantified under a magnifying glass with a 30–50 magnification.

Glomalin-related soil protein (GRSP), operationally defined as Bradford-reactive soil protein (Rillig 2004), was determined according to the method described by Wright and Upadhyaya (1998), with minor modifications. The GRSP was extracted from 1 g of soil with 8 mL of 50 mM citrate, pH 8.0, and autoclaving for 1 h at 121 °C. After that, the supernatant was separated by centrifugation at 8000*g* for 20 min and filtrated using paper Whatman No 1. The above procedure was repeated several times on the same sample until the reddish-brown color typical of GRSP disappeared from the supernatant, combining all extracts from a soil sample. The protein content in the crude extract was determined by Bradford assay (Bio Rad Protein Assay; Bio-Rad Laboratories) with bovine serum albumin as the standard.

2.5 Soil microbial activity

At the end of the experiment, soil samples from pots were analyzed for microbial activity. Soil basal respiration and dehydrogenase activity (DHA) were analyzed using moist samples to monitor microbial activity (Anderson and Domsch 1990). For soil basal respiration, 18 g (dry weight) soil was weighed in a plastic tube (perforated at the top for gas exchange). The soil moisture was then raised to 60 % of the water holding capacity, and the plastic tube was inserted in a Schott bottle containing 20 mL of 0.05 M NaOH solution and tightly closed. The blank contained no soil. The bottles were incubated for 24 h at 22 °C (Anderson and Domsch 1990). Then, the plastic tube was removed and 2 mL of 0.5 M $BaCl_2$ solution was added to the solution in Schott bottles. After precipitation of $BaCO_3$, a couple of drops of phenolphthalein

Table 1 General characteristic of the chicken manure biochar and oat hull biochar

Property	Chicken manure biochar	Oat hull biochar
P (g kg^{-1})	19.4	2.1
K (g kg^{-1})	17.2	11.3
Ca (g kg^{-1})	54.0	1.0
Mg (g kg^{-1})	5.1	1.3
Al (mg kg^{-1})	24.1	0.9
Total C (%)	29.67	69.02
Total N (%)	2.13	1.06
C/N Ratio (%)	13.92	65.11
pH (H_2O 1:5)	9.1	7.8
Electrical conductivity (1:5, uS cm^{-1})	924	789
Dry matter (%)	97.6	96.4
Total acidity (mmol g^{-1})	4.4	3.41
Carboxylic acidity (mmol g^{-1})	0	0.28
Phenolic acidity (mmol g^{-1})	4.4	3.13
BET (m^2 g^{-1})	11.51	0.16
Pore diameter (nm)	3.82	3.24
Pore volume (cm^3 g^{-1})	0.009	0.003
Zeta potential (mV)	−29.4	−40.5

indicator solution were added, and the solution was titrated with 0.06 M HCl solutions until the color turned from red to colorless. The amount of CO_2 produced (mg CO_2 kg^{-1} h^{-1}) by soil respiration was calculated based on the consumed HCl solution for titration (Bloem et al. 2006).

For DHA analysis, 3 g (dry weight basis) of fresh soil was weighed into 50-mL sterile centrifuge tubes with 3 mL of 0.5 % triphenylte-trazolium chloride (TTC) solution in 0.1 M Tris buffer (pH 7.6 - 7.8) (Singh and Singh 2005). After incubation for 24 h at 37 °C, 10 mL of methanol was added to each sample to extract produced triphenyl formazan (TPF) by DHA. The sample suspension was centrifuged, and the absorbance of the samples was measured at 485 nm against blank (sterile Milli-Q water).

2.6 PCR-DGGE of the bacterial and fungal communities

The composition on microbial communities (bacteria and fungi) was analyzed by polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE), according to the method described by Acuña et al. (2013). Total DNA of five soil samples from BC treatments was extracted using FastDNA SPIN Kit for Soil (MP Biomedicals) according to manufacturer's guide. For bacterial community analysis, fragments in 16S ribosomal RNA (16S rRNA) gene were amplified by touchdown PCR using specific primer set EUBf933-GC (5′-GCA CAA GCG GTG GAG CAT GTG G-3′) and EUBr1387 (5′-GCC CGG GAA CGT ATT CAC CG-3′), generating a 454-bp fragment (Iwamoto et al. 2000).

Fungal ITS regions (18S rRNA gene) were amplified with nested PCR using primer set ITS1F (5′-CTTGGTCATTTAGAGGAACTAA-3′) and ITS4 (5′-TCC T CCGC TTATT G ATATGC-3′) for first round of PCR. One microliter from first PCR was used a template to second PCR using primer set ITS1F (5′-CTTGGTCATTTAGAGGAACTAA-3′) and ITS2-GC (5′-TCCTCCGC TTATTGAT ATGC-3′).

The PCR program to amplify the 16S rRNA and 18S rRNA genes was the same as described by Iwamoto et al. (2000). The PCR products from each of the replicates soil from BC samples was visualized on an agarose gel stained with ethidium bromide (1 %, *w*/*v*). DGGE analysis was performed using a DCode system (Bio-Rad Laboratories, Inc.). The PCR product (20 μL) was loaded onto a 6 % (*w*/*v*) polyacrylamide gel with linear denaturating gradient (urea and formamide) ranging from 50 to 70 % for 16S rRNA and from 35 to 65 % for the 18S rRNA gene. The electrophoresis was run for 12 h at 100 V. The banding patterns were visualized by staining the gel 1:10,000 with (*v*/*v*) SYBR Gold (Molecular Probes, Invitrogen Co.) for 30 min followed by image capture using an GelDoc-IT TS2 imaging system (Analytik Jena AG company, Jena, Germany).

