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Biochar amendment increases bacterial diversity and vegetation cover in trace element-polluted soils: A long-term field experiment

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ABSTRACT

Application of biochar has been widely suggested as a remediation tool for trace element-polluted soils, but the impact of biochar on microbial communities and on native plants remain largely unknown. To overcome this knowledge gap, biochar produced from rice husk and olive pit were applied at a rate of 8 t ha^{-1} into a soil with two contrasting levels of trace elements (high and moderate) to study their effects on soil microbial community composition, vegetation cover and soil properties after 1, 6, 12 and 20 months under field conditions. Differences in bacterial community composition were studied using the Illumina Miseq technology of the 16S rRNA gene. Although variations in soil properties and ecological function were seasonal and soil-type dependent, biochar application enhanced soil properties and vegetation cover in the moderately polluted soil (MPS), and increased microbial diversity as well as vegetation cover in the highly polluted soil (HPS). Enzymatic activities and soil respiration rates were not modified with the application of biochar, but increased total carbon content of soils. The application of biochar from crop residues to trace-element contaminated soils provided environmental benefits, including plant diversity and growth, as well as the increase of bacterial diversity and carbon sequestration.

1. Introduction

Trace element-polluted soils is a worldwide concern comprising 37% of the degraded soils in the European Union (EEA, 2007). Ex-situ decontamination of polluted soils is generally unfeasible due to land size and soil contamination levels, which are difficult to effectively and economically reduce with conventional soil remediation procedures (Tack et al., 2018). Biochar, the C-rich porous solid residue produced by the thermal conversion of biomass under the partial or total absence of oxygen (pyrolysis, e.g. Hagemann et al., 2018), has the ability to immobilize trace elements and increase the pH of acidic soils reducing trace element mobility and bioavailability. Karer et al. (2015) reported a decrease in NH_4NO_3-extractable fraction of Pb, Zn and Cd with biochar amendment, but an increase of Cu. Beesley et al. (2010) also reported an immobilization of Cd and Zn and a mobilization of Cu after biochar application. Oustriere et al. (2017) showed long-term Cu stabilization due to biochar addition into a contaminated soil, whereas Uchimiya et al. (2012) reported Cu immobilization but mobilization of Sb. These discrepancies are probably due to the complexity of immobilization mechanisms and different biochar compositions and properties, but also due to differences in the soil properties, e.g. in pH. In fact, previous studies already demonstrated that the efficacy of biochar as a soil amendment greatly depends on its pyrolysis conditions and feedstock (Campos et al., 2020; De la Rosa et al., 2014). For instance, Kammann et al. (2012) showed a significant increase in biomass yield after applying 50 Mg ha^{-1} of peanut hull biochar to a Luvisol. Gascó et al. (2016) reported that β-glucosidase, phosphomonoesterase and phosphodiesterase activities were lower when a sandy loam soil was incubated with 8% (w/w) of biochar produced from pig manure at 500 °C whereas the biochar produced at 300 °C increased dehydrogenase

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activity. The study of Shen et al. (2019) demonstrated that biochar produced at 500 °C was more effective in the removal of lead from soil solution than the biochar produced at 300 °C. Generally, biochar produced at 500 °C has high pH and water holding capacity, and high degree of aromatization (Campos et al., 2020).

The effects of biochar on the physical and chemical properties of agricultural soils have been profoundly studied and during the last years special attention has been paid to the study of biochar as soil amendment for the retention of contaminants (Uchimiya et al., 2011; Kumar et al., 2018; De la Rosa et al., 2019). Within this context, effects of biochar addition on soil microbiota, which play a vital role in soil ecosystem stability, soil quality and soil nutrient cycle (Lehmann et al., 2011), are highly relevant. Li et al. (2019) reported a decrease in *Actinobacteria* with biochar application into purple soil, whereas Ali et al. (2019) reported an increase for pesticide-contaminated soil. Most of these studies on soil microbial diversity in polluted soils after biochar application are pot-based experiments (Jiang et al., 2017; Han et al., 2017), which are likely to be important in the quest to constrain the numerous influencing factors, but are less realistic than field studies.

An aspect also worth further researching is the biodegradability of biochar in soils. Biochar has traditionally been considered a material of high chemical and biochemical stability, which predominantly contains C in the form of condensed aromatic rings. This fraction of C is hardly decomposed by soil biota due to its recalcitrant nature (Kuzyakov et al., 2009, 2014). Nevertheless, recent studies indicate a much lower biochemical stability (Knicker et al., 2013, De la Rosa et al., 2018). Thus, the effects of biochar application on soil CO_2 emissions are often ambiguous and previous studies reported increases, decreases or no changes (Bamminger et al., 2014; Kolb et al., 2009; Paz-Ferreiro et al., 2012). Hence and considering that changes on soil properties promoted by biochar application may affect soil microbial communities (Luo et al., 2013; Su et al., 2015; Xu et al., 2016) and soil CO_2 emissions, their assessment deserve further attention.

The application of low degradability of biochar in trace element-polluted soils would allow an effective *in situ* remediation by enhancing soil quality and improving its capability to perform soil ecological functions. To test this hypothesis, we applied rice husk (RH) and olive pit (OP) biochar into two trace element-polluted acidic soils under field conditions to study their effects on soil physicochemical properties, soil CO_2 emissions and enzymatic activities, as well as soil microbial community composition and vegetation cover after 1, 6, 12 and 20 months of biochar application.

2. Materials and methods

2.1. Biochar samples

Rice husk (RH) and olive pit (OP) were used as feedstock to produce biochar due to their great abundance in Mediterranean countries. RH is a siliceous-rich raw material with relatively low C content, while OP is a hard-wood biomass, mainly composed of cellulose and lignin. The company Orivarzea S.A. (Portugal) provided the RH biomass, whereas OP was provided by *Cooperativa Nuestra Señora de los Ángeles* (Montellano, Spain).

Prior to pyrolysis process, feedstock was dried at 40 °C during 48 h, homogenized and stored in sealed plastic bags at 4 °C. The RH and OP biochar (RHB and OPB) were produced in a continuously feed pyrolysis reactor with a screw conveyor (PYREKA, Pyreg GmbH, Dörth, Germany, cf. Hagemann et al., 2020) under N_2 flux at Agroscope Zurich (Switzerland). The pyrolysis temperature was 500 °C and the residence time was 12 min. Biochar was stored in sealed plastic bags, in cool (4 °C) and dark conditions. Biochar characteristics are shown in Table 1.

2.2. Area of study and experimental design

The field experiment was conducted at 'Las Doblas' site (37° 23′ 7.152″N, 6° 13′ 43.175″W) over a period of 20 months. This place is located close to the Guadiamar river, 10 km from the former mine "Los Frailes" close to Aznalcollar, Southern Spain. On April 25, 1998, after a major mining accident, a huge amount of toxic sludge spilled out from a tailing reservoir of this large open-pit mine, causing high levels of heavy metals to leach into the soil and groundwater. Fig. 1 shows the location of the field experiment. The area belongs to a typical dry Mediterranean climate region, with hot and extended summers, mild winters and a very pronounced variation in the precipitation rate (AEMET, 2020).

