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Sustainable Restoration of Soil Functionality in PTE-Affected Environments: Biochar Impact on Soil Chemistry, Microbiology, Biochemistry, and Plant Growth

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Abstract: Biochar can be useful for the functional recovery of soils contaminated with potentially toxic elements (PTEs), even if its effectiveness is variable and sometimes limited, and conflicting results have been recently reported. To shed some light on this regard, softwood-derived biochar was added at 2.5 (2.5-Bio) and 5.0% *w*/*w* (5.0-Bio) rates to an acidic (pH 5.74) soil contaminated by Cd (28 mg kg^{-1}), Pb (10,625 mg kg^{-1}), and Zn (3407 mg kg^{-1}). Biochar addition increased soil pH, available P and CEC, and reduced labile Cd, Pb, and Zn (e.g., by 27, 37, and 46% in 5.0-Bio vs. the unamended soil). The addition of biochar did not change the number of total heterotrophic bacteria, actinomycetes, and fungi, while it reduced the number of Pseudomonas spp. and soil microbial biomass. Dehydrogenase activity was reduced in amended soils (e.g., by ~60 and 75% in 2.5- and 5.0-Bio, respectively), while in the same soils, urease increased by 48 and 78%. Approximately 16S rRNA gene amplicon sequencing and the Biolog community-level physiological profile highlighted a significant biochar impact (especially at a 5% rate) on soil bacterial diversity. Tomato (but not triticale) yield increased in the amended soils, especially in 2.5-Bio. This biochar rate was also the most effective at reducing Cd and Pb concentrations in shoots. Overall, these results demonstrate that 2.5% (but not 5.0%) biochar can be useful to restore the soil chemical fertility of PTE-polluted soils with limited (or null) impact on soil microbial and biochemical parameters.

Keywords: biochar; labile PTE; soil culturable microorganisms; soil bacterial diversity; biochemical activity; plant growth

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1. Introduction

Soil pollution by potentially toxic elements (PTE; e.g., Pb, Zn, Cd, As, and Sb) is of growing concern worldwide due to its critical effects on soil biota, including plants, and its potential impact on public health [1–3]. Soils polluted by PTE cannot be used for agricultural purposes (due to the health risks mentioned above), not contributing to the provision of food or feed, and limiting the achievement of many of the United Nations Sustainable Development Goals (SDGs), e.g., zero hunger (SDG 2), no poverty (SDG 1), and decent work and economic growth (SDG 8) (https://sdgs.un.org/goals, accessed on 20 August 2023). Sustainable remediation of these soils is therefore urgently needed to limit PTE spread in the environment and attenuate their negative consequences for health and society. In this sense, remediation interventions can be fundamental to converting marginal lands (i.e., PTE-polluted areas) into productive ones, e.g., by cultivating high-income non-food crops.

Adding organic amendments to PTE-polluted soils is one of the sustainable remediation options to increase soil fertility, reduce labile PTE (i.e., potentially bioavailable fractions), and reduce potential health risks [4,5]. In this context, biochar, i.e., the solid material deriving from the pyrolysis of different feedstock biomasses, can have relevant implications [6]. In particular, its large surface area, high pH, presence of different functional groups, relevant porosity, surface charge, cation exchange capacity (CEC), and abundance of recalcitrant C are responsible for effective immobilization of labile PTE in soil through a variety of mechanisms such as precipitation, specific and non-specific adsorption, and diffusion within pores [7–11]. Such PTE-immobilizing capacities are greatly influenced by the feedstock nature and pyrolysis conditions, among others [12]. A recent meta-analysis showed that biochar's effectiveness in reducing PTE bioavailability in polluted soils depended mainly on soil pH (after amendment), texture, aging time, and the pyrolysis temperature of biochar [13]. In the same study, other important drivers regulating the bioavailability of PTE were identified, such as PTE species in soil, biochar feedstock, and application rate. This implies that a detailed characterization of each biochar x soil combination is needed to develop tailored solutions for soil recovery. This is essentially supported by the scientific literature of the last 10 years, which, however, has not provided so far any conclusive solutions and/or well-defined guidelines for biochar use in different soil pollution scenarios (e.g., [14]). For instance, while softwood-derived biochar was effective at immobilizing As and Cu in an aqueous solution [8], Beesley at al. [15,16] reported increased mobilization of As and Cu after soil amendment with hardwood biochar. Moreover, while a large literature reports on the PTE-immobilization capacities of biochar (e.g., [17–19]), an increase of Cu, Cd, Ni, and Zn in soil solution after biochar amendment was reported by El-Naggar et al. [20]. Additionally, similar biochars added to different soils in comparable amounts and incubation times showed varying effectiveness of PTE immobilization, e.g., Cd immobilization by maize straw biochar (pyrolyzed at ~500 °C) in two alkaline soils reached 24% in one case and 71% in the other [12]. Again, this supports the view that a customized, or case-by-case, assessment of biochar effectiveness is required and that knowledge gaps on biochar use for the recovery of PTE-polluted soils still exist.