2.7 Analysis of composition of bacterial and fungal communities

The similarities between the banding patterns in the DGGE were analyzed across clustering of DGGE banding profiles

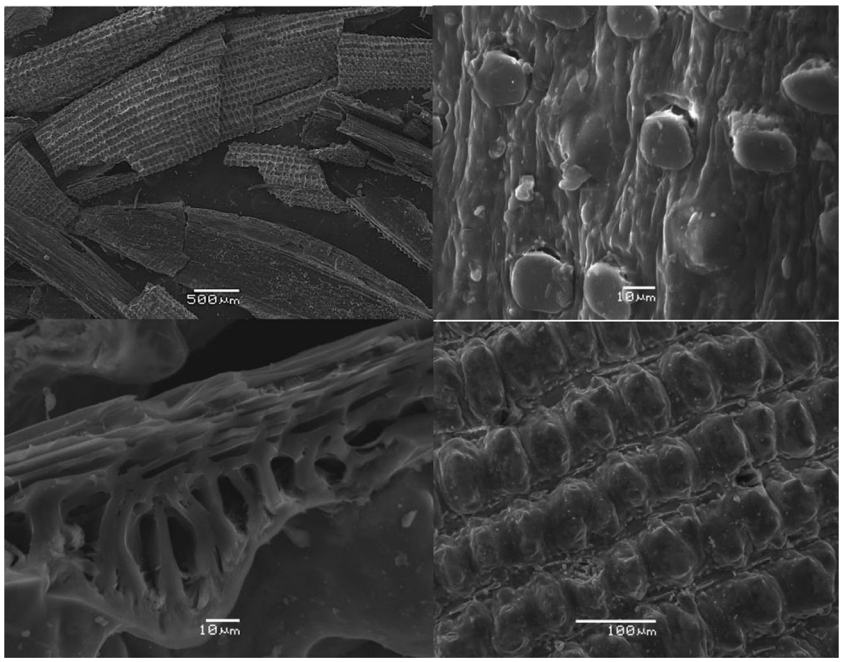

Fig. 1 Scanning electron micrographs of chicken manure biochar (CMB)

using a dendrogram with Phoretix 1D analysis software (Total Lab Ltd.). Based on the matrix obtained from Phoretix 1D analysis, the changes in the microbial community composition (presence or absence) and abundance of bacterial and fungal groups were visualized using nonlinear multidimensional scaling (NMDS) with PRIMER-v6 software (www.primer-e.com) with Bray-Curtis similarity index. Analysis of microbial community diversity by Richness, Shannon-Weaver, and Simpson indexes was also carried out according to Yang et al. (2003) and Haegeman et al. (2014), respectively.

2.8 Copper fractionation in the soil

For chemical analysis of soil samples collected after the experiment, samples were dried and analyzed for pH, electrical conductivity (EC), and metal fractionation using a sequential extraction technique. The pH and EC were measures following the protocol of Sadzawka et al. (2006) using pH electrode and a conductivity meter.

Air-dried soil samples were sequentially extracted according to Tessier et al. (1979). The reagents and operating

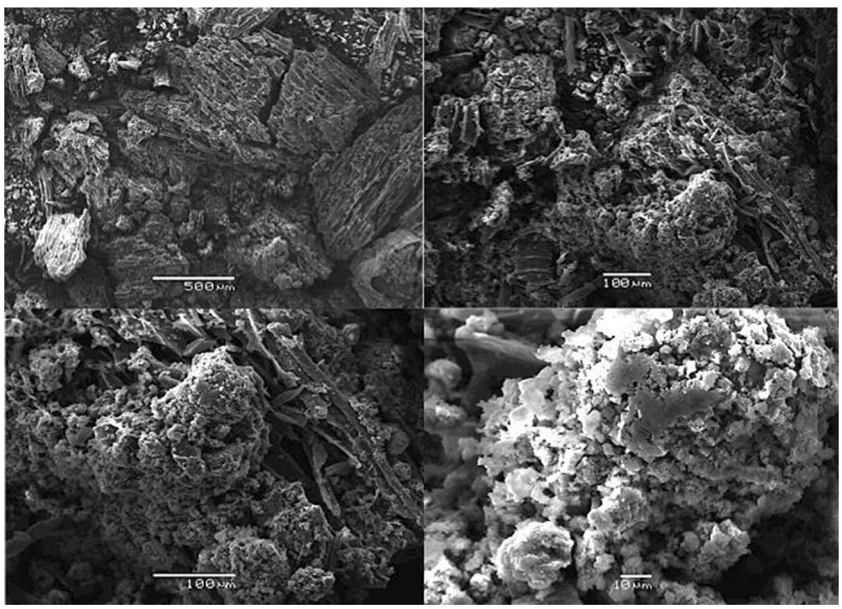

Fig. 2 Scanning electron micrographs, and energy-dispersive spectrum of oat hull biochar (OHB)

conditions for these methods are summarized in Park et al. (2011). The extraction was carried out in 50-mL polyethylene centrifuge tubes; after each extraction step, the supernatant liquid was separated from the solid phase by centrifugation at 4000 rpm for 15 min. It was then decanted into polyethylene vessels and stored at 4 °C before analysis. The remaining residue was washed with 10 mL of Milli-Q water, and the washings were discarded after centrifugation. The Cu residual fraction was measured using a Microwave Sample Preparation System Titan MPS PerkinElmer using the protocol EPA 3051A. The samples obtained were analyzed for Cu and using AA spectrometry (UNICAM Solar 969).

2.9 Statistical analyses

All experiments were conducted with three replicates. The data collected were analyzed statistically using SPSS 15 software. Tukey's multiple range test was used to compare the means of the treatments, variability in the data was expressed as the standard error, and a $p<0.05$ was considered to be statistically significant.

3 Results

3.1 Biochar properties

The properties of the BCs are presented in Table 1. The two BCs were different from each other. The CMB was strongly alkaline (9.1), while OHB was slightly alkaline (7.8). The CMB had higher amounts of major nutrients than those of OHB, which had higher carbon content than CMB. Being more porous, the surface area of CMB was higher than that of OHB. Both BCs had negative ζ potential (Figs. 1 and 2).