The sandy loam soil of the area is classified as Fluvisol (IUSS Working Group WRB, 2015). In this study, two nearby sites were selected according to their contamination level and acidity, comprising a highly polluted soil (HPS) and a moderately polluted soil (MPS). HPS is a bare soil with high acidity and concentrations of heavy metals, as previously described in Cabrera et al. (1999) and Martín-Peinado et al. (2015). These bare spots account over 200 ha of lands affected by the accumulation of residual toxic sludge of the spill. In contrast, MPS areas were subjected to a decontamination programme by the Andalusian regional government which included the removal of the toxic sludge (Arenas et al., 2008). Despite the decontamination efforts, MPS also shows relatively high concentration of Ba, Cu, Fe, Pb and Zn (Campos and De la Rosa, 2020). Soil pH, total carbon (TC) and total nitrogen (TN) contents of HPS and MPS are shown in Table 1.

In April 2018, 12 plots of 1 m × 1 m each were randomly established in HPS and MPS sites (6 plots per site). RHB and OPB were applied as produced and mixed into the first 10 cm of soil at a dose of 8 t ha^{-1} (Plots ID: RHB_HPS, OPB_HPS, RHB_MPS and OPB_MPS). In addition, control plots without amendment were stablished for both areas (C_MPS and C_HPS) but received the same mechanical treatment. For all the plots, ground vegetation (shrub and grass) was manually removed; the soil was then homogenized using a manual rake.

Four sampling campaigns were performed after 1, 6, 12 and 20 months of biochar incorporation into soils (hereafter: t_1, t_6, t_{12} and t_{20}, respectively). For each plot, five samples of soil were taken randomly from the first 10 cm depth to create a composite sample per plot. An aliquot of the composite sample was immediately used for enzymatic analyses, other aliquot was stored in sterile Whirl-pak® bags at −80 °C for DNA-based analysis and the remaining material was dried at 40 °C during 48 h, sieved (<2 mm) and stored in sealed bags at 4 °C.

Table 1
pH, total carbon (TC), total nitrogen (TN) and trace elements contents of rice husk biochar (RHB), olive pit biochar (OPB), highly polluted soil (HPS) and moderately polluted soil (MPS). Values represent means (n = 3) and standard deviation.

		pH	TC (g kg^{-1})	TN (g kg^{-1})	Ba (mg kg^{-1})	Cd (mg kg^{-1})	Cu (mg kg^{-1})	Fe (mg kg^{-1})	Ni (mg kg^{-1})	Pb (mg kg^{-1})	Sr (mg kg^{-1})	Zn (mg kg^{-1})
Biochars	**RHB**	10.17 ± 0.34	537 ± 1.0	1.6 ± 0.9	7.3	0.05	35.0	1224.2	8.5	1.7	11.6	42.6
	OPB	9.34 ± 0.19	927 ± 2.0	5.1 ± 2.4	<LOQ[a]	<LOQ	5.9	<LOQ	<LOQ	0.4	4.4	<LOQ
Soils	**HPS**	3.85 ± 0.14	7.2 ± 0.1	0.6 ± 0.1	47.1	1.28	240.6	53023.3	15.6	569.0	53.7	249.3
	MPS	4.82 ± 0.13	9.0 ± 0.6	0.8 ± 0.1	93.3	1.56	215.5	36945.7	15.6	156.5	38.6	293.5

[a] <LOQ: below limit of quantification.

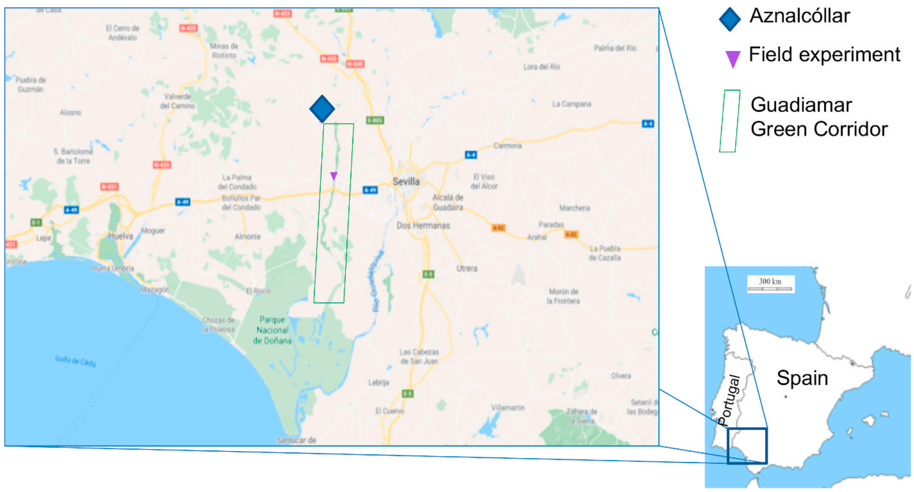

Fig. 1. Location of the field experiment, Aznalcóllar mine and Guadiamar Green Corridor. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.3. Chemical and biochemical analysis

The pH was measured in triplicates in the supernatant of a 1:5 (w/v) soil:0.01 M $CaCl_2$ solution ratio mixture after 30 min shaking and 30 min resting, using a pH meter (CRISON pH Basic 20).

The soil moisture (%) was determined on the dry weight basis: 20 g of moist soil was weighed, dried at 40 °C during 24 h and re-weighed. Total soil moisture (%) was determined for soil samples dried at 105 °C for 24 h.

Total C (TC) was obtained by dry combustion (1050 °C) using an elemental analyzer (TRUSPEC CHNS MICRO, LECO, St. Joseph, MI, USA).

The water holding capacity (WHC) was measured following the procedure and formula described in Campos et al. (2020). The WHC is expressed as the percentage relatively to the total dry weight of the sample:

$$WHC\ (\%) = \frac{\text{Water retained weight}}{\text{Initial weight of the dry sample}} \cdot 100 \qquad (1)$$

For elucidating microbial oxidative activities in soil, dehydrogenase activity was determined according to the method of Trevors (1984). Briefly, soil samples were incubated for 20 h with 1 M TRIS–HCl buffer (pH 7.5) and 2 (p-iodophenyl)-3-(p-nitrophenyl) 5-phenyl tetrazolium chloride (INT), that was used as the electron acceptor. After adding methanol, the iodonitrotetrazolium formazan (INTF) produced was measured spectrophotometrically at 490 nm.

In addition, soil β-glucosidase activity was measured according to the method of Tabatabai (1982). Briefly, 1 g of soil was incubated 1 h at 37 °C with p-nitrophenyl-β-D-glucopyranoside. After addition of $CaCl_2$, the p-nitrophenol was extracted by filtration and measured using a spectrophotometer (Jenway, model 6315, UK) at 400 nm. β-glucosidase and dehydrogenase activities were measured in both unamended and biochar-amended soils at t_1, t_6, t_{12} and t_{20}.

All chemical and biochemical analyses of the samples were performed in triplicate.

2.4. Measurement of soil CO_2 efflux (soil respiration)

Soil respiration (carbon decomposition by microorganisms and ground root respiration) was determined by measuring the CO_2 effluxes and expressed as μmol CO_2 m^{-2} s^{-1}. For each plot, 3 PVC collars (10 cm diameter and 5 cm high) were installed 3 cm into the soil and measurements were conducted in triplicate using the soil CO_2 flux chamber LI-COR 6400–09 (LI-COR, Nebraska, USA) at t_1, t_6, t_{12} and t_{20}. Soil temperature was monitored using a thermocouple probe (Li6000-09 TC, LiCor Inc) inserted to a depth of 5 cm near the soil CO_2 flux chamber.