Another aspect requiring more research efforts concerns the biochar impact on the soil microbial community and its functioning. This is an important point, as plant growth and health greatly rely on belowground microbial communities [21,22]. Although many studies reported a positive impact of biochar on soil microbial abundance, diversity, and activity (e.g., [23] and references therein), others highlighted contrasting results [24,25], making it impossible to draw general conclusions on this point. For instance, Anders et al. [26] showed that soil microbial biomass did not change after the addition of different biochars, while Andres et al. [27] reported a significant reduction. In addition, Wang et al. [28] reported a reduction in the relative abundance of fungi and bacteria when high rates of maize straw biochar were applied to the soil. Decreased soil basal respiration was noticed by Domene et al. [29] after corn stover biochar addition at the 0.2–7.0% rate, while reduced microbial activity (i.e., N mineralization) was reported by Dempster at al. [30] with increasing biochar from eucalyptus. Finally, the addition of biochar (from oak and hickory hardwood sawdust) also resulted in a null influence on soil microbial community structure [31], while many other critical effects of biochar on soil biota have been discussed elsewhere [25,32]. For instance, biochar can reduce nutrient bioavailability, thereby limiting plant growth and agricultural yield (e.g., [19,33,34]). This possibility, which mainly depends on feedstock and amendment rate, should be carefully evaluated before biochar employment in soil recovery intervention, as plant growth (PTE phytostabilizing species in particular) can be essential to reducing labile PTE and their spread into the environment [18,35,36].

The aim of this study was therefore to gain new knowledge on the effectiveness of softwood biochar in the functional recovery of a PTE-polluted soil from the dismissed Montevecchio mine in Sardinia, where Zn and Pb were extracted from galena (PbS) and sphalerite (Zn,Fe)S for more than one century [37]. In particular, PTE mobility was evaluated through sequential extraction in the polluted soil and in the same soil amended with

two biochar rates. Soil microbial and biochemical parameters (e.g., soil microbial biomass, number of culturable microorganisms, community-level physiological profile, enzyme activities, amplicon sequencing analysis) were also addressed in the same soils. Finally, the biochar impact on soil fertility, plant growth, and PTE uptake was considered using different plant species, i.e., triticale and tomato.

2. Materials and Methods

2.1. Soil Origin, Biochar, and Mesocosms Set Up

The soil used in this study was sampled in the vicinity of the dismissed Montevecchio mine (39° 33′ 35″ N; 8° 25′ 29″ E) in Southwestern Sardinia (Italy). The mine was exploited for more than a century (1848–1991) to extract Pb and Zn from galena and sphalerite [37]. The mining area (i.e., Montevecchio-Ingurtosu) includes about 150 dumps, accounting for approx. 8 Mm^3 of waste dumps and tailings. In addition, approx. 7 Mm^3 of tailings are dispersed in a large area around the mining site [38]. Due to limited management and securing of dumps and tailings, the area is characterized by significant PTE pollution consequent to the weathering of metal sulfides. Different soil samples (upper 30 cm) were collected near the mining site, pooled in the laboratory (150 kg in total), and sieved to <2 mm before mesocosms were set up. The soil was acidic (pH 5.74) and had a sandy loam texture (USDA classification: 28% coarse sand, 41.5% fine sand, 15.8% silt, 14.7 clay; [39]).