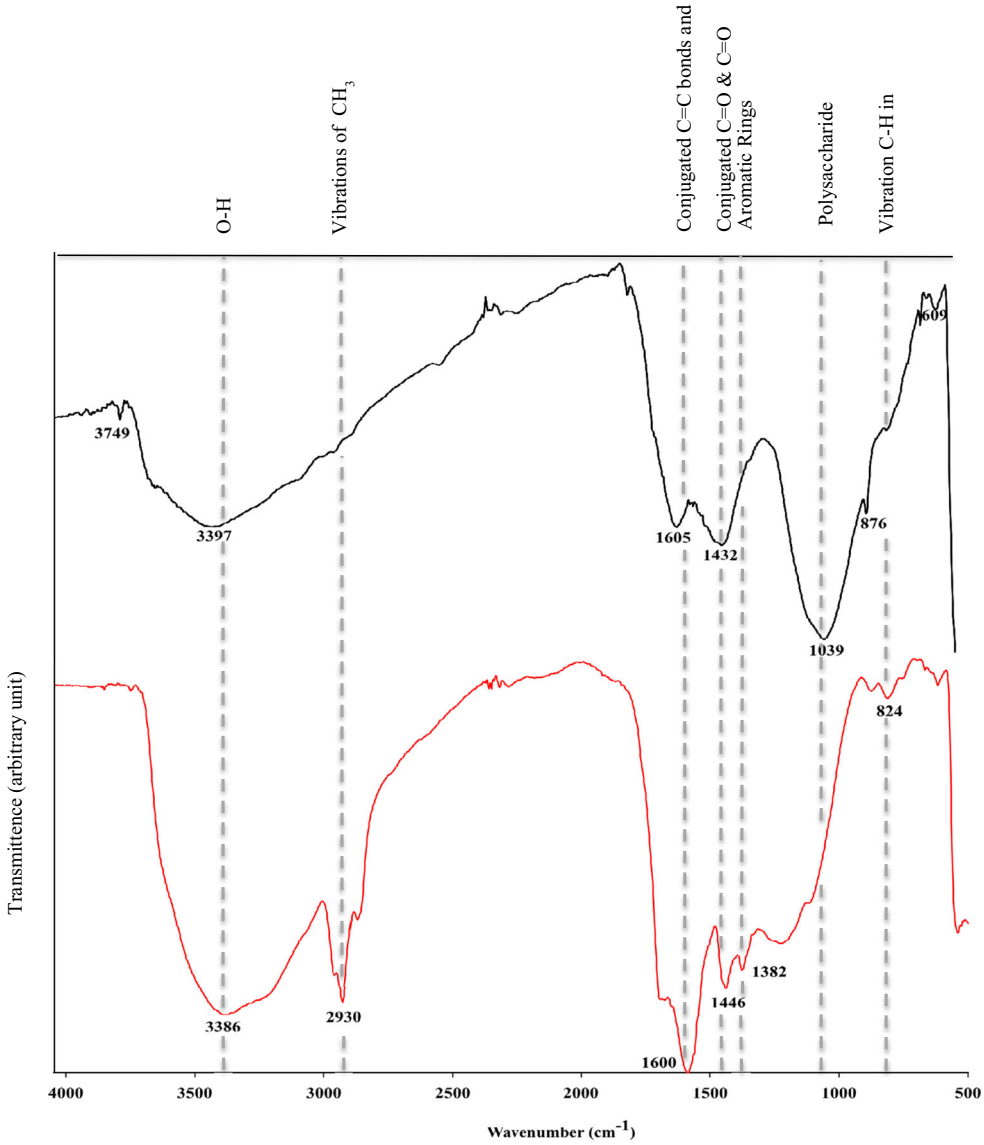

Fig. 3 FTIR spectra for chicken manure biochar (CMB) and oat hull biochar (OHB)

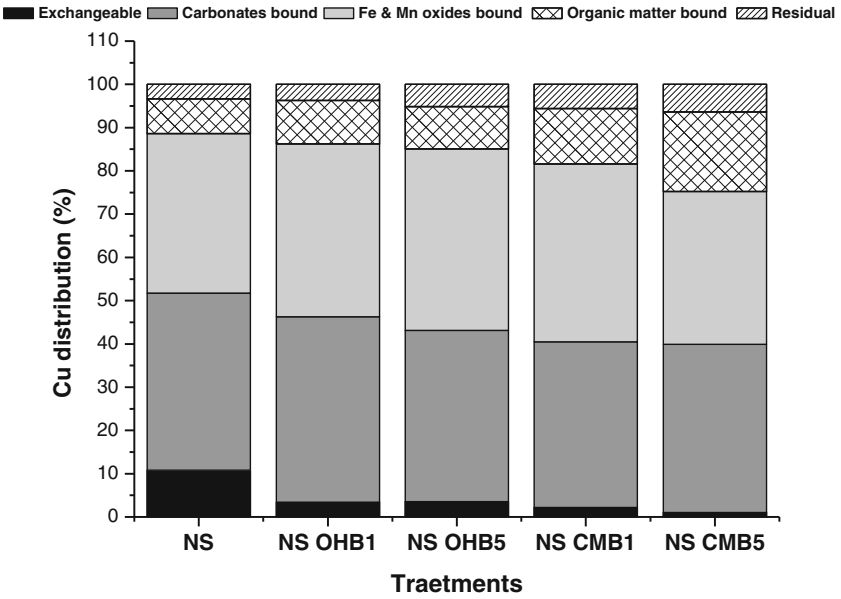

Fig. 4 Copper fractions in soil after pot experiment under the influence of chicken manure biochar (CMB) or oat hull biochar (OHB) applied at doses of 0, 1, and 5 % *w/w*

Functional groups of BCs were identified using FTIR spectrophotometry (Fig. 3). The peaks at 3386 and 3397 cm^{-1} indicate the presence of hydroxyl groups (Cheng et al. 2008). The bands between 1605 and 1432 cm^{-1} correspond to the stretching vibrations of conjugated C=C bonds in aromatic rings (Cao and Harris, 2010) and carbonyl groups (Hsu et al. 2009). The bands at 876 and 824 are due to the contribution from C–H bond vibration in aromatic compounds (Moreno-Castilla et al. 2000).

3.2 Soil pH and copper fractions

Both BCs increased the soil pH of all treatments. The CMB1 and CMB5 increased the soil pH by 0.94 units compared to that of control. The increase of soil pH by OHB was slightly higher than by CMB.