2.5. Effects on vegetation

The vegetation species were carefully identified and the number of individuals per plot were accounted at t_{12}. Subsequently, the total plant biomass was determined by harvesting and measuring the fresh weight per plot.

In order to determine the percentage of vegetation cover at time t_{20}, high resolution photographs were taken for each plot using a digital camera (Canon Inc., Canon 7D, Japan) installed on a tripod at a height of 1.5 m. Digital images were then analysed using the open source image-processing software Image J. The area covered by green plants was selected by adjusting the hue levels in the colour threshold tool. The percentage of vegetation cover was determined by using the following equation (2):

$$\%\ \text{vegetation cover} = \frac{\text{green area}}{\text{total area}} \cdot 100 \qquad (2)$$

2.6. Soil DNA isolation and sequencing

Total DNA was extracted from soil samples using the DNeasy PowerSoil DNA isolation kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. DNA quality and quantity were tested. As a standard procedure, 1.5% agarose gel electrophoresis was performed with 1 μL of gDNA of each sample to test the integrity and purity. DNA concentrations were verified using a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA). Qubit broad-range reagent was used for determining the DNA concentration of MPS samples, whereas Qubit high-sensitivity reagent was needed for HPS samples due to their low amount of DNA.

Library construction was performed according to the Illumina 16S Metagenomic Sequencing Library preparation protocol by STAB VIDA Sequencing Services (Portugal). MiSeq Reagent Kit v3 in the Illumina MiSeq platform was used for sequencing new generated DNA fragments. 300 bp paired-end sequencing reads were used.

The microbial community composition and diversity (alpha and beta-diversity) were determined after bioinformatics processing of the 16S rRNA gene sequences. Sequence quality control was performed using QIIME2 v2019.1.0 (Bolyen et al., 2019). The reads were denoised using the DADA2 plugin, organized in operational taxonomic units (OTUs) and classified by taxon using the SILVA database, with a clustering threshold of 97% similarity. OTUs were considered as significant only when they contained at least 10 sequence reads. This procedure results in an abundance table with taxonomy information, which were further analysed and visualized using the online web-tool Calypso (Zakrzewski et al., 2017).

The raw reads were deposited in NCBI Sequence Read Archive (SRA) database (https://submit.ncbi.nlm.nih.gov/about/sra/) under the accession number PRJNA637319.

2.7. Data analysis

Data of soil and biochar characteristics are expressed as mean ± standard error (SE) of triplicate measurements. Data of the samplings are expressed as mean ± SE of the five composite samples per treatment. Number of species and number of individuals are expressed as median.

Shapiro-Wilk test was used to verify normality and Levene test was used to test homoscedasticity of the data. Normal distributed response variables were analysed by one-way ANOVA followed by the Tukey's Honestly Significant Difference test. The level of significance used was 0.05. When response variables were non-normal, Kruskal Wallis followed by Mann Whitney U tests were conducted. Pearson correlation ($p < 0.05$) was conducted to examine relationships between soil properties, soil microbial community and vegetation data. Statistical analyses were carried out using IBM SPSS Statistics 26.0 (SPSS, Chicago, USA) for Windows.

3. Results

3.1. Soil pH and moisture

Soil pH of HPS samples were more acidic than MPS (3.57–3.77 and 4.18–5.11, respectively) (Table 2). Biochar addition did not significantly enhance soil pH of HPS. For MPS, biochar amendment clearly increased soil pH, but this increase was mitigated over the time span of the experiment.

Soil moisture determined by drying at 40 °C was greater for MPS than HPS samples with comparable treatments (Table S1). Biochar addition augmented soil moisture (40 °C) at t_6 and to a lesser extent at t_{20}. As expected, seasonal changes modified the soil moisture at 40 °C and 105 °C, with a significant drop at t_{12}, followed by a considerable increase during the autumn (t_{20}). MPS showed a greater WHC (%) than HPS, and were affected by seasonal changes, showing in general a similar trend as the soil moisture content (Table 2).

3.2. Total carbon content and soil respiration

Biochar addition caused a non-significant increase ($p > 0.05$) of the C content of the amended soils when compared with control plots (Table 2). At t_{12}, the C content significantly increased due to OPB amendment in HPS samples (9.6–16.6 g kg^{-1}).

The CO_2 emission rates were always higher in MPS than in HPS. The latter showed very low respiration rates. Significant differences were not observed with biochar application into the soils, nor differences between both biochar samples for the same soil type (Table 2).

3.3. Soil enzymatic activities

β-Glucosidase activity at t_1 of MPS was greater than HPS (0.69–1.45 *vs* 0.17–0.39 μmol PNF g^{-1} h^{-1}) (Fig. 2). At this time, the HPS control soil showed a greater β-glucosidase activity than biochar amended soils. Nevertheless, this difference disappeared at t_6 and t_{12}. This enzymatic activity was greater for both amended treatments than for control in MPS at t_1. At t_6, only OPB addition maintained a greater β-glucosidase activity than control soils and at t_{20} no significant differences were observed.

MPS plots showed greater dehydrogenase activity than in HPS in all the cases. Similarly to the trend observed for β-glucosidase activity, dehydrogenase activity showed seasonal changes and during the first 6 months of the experiment MPS soils amended with biochar showed lower values than control soils.

3.4. Effects on vegetation development

A total of 14 different species were observed in MPS plots which were not found in HPS plots (*Echium gaditanum, Lotus parviflorus, Trifolium arvense, Ornithopus compresus, Anagallis arvensis, Bartsia trixago, Trifolium* sp.*, Vulpia ciliata, Trifolium vesiculosum, Trifolium striatum, Hypochaeris glabra, Astragalus pelecinus, Trifolium campestre, Spergularia media, Silene sclerocarpa* and *Petrorhagia nanteuilii*). In contrast, solely one plant species (*Sonchus oleraceous*) was found in HPS plots which was not found in MPS (Table S2). *Rosmarinus officinalis, Chamaemelum mixtum, Agrostis truncatula, Spergularia rubra, Logfia minima* and *Cynodon dactylon* were found in HPS and MPS plots. Furthermore, biochar application enhanced vegetation diversity, as *Trifolium campestre, Spergularia media, Silene scleorocarpa* and *Petrorhagia nanteuilii* solely grew in MPS biochar-amended plots. *Logfia minima* was strictly found in OPB plots, but not in the unamended ones (Table S2).

Fig. 3a and b shows the average number of different plant species and individuals, respectively, in biochar-amended and unamended plots 12 months after the setup of the experiment. A greater diversity of vegetation species was observed in MPS than in HPS plots (Fig. 3a), as also occurred for the number of individuals (Fig. 3b). One-way ANOVA showed that the number of individuals per square meter (Fig. 3b) and vegetation cover (Fig. 3c) in HPS were significantly ($p < 0.05$) lower than in MPS. The application of OPB in HPS plots significantly increased

Table 2
Changes in soil characteristics, soil total carbon (TC) and soil respiration during the field experiment (t_1: 1 month-spring, t_6: 6 months-autumn, t_{12}: 12 months-spring, t_{20}: 20 months-autumn).