The BC addition reduced easily exchangeable fraction and increased the organic matter and residual bound fraction of Cu in all treatments (Fig. 4). Copper was strongly bound in the control soil because ~50 % of its total content was associated with Fe and Mn oxides, organic matter, and residual bound fractions (Fig. 4). The CMB1 and CMB5 decreased the exchangeable fraction of Cu by 80 and 90 %. Similarly, OHB1 and OHB5 also decreased exchangeable fraction of Cu by 68 and 62 %. In contrast, both BCs increased the organic matter and residual fraction of Cu, but the performance of CMB was better than OHB.

3.3 Plant growth and metal uptake

The CMB1 treatment nearly doubled the shoot and root biomass of *O. picensis* compared to that of the control, i.e., NS

Table 2 Shoot and root biomass production Cu concentration in plant tissues of *Oenothera picensis* grown in soils amended with chicken manure biochar (CMB) and oat hull biochar (OHB) at doses of 0, 1, and 5 % (*w/w*)

Treatment code	BC (%)	Biomass (g)		Cu concentration (μg g^{-1})	
		Shoot	Root	Shoot	Root
NS (control)	0	2.23 ± 0.24^{c}	0.33 ± 0.05^{c}	72.7 ± 1.2^{b}	1664 ± 54^{a}
CMB	1	4.93 ± 0.04^{b}	0.61 ± 0.03^{bc}	74.1 ± 4.9^{b}	931 ± 203^{bc}
OHB	1	4.79 ± 0.79^{b}	0.82 ± 0.07^{ab}	90.1 ± 2.4^{a}	1494 ± 133^{ab}
CMB	5	6.87 ± 0.45^{a}	0.92 ± 0.06^{a}	71.2 ± 1.4^{b}	632 ± 25^{c}
OHB	5	4.85 ± 0.43^{b}	0.88 ± 0.12^{ab}	64.1 ± 3.0^{c}	1212 ± 150^{abc}

Each value represents the mean of three replicates±standard error. Different letters within a column indicate a significant difference at $p < 0.05$ according to Tukey's multiple range tests using SPSS 15 software

BC biochar

Table 3 The pH, electrical conductivity (EC), basal respiration, and dehydrogenase activity (DHA) in soils after application of chicken manure biochar (CMB) or oat hull biochar (OHB) at doses of 0, 1, and 5 % *w/w*

Treatment code	BC (%)	pH	EC (μS cm^{-1})	Basal respiration (mg $CO_2 kg^{-1}$ h^{-1})	DHA (mg TPF kg^{-1} 24 h^{-1})
NS (control)	0	5.35 ± 0.01^{c}	672 ± 14^{a}	4.42 ± 0.51^{c}	4.66 ± 0.09^{c}
CMB	1	6.29 ± 0.05^{b}	626 ± 66^{a}	5.24 ± 0.6^{b}	7.63 ± 0.48^{b}
OHB	1	6.57 ± 0.12^{ab}	566 ± 31^{a}	5.01 ± 0.47^{b}	7.82 ± 0.64^{a}
CMB	5	6.45 ± 0.21^{b}	663 ± 36^{a}	6.05 ± 0.47^{a}	31.3 ± 3.39^{a}
OHB	5	7.04 ± 0.09^{a}	583 ± 67^{a}	5.7 ± 0.42^{b}	8.0 ± 0.50^{b}

Each value represents the mean of three replicates±standard error. The different letters within a column indicate a significant difference at $p<0.05$ according to Tukey's multiple range tests using SPSS 15 software

BC biochar, *EC* electrical conductivity, *DHA* dehydrogenase activity, *TPF* triphenyl formazan

(Table 2). This effect nearly tripled for the CMB5 treatment. Both OHB treatments increased the shoot and root biomass of *O. picensis* more than two times compared to that of the control. Both CMB treatments decreased root Cu concentration to ~60 % (significant) and ~40 %, respectively, compared to that of the control (Table 2). The shoot Cu concentration did not change for CMB treatments. In both OMB treatments, root Cu concentrations decreased slightly, whereas in OMB1, shoot Cu concentration increased significantly, while in OMB5, shoot Cu concentration decreased significantly.

3.4 Soil microbial activity

The microbial activities and DHA increased in all BC treatments compared to control (Table 3). The CMB increased the soil basal respiration by ~19 and ~37 %, respectively, for the