	pH			WHC (%)			TC (g kg^{-1})			Soil respiration (μmol CO_2 m^{-2} s^{-1})		
Sample	t_6	t_{12}	t_{20}	t_6	t_{12}	t_{20}	t_6	t_{12}	t_{20}	t_6	t_{12}	t_{20}
C_HPS	3.57 ± 0.13a	3.59 ± 0.08a	3.52 ± 0.20a	20±7a	14±5a	32±2a	7.9 ± 0.6a	9.6 ± 1.3a	12.3 ± 2.7a	1.0 ± 0.2a	1.0 ± 0.2a	0.9 ± 0.2a
RHB_HPS	3.64 ± 0.18a	3.68 ± 0.21a	3.77 ± 0.17a	23±1a	20±2 ab	26±4a	12.9 ± 4.8a	14.9 ± 4.7 ab	16.7 ± 1.5a	1.0 ± 0.3a	0.8 ± 0.2a	0.6 ± 0.3a
OPB_HPS	3.63 ± 0.03a	3.64 ± 0.04a	3.69 ± 0.17a	23±5a	17±5 ab	30±4a	13.6±8a	16.6 ± 4.5b	18.4 ± 6.1a	1.2 ± 0.4a	0.9 ± 0.2a	1.2 ± 0.6a
C_MPS	4.18 ± 0.21a	4.8 ± 0.10b	4.87 ± 0.20b	32±2b	23±5 ab	47±6 ab	12.4 ± 3.5a	9.5 ± 0.3a	21.7 ± 1.3a	2.9 ± 0.2b	1.2 ± 0.8a	3.9 ± 0.2b
RHB_MPS	4.75 ± 0.01b	5.02 ± 0.22b	5.07 ± 0.18b	39±1b	25±8b	52±2b	12.2 ± 1.7a	14.3 ± 3.2 ab	19.9 ± 9.2a	2.7 ± 0.3b	1.3 ± 0.7a	3.3 ± 0.4b
OPB_MPS	4.74 ± 0.54b	4.86 ± 0.50b	5.11 ± 0.20b	38±7b	19±7 ab	67±3c	17.0 ± 7.9a	12.7 ± 0.3 ab	20.3 ± 10.2a	3.1 ± 0.3b	0.5 ± 0.1a	3.7 ± 1.0b

WHC: Water holding capacity. Different letters within a column indicate significant differences between treatments ($p < 0.05$) based on a one-way ANOVA test followed by the Tukey HSD test.

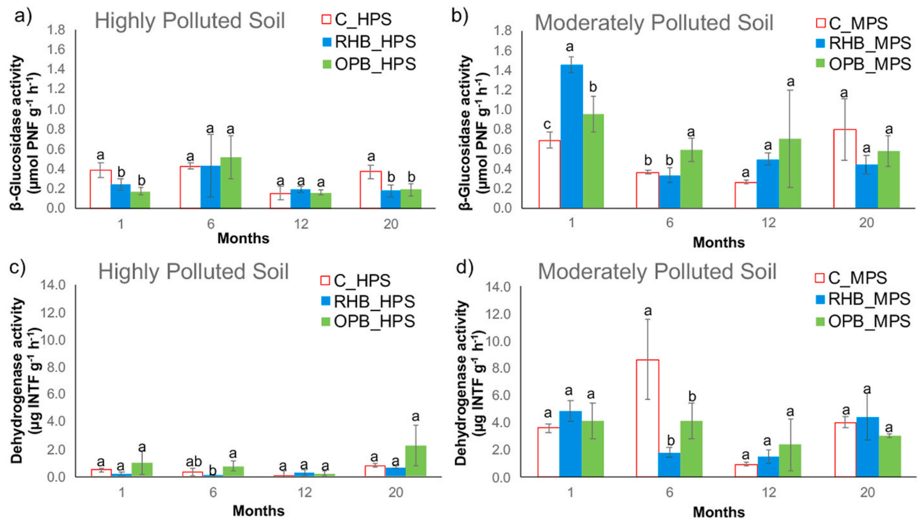

Fig. 2. Enzymatic activities in control and biochar amended soils. a) β-Glucosidase activity in Highly Polluted Soil, b) β-Glucosidase activity in Moderately Polluted Soil, c) Dehydrogenase activity in Highly Polluted Soil and d) Dehydrogenase activity in Moderately Polluted Soil. Different letters for each sampling period indicate significant differences between treatments ($p < 0.05$) based on one-way ANOVA test followed by the Tukey HSD test.

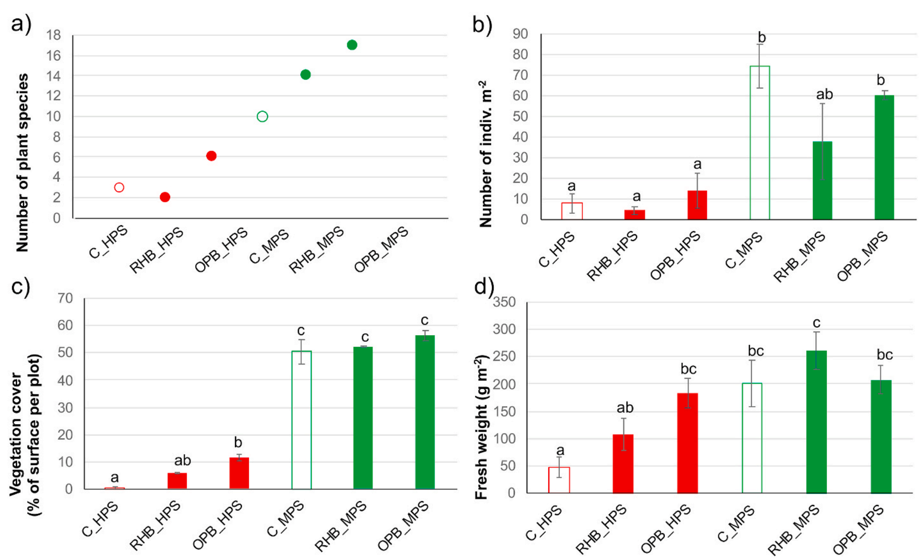

Fig. 3. a) Number of different vegetation species per plot at t_{12}. b) Number of plants per m^2 in control and biochar amended plots at t_{12}. c) Average of vegetation cover (%) per plot. d) Average fresh weight of plants per plot. Different letters indicate significant differences between treatments ($p < 0.05$) based on one-way ANOVA test followed by the Tukey HSD test.

the area of vegetation cover in comparison with the control plots (C_HPS; Fig. 3c). In contrast, although an increase in vegetation cover was observed for HPS amended with RHB, it was not statistically significant (Fig. 3c).

Concerning the fresh weight per plot (Fig. 3d), it increased significantly due to OPB application into HPS, but no significant differences were found for MPS plots. However, the increase of plant fresh weight in OPB_HPS was statistically similar to OPB_MPS. For MPS plots, the application of biochar did not promote statistical differences ($p > 0.05$) among samples for all parameters (Fig. 3 b–d).

3.5. Pearson correlations of soil properties

Table S3 shows that pH was positively correlated with soil moisture measured at 40 °C and soil respiration after 6 months ($p < 0.05$; Pearson coefficients were 0.877 and 0.917, respectively) and 20 months after setup ($p < 0.05$; Pearson coefficients were 0.903 and 0.964, respectively). Soil moisture correlated with WHC at month 6 of the experiment ($p < 0.05$; Pearson coefficient 0.835) and with soil respiration after 20 months ($p < 0.05$; Pearson coefficient 0.862). Nevertheless, 12 months after biochar application, only the pH was negatively correlated with soil moisture measured at 40 °C ($p < 0.05$; Pearson coefficient −0.984), indicating variability of soil properties with time and climate conditions.

Pearson correlations were performed between vegetation results and between vegetation results and soil properties (Table S4). Fresh weight correlated positively with dry weight, number of species and number of individuals ($p < 0.05$; Pearson coefficients between 0.839 and 0.877). Plots with greater number of species also showed greater number of individuals ($p < 0.05$; Pearson coefficient 0.959). Positive correlation was found between pH and fresh weight, number of individuals and number of species ($p < 0.05$, Pearson coefficients 0.851, 0.867 and 0.949, respectively). In addition, positive correlation was found between WHC and fresh weight ($p < 0.05$; Pearson coefficient 0.870).

Negative correlations were found between soil moisture and vegetation results.

3.6. Bacterial community composition

3.6.1. Sequence data

The number of raw sequence reads ranged from 113072 to 237356 for samples collected at t_6, from 377786 to 704600 for t_{12} and from 291568 to 415478 for t_{20}. After quality filtering and denoising, a total of 2354783 paired-end sequences were obtained for all samples. These sequences were clustered into 16964 OTUs at 97% similarity, containing both assigned and non-identified bacteria. Samples from t_{12} showed the highest number of OTUs (8000), followed by t_{20} (4863) and t_6 with 4101 OTUs. For all the samples, the rarefaction curves reached the plateau (data not shown), suggesting that we obtained a good representation of the microbial communities from both soil types (HPS and MPS).

3.6.2. Differences between HPS and MPS

The microbial communities from all the samples were almost exclusively composed of bacteria, with the exception of the control sample HPS (C_HPS), where Archaea accounted for 0.47%, being represented by the *Thaumarchaeota* and *Euryarchaeota* phyla (Table S5).

Differences in the taxonomic composition were clearly observed between both types of soil and between sampling campaigns, particularly between t_6 and t_{12} (Fig. 4 and Table S5). MPS plots at t_6 showed to be more diverse at the phylum level than HPS. At t_6, the most abundant phyla found in MPS plots were *Proteobacteria*, *Planctomycetes*, *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, *Gemmatimonadetes* and *Verrucomicrobia*, whereas in HPS *Chloroflexi*, *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Firmicutes* and *Saccharibacteria* were the most abundant (Fig. 4a).

The *Planctomycetes* phylum was mostly observed in MPS and in very low relative abundance in HPS. *Bacteroidetes*, *Gemmatimonadetes* and *Verrumicrobia* were solely found in MPS (amended and control plots), whereas *Firmicutes* was mostly found in HPS (7% in C_HPS and 2–3% in amended HPS samples). Interestingly, *Saccharibacteria* was solely found in the amended HPS plots.

After 1 year (t_{12}), the relative abundance of bacterial phyla changed considerably in comparison with t_6 (Fig. 4b). *Actinobacteria*, *Chloroflexi*, *Proteobacteria*, *Acidobacteria*, *Firmicutes* and *Planctomycetes* contributed to 80% and 90% of the total bacterial sequences in MPS and HPS, respectively. *Verrumicrobia* and *Gemmatimonadetes* were solely found in MPS (amended and control plots). *Patescibacteria* was found in the control and amended MPS samples, as well as in amended HPS.

Differences in microbial community composition at the phylum level were not significantly observed between t_{12} and t_{20} (Fig. 4b and c). At t_{20} the predominant phyla were *Actinobacteria*, *Chloroflexi*, *Proteobacteria*, *Acidobacteria*, *Firmicutes*, *Planctomycetes* and *Bacteroidetes* for both soils (Fig. 4c).

Remarkable differences in microbial community composition at the order level were noticeable between HPS and MPS plots, as the bacterial sequences in MPS were almost absent in HPS samples (Fig. S1).

Principal components analysis (PCA) was computed to explain differences between samples (Fig. 5). At t_6, the first two components explained 95% of the variation observed (Fig. 5a). The plot of the loadings of PC-1 *vs* PC-2 defined two clusters, corresponding to each soil type. This showed that microbial diversity from HPS samples is significantly different from MPS samples. However, within cluster 1 (HPS samples), PC2 significantly separates the HPS control sample from the biochar-treated HPS samples along the projected plane (Fig. 5a). In contrast, no significant differences of bacterial community composition were noted between amended and unamended MPS plots at t_6.

At t_{12} (Fig. 5b), the plot also displays clear discrimination between both types of soils, but in addition contains separation within cluster 1 among biochar treatments.

At t_{20} (Fig. 5c), PC-1 (70%) vs PC-2 (12%) scores of the control and biochar-treated soils also define two clusters, reinforcing that microbial diversity from both soil types is significantly different along the time span of the field experiment. However, PC-2 discriminates samples within each cluster. Specifically, samples treated with OPB for both types of soils were separated from their corresponding control and RHB-treated samples (Fig. 5c), revealing changes in the microbial diversity for OPB-treated soils after 20 months of incubation.

Venn diagrams were plotted to calculate the number of unique and shared OTUs among the HPS and MPS samples at t_6, t_{12} and t_{20} (Fig. S2). Interestingly, the number of shared taxa between treatments (control and amended plots) was remarkably higher than the number of unique taxa, at t_6, t_{12} and t_{20}, especially in MPS.

The number of shared taxa in HPS increased over time (Figs. S2c and e). The largest OTUs numbers were shared between all the MPS samples (260 at t_6, 504 at t_{12} and 330 at t_{20}), whereas the unique taxa ranged between 5 (at t_6 for C_MPS) and 104 (at t_{12} for C_MPS). At t_6, 34 OTUs (50%) were uniquely present in HPS plots, while in MPS 56 OTUs (16%) corresponded to unique taxa (Fig. S2a). At t_{20} (Figs. S2e and f), the percentage of unique taxa was 45% in HPS against 34% in MPS, suggesting that the bacterial communities became in general more similar between the two soil types over time. Focusing on the biochar treatments, there were more overlapped OTUs among the amended plots than between biochar-treated plots and the controls at t_6 (Figs. S2a and b) and t_{12} (Figs. S2c and d).

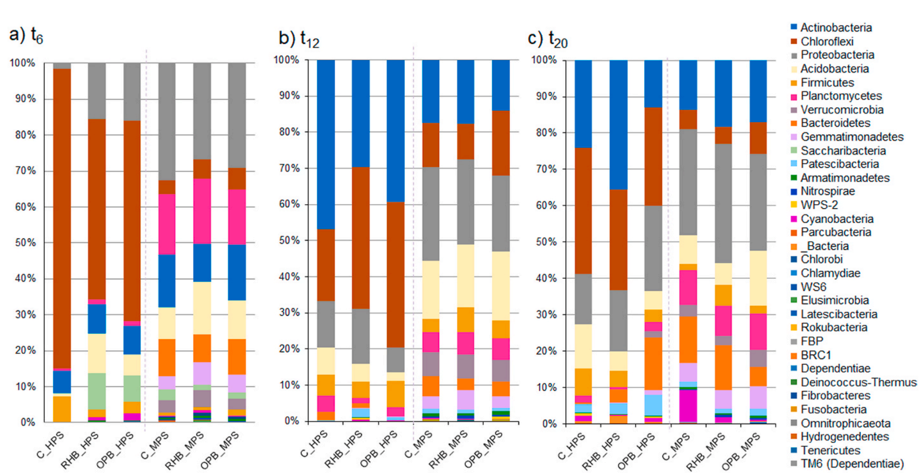

Fig. 4. Relative abundance of the OTUs at the phylum level in the control (C_HPS and C_MPS) and biochar-amended soils (RHB_HPS, OPB_HPS, RHB_MPS and OPB_MPS) at: a) t_6 (6 months after biochar application into soils); b) t_{12} (12 months), and c) t_{20} (20 months after biochar application).