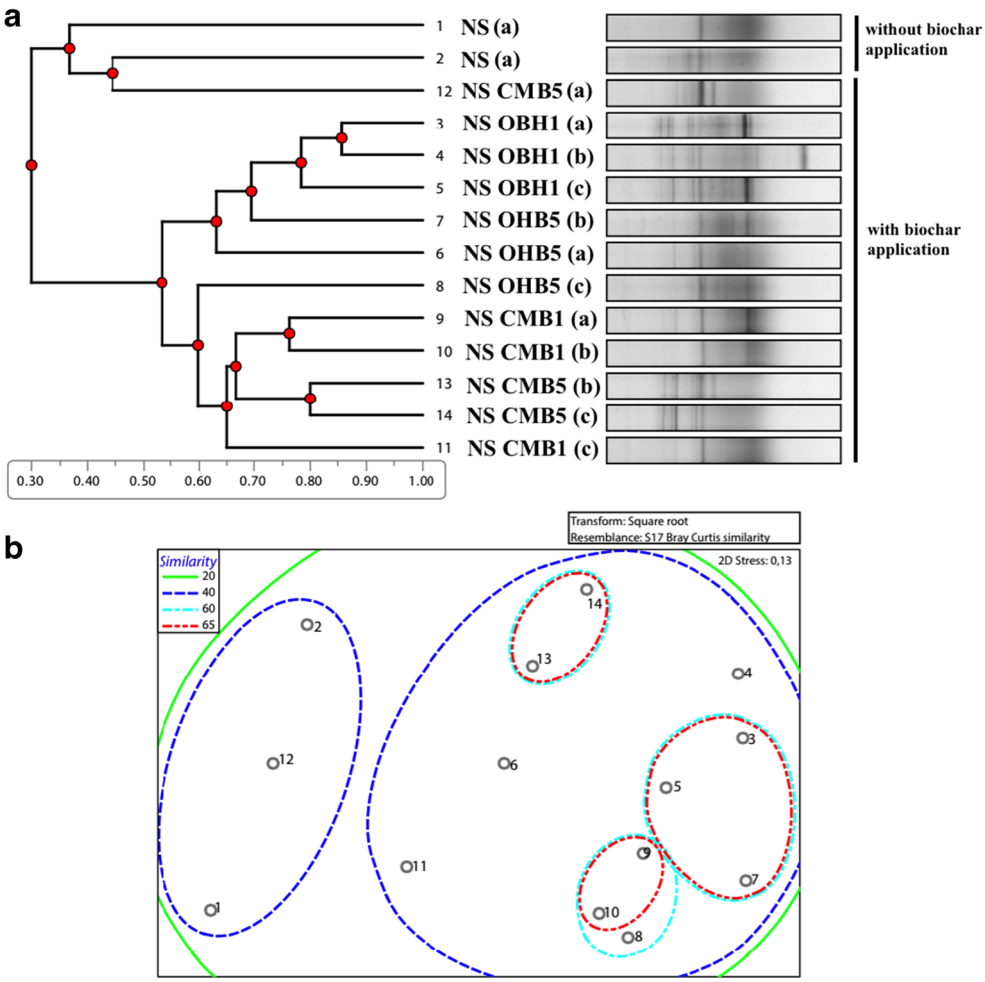

Fig. 5 **a** Dendrogram of denaturing gradient gel electrophoresis (DGGE) profiles and **b** nonmetric muldimendional scale (nMDS) of fungal communities in a Cu-contaminated soils with the addition of chicken manure biochar (CMB) or oat hull biochar (OHB) applied at doses of 0 (NS), 1, and 5 % *w/w*

low and high doses. Similarly, the OHB increased soil microbial activity by ~13 and ~29 %, respectively, for the low and high doses. The CMB increased DHA by ~62 and ~574 %, respectively, for the low and high doses. Similarly, the OHB increased soil microbial activity by ~68 and ~72 %, respectively, for the low and high doses.

3.5 Composition of bacterial and fungal communities

Analyses of DGGE profiles are shown in Figs. 5 and 6. The results show an unequal intensity of dominant bands in both DGGE profiles of BC-treated soils in respect to control, either for bacterial and fungal communities. According to this difference, UPGMA clustering analysis of bacterial communities (Fig. 6) revealed the existence of 65 % similarity between CMB1, OHB5, and OHB1, whereas CMB5 shown 55 % similarity with respect to control. The composition of fungal community (Fig. 5) showed a similarity around to 50 % among all the treatments. However, the most significant separation (30 % similarity) was observed between replicates of CMB5 and control for the fungal community. These results suggest that the type and dose of BC produce changes for fungal community structure, and its impact could be major than those for bacteria community among the different BC treatments (Figs. 5a and 6a). The nonmetric muldimendional scale (nMDS) analysis also indicated 40 and 20 % similarity for bacterial and fungal communities, respectively, under BC treatments with respect to control (Figs. 5b and 6b), suggesting a high variation in dominant microbial genera.

In addition, bacterial and fungal diversity was expressed by the Richness (S), Shannon-Wiener (H), and Simpson (D) indices (Figs. 7 and 8). The results indicated no significant changes in S, H, and D indices for bacterial diversity (Fig. 7). For fungi, the index values were different among the BC-treated soils in respect to control (Fig. 8b); however, these differences were not statistically significantly ($p<0.05$). Diversity indices were higher in fungi than in bacteria under BC treatments (Fig. 7).