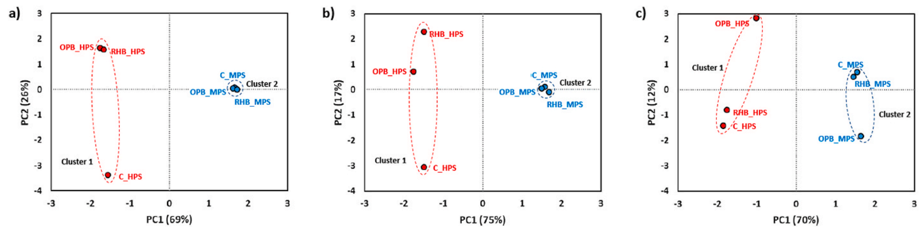

Fig. 5. Assessment of bacterial diversity using principal component analysis (PCA) of highly polluted soil (HPS) and moderately polluted soil (MPS) at: a) t_6; b) t_{12}, and c) t_{20}. RHB and OPB represent biochars derived from rice husk and olive pit, respectively, whereas C_HPS and C_MPS correspond to control soils.

3.6.3. Impact of biochar amendment on HPS and MPS

At month 6 (Fig. 4a), the relative abundance of soil microbiota from the HPS control plot (C_HPS) differed markedly from those treated with biochar (RHB_HPS and OPB_HPS). The *Chloroflexi* phylum was the most abundant in the C_HPS plot, but decreased notably from 83% to 50% in the biochar-amended HPS samples, as well as *Firmicutes*. In contrast, the relative abundance of *Proteobacteria* in HPS increased from 2 to 16% with biochar application, as well as *Acidobacteria* (Fig. 4a and Table S5).

For MPS plots, no significant changes were noted on the relative abundance of soil microbiota between biochar-amended and unamended plots.

After 1 year of biochar application into HPS (Fig. 4b), the relative abundance of *Actinobacteria* slightly decreased with biochar amendment from 47 to 30–37%. *Chloroflexi* increased from 20% in the control to 40% in the biochar-amended HPS samples.

At t_{20} (Fig. 4c), an increase was observed on the relative abundance of *Proteobacteria* (from 14 to 17–23%) and *Bacteroidetes* (from 0.4 to 4–14%) for HPS plots amended with biochar. In contrast, biochar application reduced the relative abundance of *Chloroflexi* (from 35 to 27–28%), *Acidobacteria* (from 12 to 5%) and *Firmicutes* (from 7 to 3–5%) in the HPS samples.

In MPS plots at t_{20}, the application of biochar increased the relative abundance of *Actinobacteria* (from 14 to 17–18%) and reduced the abundance of cyanobacteria (from 9 to 0.4–1%).

At the order level, the most abundant taxa, representing *Chloroflexi* in C_HPS at t_6, belonged to the order *Ktedonobacterales* (71%), followed by the enigmatic phylotypes JG30-KF-AS9 and B12-WMSP1 also within the class *Ktedonobacteria*, both contributing to 9% of the total bacterial sequences (Fig. S1a). The relative abundance of this Ktedonobacterial community was almost reduced by half (from 80% to 46%) with the incorporation of biochar. However, the relative contribution of B12-WMSP1 and JG30-KF-AS9 increased significantly from 4% to 32–37% and from 5% to 12–16%, respectively. In contrast, a sharp decrease was observed for members of the order *Ktedonobacterales* (from 71% to 2% in RHB_HPS and 0.5% in OPB_HPS). The abundances of *Rhodospirillales* (within the Proteobacteria phylum) and *Acidobacteriales* (within *Acidobacteria*) were also higher in biochar-amended HPS. The order *Bacillales*, belonging to the phylum *Firmicutes* and solely represented by the genus *Alicyclobacillus* in C_HPS, decreased from 7% to 3% in OPB-amended HPS and to 1.6% in RHB-amended HPS.

The most abundant orders found across all treatments in MPS plots at t_6 (Fig. S1a) were *Tepidisphaerales* (within *Planctomycetes*), *Sphingomonadales* (within *Alphaproteobacteria*), *Shingobacteriales* (within *Bacteroidetes*), *Burkholderiales* (within *Betaproteobacteria*) and *Rhizobiales* (within *Alphaproteobacteria*).

At t_{12} and t_{20} (Figs. S1b and c), a greater bacterial diversity at the order level is observed for all the treatments in comparison with t_6, and bacterial communities in biochar-amended plots became more similar to their corresponding control plots, particularly at t_{20} (Fig. S1c). Nevertheless, at t_{12}, the relative abundance of B12-WMSP1, representing the Ktedonobacterial community, was higher in the biochar-amended HPS plots (Fig. S1b), as also observed at t_6. The order *Frankiales*, belonging to the *Actinobacteria* phylum, increased markedly at t_{12} across all treatments, independently of biochar application. It is also worth noting the increase of *Acetobacterales*, representing *Alphaproteobacteria*, with biochar amendments in HPS plots. At t_{20}, communities in biochar-amended plots became in general more similar to their corresponding control plots (Fig. S1c). However, after 20 months differences in the relative abundance of bacterial taxa within MPS samples were noticed for OPB-treated MPS (Fig. S1c).

3.7. Bacterial diversity

The diversity of microbial community structure in the HPS and MPS samples was estimated by alpha diversity and richness indices, revealing values significantly different between the samples. MPS samples (control and amended) showed higher alpha diversity (Shannon and Simpson) and richness (Chao1 and OTU count) than HPS samples (Table 3). Shannon index values ranged from 2.00 to 4.24 in HPS samples, with an average of 3.23, and from 4.47 to 5.33 in MPS samples, with an average of 4.73.

The observed Simpson index of diversity ranged from 0.71 to 0.97, with an average of 0.91 for HPS samples, and from 0.97 to 0.99, with an average of 0.98 for MPS (Table 3).

Shannon and Simpson index values increased in the HPS due to biochar addition at t_6. This increase in alpha diversity indices was also observed for MPS plots at t_6. Regardless of the presence or absence of biochar, alpha diversity increased through the time span of the experiment (Table 3).

3.8. Correlation between soil properties and microbial community composition

Soil physicochemical properties and bacterial abundance variables were used to generate correlation heatmaps for t_6, t_{12} and t_{20} (Fig. 6). At t_6, pH, WHC and soil respiration were significantly ($p < 0.05$) and positively correlated with the most abundant bacterial phyla found in MPS plots (*Gemmatimonadetes*, *Planctomycetes*, *Verrucomicrobia*, *Bacteroidetes*), and negatively correlated with *Chloroflexi* and *Firmicutes*, which were the most abundant phylum in HPS plots (Fig. 6a and Table S6). Soil moisture measured at 40 °C and total moisture (measured at 105 °C) were also positively correlated with most of bacterial phyla commonly found in MPS samples, particularly *Proteobacteria* and *Acidobacteria*. Soil TC and dehydrogenase activity showed weak correlation (positive or negative) with the most abundant phyla retrieved in the soil samples. Similarly, glucosidase activity showed almost no correlation with soil microbial communities (Fig. 6a).

At t_{12}, the most abundant phyla detected in MPS plots were strongly and positively correlated with soil pH, WHC, dehydrogenase and glucosidase activities, as well as with the botanical variables measured at t_{12} (fresh weight, number of plant species and individuals), but negatively correlated with *Actinobacteria* (Fig. 6b and Table S6). *Chloroflexi* was negatively correlated with pH, whereas it was positively correlated with soil moistures measured at 40 and 105 °C ($p < 0.05$). Soil respiration and TC showed no significant relationship with soil microbial communities.

At t_{20}, all the soil physicochemical parameters measured in this study were positively correlated with MPS microbial communities, and

Table 3
Alpha-diversity indices of microbial community structure in the unamended and biochar-amended HPS and MPS samples. The diversity indices (Shannon and Simpson index) and richness index (Chao1 and OTUs) were determined at 97% sequence similarity.

Sample ID	No. OTUs	Alpha-diversity								
		Shannon			Simpson			Chao1		
		t_6	t_{12}	t_{20}	t_6	t_{12}	t_{20}	t_6	t_{12}	t_{20}
C_HPS	426	2.00	3.23	3.84	0.71	0.91	0.96	52	142	232
RHB_HPS	468	2.76	3.42	3.33	0.86	0.91	0.91	76	193	199
OPB_HPS	488	2.62	2.84	4.24	0.83	0.86	0.97	73	173	242
C_MPS	1475	4.47	5.33	4.72	0.97	0.99	0.98	291	702	482
RHB_MPS	1493	4.62	5.20	4.73	0.98	0.99	0.98	359	703	431
OPB_MPS	1530	4.61	5.18	5.16	0.98	0.99	0.99	341	646	543

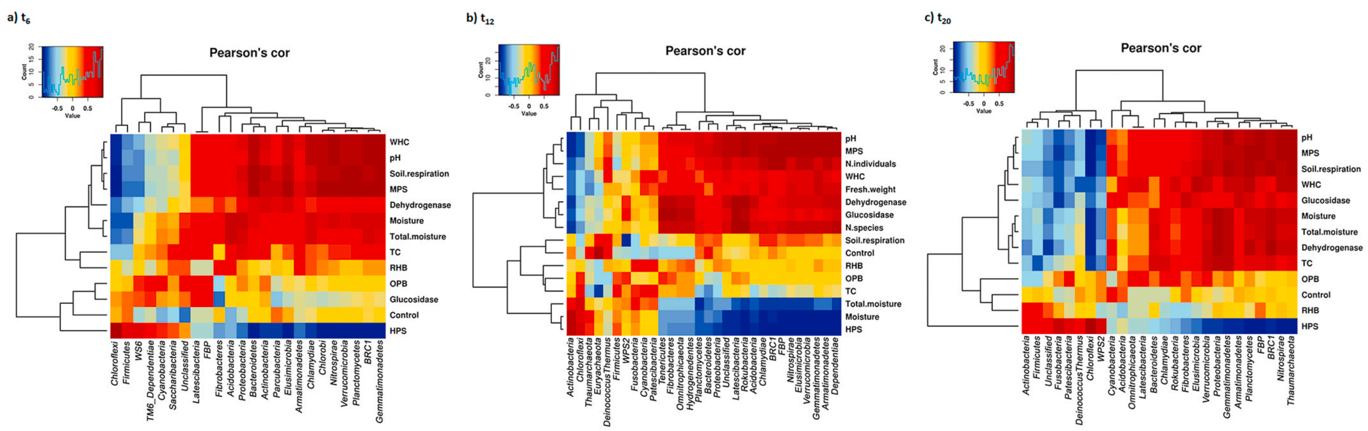

Fig. 6. Correlation heatmaps between soil physicochemical properties (pH, WHC, TC, moisture, soil respiration, β-Glucosidase, Dehydrogenase) and bacterial abundance for a) t_6; b) t_{12}, and c) t_{20}.

negatively correlated with *Chloroflexi*, which was almost exclusively found in HPS samples (Fig. 6c and Table S6).

4. Discussion

Physical, chemical and biological parameters were monitored for 6, 12 and 20 months in biochar-amended soils with two different levels of trace-element contamination under field conditions. These parameters (pH, carbon content, WHC, soil moisture, enzymatic activities, soil respiration, vegetation cover and microbial diversity) were selected to integrate the three types of soil quality indicators, which allow assessing the capability of a soil to perform its ecological functions (Arias et al., 2005).

Soil properties and, consequently, their plant and microbial diversity were very different in HPS and MPS, independently of biochar addition. The soil properties of HPS plots measured before biochar application indicated a very degraded soil with extreme difficulties to sustain ecological functions. Although biochar application induced changes on soil properties, climatic conditions need to be considered, as changes between samplings were notable.

The dehydrogenase activity (DHA) has been also proposed as a good indicator of the toxicity of trace elements (Dick et al., 1996). Under acidic conditions, this enzymatic activity can be inhibited due to the destruction of ion and hydrogen bonds in the enzyme active centre and the alteration of its three-dimensional shape (Frankenberger and Johanson, 1982). This explains the greater values of dehydrogenase activity observed for the less acidic MPS plots, in comparison with HPS, and the positive correlation between soil pH and dehydrogenase activity (Fig. S3). The low β-glucosidase activity measured for all HPS plots, regardless of biochar addition, can be related to soil pH (Eivazi and Tabatai, 1990), and the low abundance of labile organic matter (Ferraz de Almeida et al., 2015). This low enzymatic activity indicates a high recalcitrance of the applied biochar in HPS, as biochar has condensed aromatic structures that make them less available to microbial degradation (Elzobair et al., 2016; Günal et al., 2018; Sohi et al., 2010). Despite of the increase of C content in soils caused by biochar addition, respiration measurements showed that the application of OPB or RHB to the Fluvisol did not modify CO_2 emission rates. This is similar to the findings previously reported by other authors (Sun et al., 2014; Phongthep et al., 2017). In this study, no priming effect is found and a high stability of both sorts of biochar can be predicted. Considering that soil metal pollution is a significant environmental issue, the use of biochar is worthwhile for the remediation of trace element-polluted soils.

As expected, MPS plots showed greater diversity and abundance of vegetation species than HPS (Fig. 3). Comparing both biochar treatments, the application of OPB enhanced not only plant diversity but also the primary productivity in HPS.

The combination of digital image analysis, for measuring the total area of soil covered by the vegetation canopy, and the plant fresh weight approach, which provides information on the plant yield, gave a rather good presentation of the effect of biochar addition on the vegetation production (Fig. 3c and d). It is interesting to note that the application of OPB in HPS plots promoted a significant increase of fresh weight, reaching values similar to those observed for MPS (Fig. 3d), while the vegetation cover of OPB-HPS was five times lower than the unamended and amended MPS plots (Fig. 3c). This is explained by the presence of different plant species in OPB_HPS and MPS plots (Table S2). In MPS, the plant species greatly covered the soil surface (high vegetation coverage area), but their stem diameter and height were much smaller than the species found in HPS plots. In OPB-treated HPS plots, few plants were found but they displayed greater height and stem diameter, and less vegetation coverage.

The positive correlations obtained between plant data (number of species and individuals, and fresh weight) and soil pH (Table S4), demonstrate that biochar is able to enhance the properties of acidic soils, favouring the recovery of degraded polluted soils due to the spill of heavy metals.