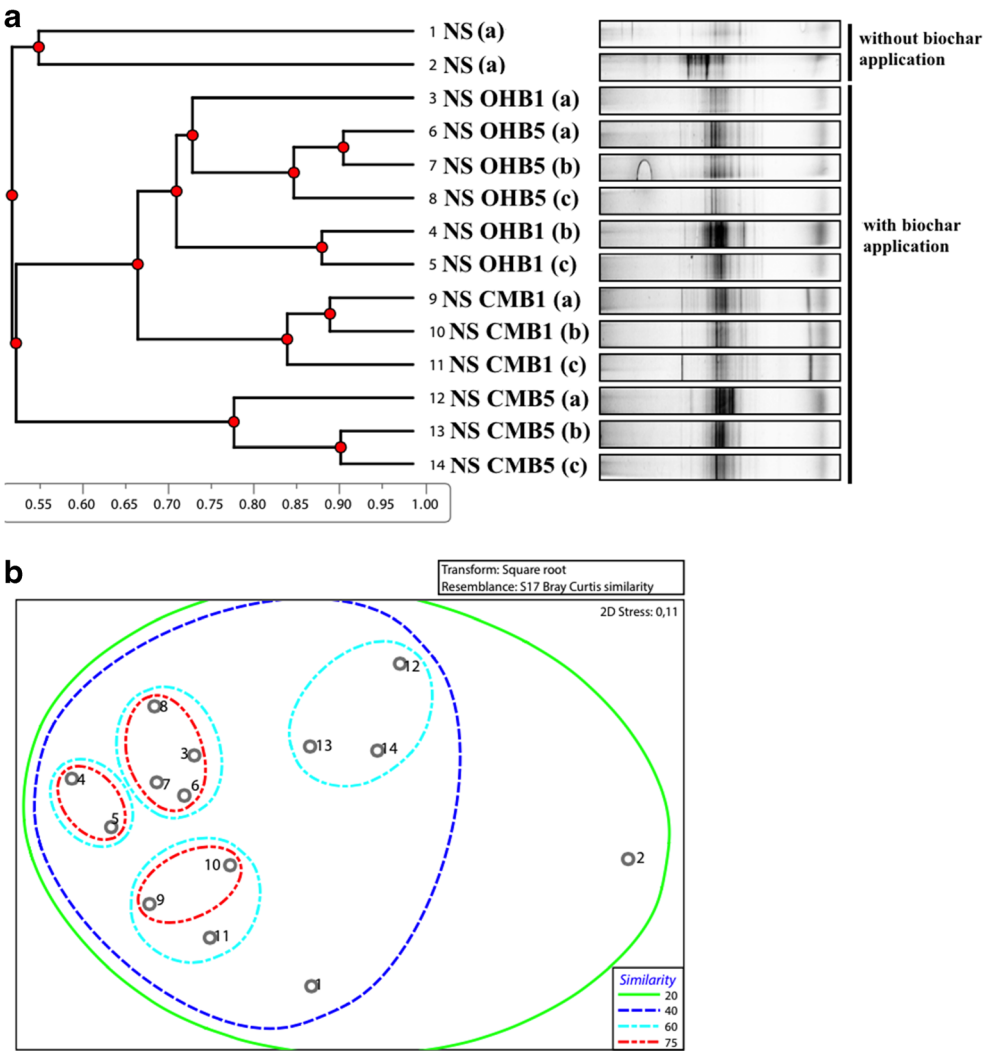

Fig. 6 **a** Dendrogram of denaturing gradient gel electrophoresis (DGGE) profiles and **b** nonmetric muldimendional scale (nMDS) of bacterial communities in a Cu-contaminated soils with the addition of chicken manure biochar (CMB) or oat hull biochar (OHB) applied at doses of 0 (NS), 1, and 5 % *w*/*w*

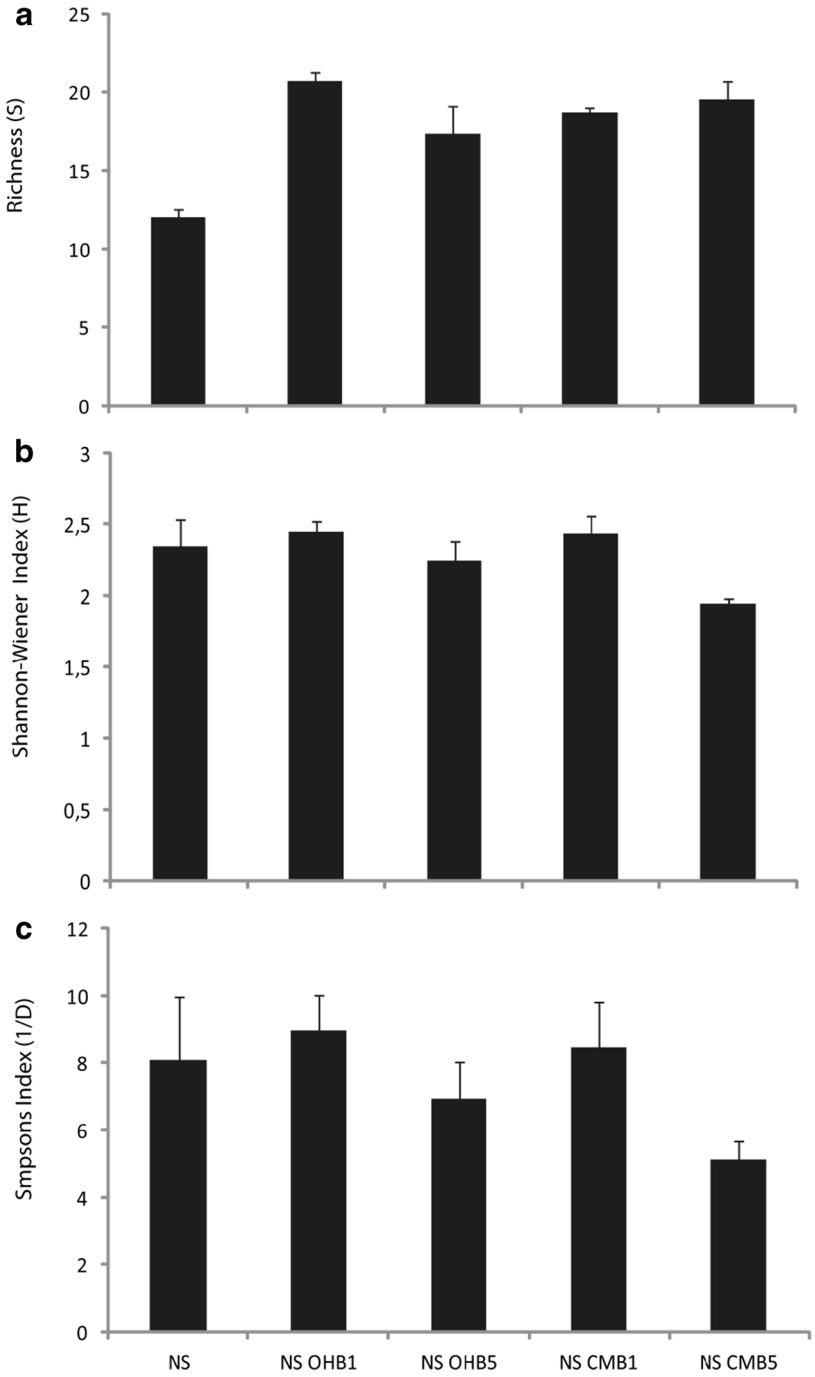

Fig. 7 **a** Genetic richness, **b** Shannon-Wiener index, and **c** Simpson index of bacterial communities in Cu-contaminated soils with the addition of chicken manure biochar (CMB) and oat hull biochar (OHB) applied at doses of 0, 1, and 5 % *w/w*

3.6 Arbuscular mycorrhizal fungi

Root colonization, spore number, and total GRSP production of arbuscular mycorrhizal fungi increased by ~3, ~2, and ~3 times, respectively, by OMB1 compared to those of control (Table 4). Only the total GRSP production increased in OMB5 treatment. The rest of the treatments only slightly increased the value of these AMF parameters.

4 Discussion

It has been widely reported that BC increases water retention, specific surface area, CEC, and nutrient availability in agricultural soils (Ippolito et al. 2012) and in metal-polluted soil (Park et al. 2011; Lu et al. 2015). Although OHB had a low surface area (0.16 $m^2\ g^{-1}$) compared to that of CMB (11.51 $m^2\ g^{-1}$), other properties such as the pore structure

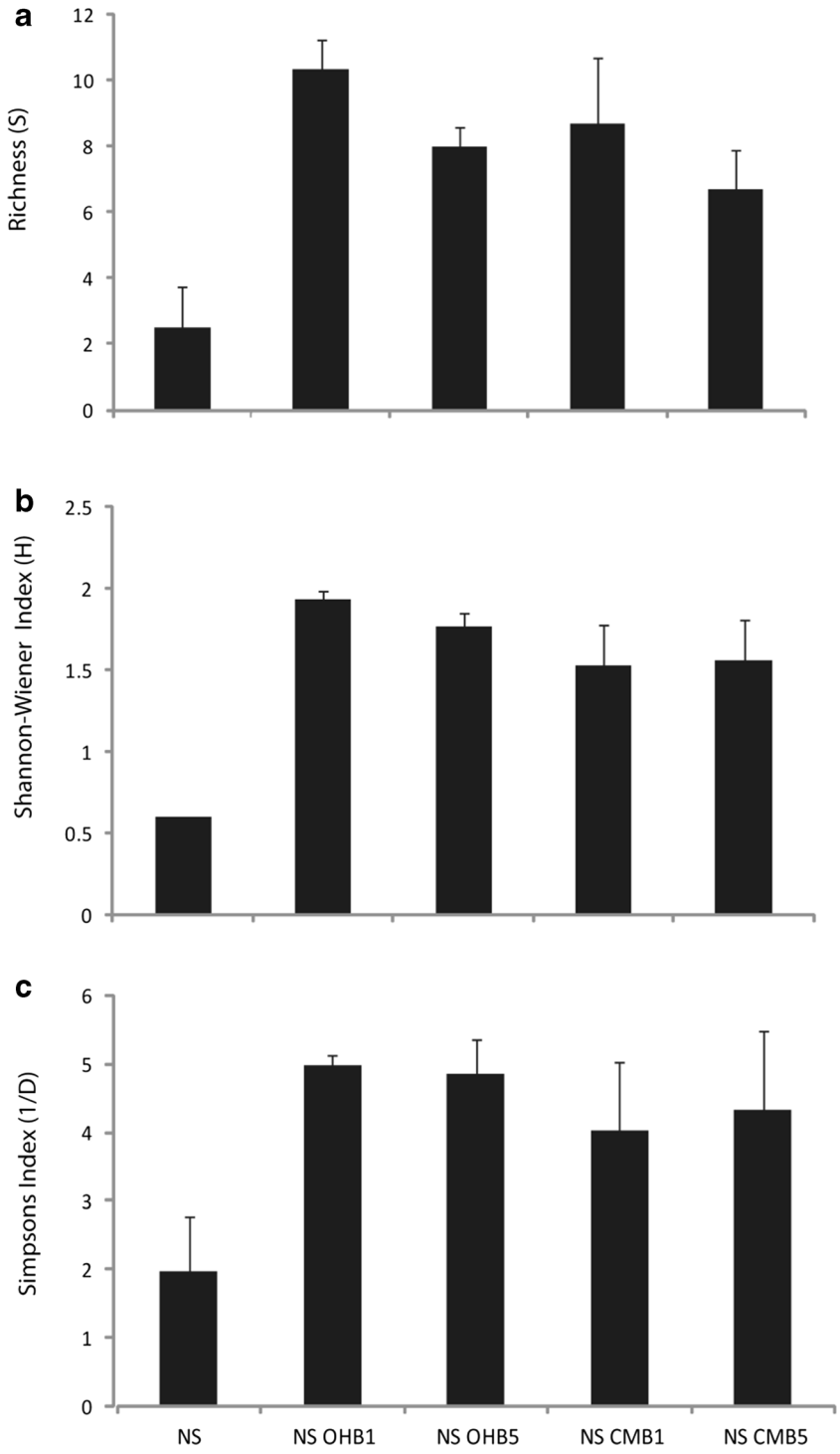

Fig. 8 **a** Genetic richness, **b** Shannon-Wiener index, and **c** Simpson index of fungal communities in Cu-contaminated soils with the addition of chicken manure biochar (CMB) and oat hull biochar (OHB) applied at doses of 0 (NS), 1, and 5 % *w/w*

and volume, the presence of functional groups especially the phenolic ones that have high affinity for Cu (Meier et al. 2012a), and a negative ζ potential could indicate that the Cu immobilization by OHB could also be significant (Table 1 and Fig. 4).

The results showed that both BCs are alkaline, and all their treatments increased soil pH (Table 3). Several studies found that biochar can induce a liming effect due to dissolution of metal carbonates (e.g., calcite) and hydroxides in these amendments (Kookana et al. 2011; Lucchini et al. 2014). The increase in pH contributes to the reduction of the mobility of the Cu in contaminated soils (Park et al. 2011; Lucchini et al. 2014). However, the BC addition did not produce changes in EC, which could indicate that the pH is the principal

chemical factor governing Cu availability in this soil. The plant growth varied with the type and doses of BC. The CMB induced a better growth compared to OHB probably due to a different physiochemical composition inherited from its feedstock. The increase in shoot and root biomass may mainly be attributed to two factors: first, the contribution of CMB with major nutrients, which were higher in this biochar compared to those in OHB, to soil and plant (Lu et al. 2015), and second, the reduction of Cu toxicity through immobilization or precipitation of Cu by BC-derived phosphates (Park et al. 2011).

The BCs did not produce uniform effects in shoot Cu concentration. Beesley et al. (2010) suggested that Cu forms complex with BC-derived dissolved organic carbon (DOC), thereby resulting in the mobilization of this metal. This may explain why, in some cases, the BC addition did not always reduce the shoot Cu concentrations.