Changes in soil microbial community were also assessed in the biochar-amended and untreated soils to inform about soil quality and biochar potential to restore soil functionality. Monitoring microbial diversity by 16S rRNA gene NGS-based analyses after 6, 12 and 20 months of biochar addition showed changes in the soil microbial community structure, particularly in HPS plots after 6 months of soil amendment with biochar. However, after 12 and 20 months, we did not find consistent phylum or order-level responses to biochar amendments (Fig. 4 and Fig. S1), as the treated plots showed higher similarity over control soils, as also reported by Song et al. (2017). Similarly, Shannon and Simpson index values indicated that the addition of RH and OP biochar solely promoted soil bacterial diversity in HPS at t_6 (Table 3). These findings suggest that the type and dosages of biochar applied into HPS had a short-term effect on the distribution of microbial communities, which was dissipated over time.

From the microbial community structure displayed in Fig. 4, we drew the conclusion that biochar addition significantly decreased the relative abundance of members of the *Chloroflexi* phylum in HPS-amended plots at t_6, probably due to changes in soil pH and elements immobilization as *Chloroflexi* have preference for extreme environments (Soo et al., 2009; Yabe et al., 2017). This phylum was mainly represented by the order *Ktedonobacterales* (with 71% of relative abundance in C_HPS) from the class *Ktedonobacteria*, which are filamentous bacteria that inhabit forest and garden soils at low abundances, as well as extreme environments such as geothermal areas and caves (Yabe et al., 2017). The relative abundance of the Ktedonobacterial community (80%) in the HPS control plots abruptly declined (from 80% to 46%) with the incorporation of biochar (Fig. S1a), probably due to changes in pH, which explains the negative correlation between pH and *Chloroflexi* in HPS (Fig. 6a). This decline in *Chloroflexi* abundances after biochar application was also previously reported by several authors (Nielsen et al., 2014; Xu et al., 2014; Ali et al., 2019; Li et al., 2019). However, Chen et al. (2019) showed an increase in *Chloroflexi* with the application of 10% of biochar to calcareous soils.

The relative abundance of *Firmicutes* was also reduced in HPS after 6 months of biochar application. *Firmicutes* can adapt to low nutrient environments and thrive in extreme conditions by forming spores (Bai et al., 2017; Li et al., 2014). Cole et al. (2019) also found a decline in relative abundance of *Firmicutes* with biochar application. However, Ali et al. (2019) reported an increase when a biochar produced from sewage sludge was applied.

Conversely, the increase of *Proteobacteria* observed in HPS-amended samples at t_6 is probably explained by their heterotrophic nature, as biochar increases soil carbon content and nutrient conditions of poor-nutrient soils as HPS. Ali et al. (2019), Cole et al. (2019), Li et al. (2019) and Su et al. (2015) also reported greater abundances of *Proteobacteria* in amended soils than in control soils, obtaining good correlation between *Proteobacteria* and labile C content.

In addition to *Proteobacteria*, the relative abundance of *Acidobacteria* slightly increased after 6 months of biochar addition into HPS, as also reported by Cole et al. (2019). However, Li et al. (2019) and Fan et al. (2020) reported a decrease of *Acidobacteria* after biochar application, but Jenkins et al. (2017) found an increase of *Acidobacteria* even in control soils without biochar treatment, indicating variations by weather conditions. This is in accordance with our results for control HPS over time.

In this study, the relative abundance of *Planctomycetes*, *Bacteroidetes*, *Gemmatimonadetes* and *Verrucomicrobia* did not depend on biochar application but on soil type and seasonal changes. *Planctomycetes* were more abundant in MPS plots than in HPS and their relative abundance varied with different seasons. Rice husk biochar only slightly reduced *Planctomycetes* after 12 months of application into HPS, which is in accordance with the findings of Noyce et al. (2016) when low pH soil was amended with wood chips biochar. However, Ali et al. (2019) showed an increase in *Planctomycetes* abundance in a contaminated-agricultural soil after the application of rice straw biochar.

Chen et al. (2019) observed that the relative abundance of *Bacteroidetes* was higher in the control soil than in the biochar-amended soil, attributing these changes to the initial high pH and nutrient levels in the studied calcareous soils. However, Hu et al. (2014) solely detected *Bacteroidetes* in the biochar amended soil. In this study, *Bacteroidetes* were found in control and amended MPS plots, but not in HPS. It could be due to their copiotrophic nature and capability for living in rhizosphere conditions (Shen et al., 2018), as plant growth was solely observed in MPS plots at t_6. Khodadad et al. (2011) reported an increase of *Gemmatimonadetes* in soils with natural or added pyrogenic carbon, suggesting an active role of these microorganisms in soil pyrogenic C metabolism. We observed a small increase when RHB was applied, which could indicate that this biochar could be more accessible than OPB for these group of bacteria.

Verrucomicrobia was only found in MPS plots, suggesting that its presence was dependent on the soil type, instead of biochar application. In fact, Chen et al. (2019) observed that *Verrucomicrobia* was greater in control than in biochar-amended soils. Nevertheless, Fan et al. (2020) reported an increase in *Verrucomicrobia* phylum in soils amended with biochar.

Actinobacteria are possibly involved in the redistribution of consumed C or in the degradation of more recalcitrant compounds (Blagodatskaya and Kuzyakov, 2008). Cole et al. (2019) and Khodadad et al. (2011) reported an increase in A*ctinobacteria* in soils with natural or added pyrogenic carbon. However, Li et al. (2019) reported a decrease in the relative abundance of *Actinobacteria* after biochar addition to soil. Our results are more in accordance with this decline, particularly in RHB-amended MPS plots at t_6 and in HPS plots at t_{12}. Jenkins et al. (2017) found an increase of *Actinobacteria* even in control soils without biochar, indicating variations due to weather conditions. In this study, the relative abundance of *Actinobacteria* also seemed to be related to seasonal changes particularly in the case of HPS plots (Fig. 4).

In summary, the effects of biochar on soil bacterial communities are not unanimously explained, as numerous other factors, such as soil type, pH, moisture and biochar feedstock are likely to structure microbial communities (Chen et al., 2019; Jenkins et al., 2017). In addition, environmental conditions and long-term biochar application may have more influence in soil microbial communities than biochar types. It is worth mentioning that this variability in soil microbial communities is mostly found in field experiments, whereas in pot incubation experiments parameters are constrained (Hu et al., 2014; Xu et al., 2014). Overall, the changes in the soil bacterial richness and diversity after soil amendment application were correlated with changes in soil pH (Fig. 6), as the incorporation of biochar increased pH, and bacterial diversity, as well as plant growth.

5. Conclusions

This field study conducted on polluted acidic soils has shown that the addition of biochar allowed the recovery of plant cover and increased plant biodiversity, particularly in moderately contaminated soils (MPS). Biochar application did not modify soil CO_2 emissions, nor significantly increase enzymatic activity beyond the first six months of biochar application, which points to a great stability of the tested olive pit and rice husk biochar (OPB and RHB) and their ability to be used for carbon sequestration in degraded soils. Findings from 16S rRNA gene next-generation sequencing revealed that the incorporation of biochar modified the soil microbial community in the highly polluted soil (HPS). Bacterial diversity was found to be site-specific as the properties differed among the studied soils. We conclude that the application of biochar from crop residues to trace-element polluted soils participated in soil conditioning, promoting plant development, increasing bacterial diversity and soil carbon stabilization. This suggested that the application of biochar is important in the ecological restoration of these degraded soils. Our results showed that long-term experiments under field conditions are essential in the quest to investigate the performance of biochar without constraining environmental parameters, as seasonal changes were remarkable in this study. This knowledge could help to fully understand the impact of biochar on global nutrient cycles and on the recovery of soil ecological functions.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2020.108014.

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