Both CMB and OHB reduced the Cu concentration in *O. picensis* roots. These decrease in Cu can be attributed to both the reduction of exchangeable fraction of Cu in soil and "dilution effect" due to increase in plant biomass (Park et al. 2011). However, plant, soil, and/or microbial interactions could also explain the Cu concentrations in roots (Table 4; Figs. 5 and 6). In this sense, the increase in the microbial activity as a result of BC application might primarily be attributed to the decrease of Cu toxicity together with an increase in available nutrients (Pietikäinen et al. 2000). Park et al. (2011) observed an increase in CO_2 emission in soils contaminated with Cu, Cd, and Pb after the application of poultry litter and green waste BCs, and the change was influenced by the rate of BC. Similar results were obtained by Lu et al. (2015), but the mechanism for amendment-induced stimulation of microbial activity was not fully understood (Warnock et al. 2007). Probably, complex interactions involving physical, chemical, and biological processes contributed to microbial activity (Pietikäinen et al. 2000). Meier et al. (2011, 2015) suggested that an amendment in Cu-contaminated soil promoted the microbial activity mainly through the symbiosis established between plant and AMF, highlighting mechanisms such as (a) alteration of physicochemical properties of the soil, (b) indirect effects on mycorrhizae via effects on other soil microorganisms, (c) the existence of a specificity between metallophytes (e.g., *O. picensis*) and some native AMF ecotypes in the contaminated soils, (d) provision of refugee from fungal grazers, and (e) the presence of metal tolerant structures (spores) that store Cu thus decreasing its availability and improving the habitat for other less tolerant microorganisms (Cornejo et al. 2013). These mechanisms are observed in this study (Table 4), which indicated the existence of interactions between the BCs and AMF. However, other microbial mechanisms are also present as our results demonstrated that BC induced differences in DGGE profiles for bacterial and fungal community structure, which was affected by type and doses of BCs (Figs. 5 and 6). For instance, Pietikäinen et al. (2000) found that BCs can sorb DOC from the adjacent substrate and microorganisms partly metabolize the adsorbed DOC, which results in the microbial stimulation. Our results are in concordance with previous findings that BC surfaces provide a good habitat for microorganisms, and the available nutrients in BCs induce the microbial compositional changes in the BC-amended soil (Anderson et al. 2011). A major change in microbial community composition was observed in fungi profiles, where low similarity (20 %) between BC soil and control samples was obtained from nMDS analysis. Those results are in concordance with other studies where fungi were significantly affected by BC incorporation in the soil compared with bacteria (Rutigliano et al. 2014). In contrast, studies conducted by Gomez et al. (2014) indicated that BC addition at high dose (20 %) altered the Gram-negative bacteria dominated community rather than fungal community; however, the variation could be attributed to the longer incubation time (1 year), the difference in BC feedstock (oak), and soils used.

Finally, in this study, we found that BCs are effective in immobilizing Cu, and the effectiveness depended on the dose (Fig. 4). The BCs decreased available Cu by reducing the exchangeable Cu fraction as high as about 5 and 10 times for OHB5 and CMB5, respectively, through mainly binding the Cu to organic matter and residual phases. Increase in the pH of (acidic) soil by BC played a role in the immobilization

Table 4 Root colonization (micorrhization), spore number, and total glomalin production of arbuscular mycorrhizal fungi in a Cu-contaminated soil after the application of chicken manure biochar (CMB) or oat hull biochar (OHB) at doses of 0, 1, and 5 % *w*/*w*

Treatment code	BC (%)	Micorrhization (%)	Spores (20 g soil)	Total GRSP (mg glomalin g soil^{-1})
NS (control)	0	12±0.5^{b}	10.4±0.34^{b}	0.71±0.04^{c}
CMB	1	12.7±1.0^{b}	12.7±1.5^{b}	0.89±0.03^{c}
OHB	1	36±1.5^{a}	23.4±2.9^{a}	2.01±0.1^{a}
CMB	5	32±2.7^{ab}	15.8±0.7^{b}	0.77±0.13^{c}
OHB	5	26.3±0.6^{ab}	15.3±4.5^{b}	1.43±0.04^{b}

Each value represents the mean of three replicates±standard error. The different letters within a column indicate a significant difference at $p<0.05$ according to Tukey's multiple range tests using SPSS 15 software

BC biochar, *GRSP* glomalin-related soil protein

of Cu in this study (Kuo and Baker 1980). According to this study, the sorption of Cu by soils (sandy loam, silt loam, and silty clay loam) increases soil pH up to 6 due to sorption by organic matter and Fe oxides; thereafter, the sorption decreases with the increase in pH due to formation of soluble organo-metallic complexes. It has been noted that both CMB and OHB increased the soil pH, but the performance of CMB was slightly better than OHB. On the other hand, both CMB and OHB increased the organic matter and residual fraction of Cu, yet the performance of CMB was slightly better than that of OHB. The CMB1 and CMB5 increased the soil pH to 6.29 and 6.45, respectively, from 5.35 (NS), whereas OMB1 and OMB5 increased the soil pH to 6.57 and 7.04, respectively. Since OHB treatments shifted the pH farther from 6, they will cause lesser sorption of Cu in soils and, thus, performance of CMB should be better in assisting sorption of Cu in soils. This can be clearly seen in Fig. 4, which shows that the Cu fractions in organic matter and residual phases are higher for CMB in respect to OHB (and NS). It is also possible that AMF assisted in immobilizing parts of Cu content by secreting chelating substances in soil, binding of metals to biopolymers in the cell wall such as glomalin, and/or superficial binding in the plasmatic membrane (Meier et al. 2012c).

5 Conclusions

Despite being a metalophyte, *O. picensis* could not uptake Cu efficiently. Root and shoot Cu concentrations decreased or changed insignificantly in most BC treatments. In general, CMB was more effective in immobilizing Cu in soils. Both the BCs reduced the bioavailable Cu of soils by 5 and 10 times at the highest doses by reducing its exchangeable fraction and increasing its organic matter and residual fractions. The above effects varied with the type and the dose of BC. The microbial activities and DHA increased in all BC treatments producing changes in bacterial and fungal communities; however, their diversity and richness were not affected. The application of CMB enhanced the spore number and root colonization. The synergetic effect of BCs and AMF on plant might have assisted in the increase in plant biomass. The BCs influenced the soil biophysicochemical properties, improved habitat for microorganisms, and enhanced plant growth in Cu-contaminated soil. Therefore, the BCs can be used to enhance remediation of Cu-contaminated soils.

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