The softwood-biochar used in this study (from elder, beech, and poplar pyrolyzed at 700 °C) was kindly provided by Ronda S.p.A. (Zanè, Italy). A detailed physico-chemical characterization of this biochar was previously described by Pinna et al. [8] and reported in Table S1. Briefly, the biochar was alkaline (pH 9.3) with a pH_{PZC} = 5.0, had approx. 60% of total C, 85 mg kg^{-1} of available P, and a CEC of 19 $cmol_{(+)}$ kg^{-1}. The content of total N and dissolved organic carbon (DOC) was low, i.e., 0.3% and 0.02 mg kg^{-1} respectively. Biochar acidity was mainly due to phenolic groups (2.1 $cmol_{(+)}$ kg^{-1}) rather than carboxylic ones (0.14 $cmol_{(+)}$ kg^{-1}), while Pb, Cd, and Zn were not detected.

Triplicate mesocosms (approx. 15 kg each) were set up in plastic containers for control soil (C soil), soil amended with 2.5% biochar (2.5-Bio), and soil amended with 5.0% biochar (5.0-Bio). Such biochar rates were established based on previous studies carried out on soils with a PTE pollution status comparable to the one investigated here (e.g., [18,40]). Before addition to soil, biochar was sieved to <2 mm. Mesocosms were left to equilibrate for 3 months at 20–22 °C and a constant humidity level (i.e., 40% of their water holding capacity). During this time, they were mixed weekly to favor soil-amendment contact.

2.2. Soil Chemical Analyses

After the contact period, physico-chemical analyses were carried out on duplicate soil samples from each mesocosm. Soil pH and electrical conductivity (EC) were determined in 1:2.5 and 1:5 soil-to-water suspensions; available P and cation exchange capacity (CEC) were determined using the Olsen and the $BaCl_2$-triethanolamine methods, respectively, according to Gazzetta Ufficiale n. 84 [41]. Soil organic C and total N were determined using a Leco CHN628 CHN analyzer and a Soil LCRM Leco part no. 502–697 as a calibration sample. DOC was quantified following Manzano et al. [19], while pseudo-total PTE (i.e., Pb, Cd, and Zn) was determined after microwave mineralization of soil samples (as previously reported) using a Perkin Elmer AAnalyst 400 HGA 900 atomic adsorption spectrometer (FAAS) for Zn and a Perkin Elmer AAnalyst 400 equipped with an HGA 900 graphite furnace (GFAAS) for Pb and Cd. The NIST-SRM 2711-certified reference soil was included for quality assurance.

2.3. Mobility of Pb, Cd, and Zn in Soil

The mobility of Pb, Cd, and Zn in each mesocosm was evaluated (after the contact period) through the sequential extraction procedure described by Basta and Gradwohl [42]. Readily soluble and exchangeable fractions (labile PTE) were quantified after the extraction of duplicate soil samples (1 g each) from each mesocosm with 0.5 M $Ca(NO_3)_2$ solution,

acid-soluble fractions, and those weakly complexed by soil colloids were quantified after extraction with 1 M NaOAc (pH 5); finally, surface-complexed and precipitated PTE fractions were quantified after extraction with Na_2EDTA (pH 7). Residual PTE (i.e., very insoluble and occluded fractions) were quantified after microwave soil mineralization as described for pseudo-total PTE. After each extraction step, the soil suspensions were centrifuged (3500 rpm for 10 min), and the PTE concentration in the filtered supernatant (0.45 µm cellulose acetate filters) was determined using FAAS and GFAAS as already reported.

2.4. Culturable Microorganisms and Soil Microbial Biomass

After the contact period, the number of total culturable heterotrophic bacteria, fungi, actinomycetes, and *Pseudomonas* spp. was determined in duplicate soil samples (10 g) from each mesocosm as previously described [2]. Briefly, soil samples were serially 10-fold diluted using a 0.89% NaCl solution, and aliquots (100 µL) of the resulting suspensions were used to inoculate Petri dishes containing the following microbiological growth media: 1:10 Tryptone Soy Agar (for heterotrophic bacteria; Microbiol, Cagliari, Italy); Rose Bengal Chloramphenicol Agar (for fungi; Biolife, Monza, Italy); Actinomycetes Isolation Agar Glycerol (for actinomycetes; Difco, Milan, Italy); Pseudomonas Selective Agar (for *Pseudomonas* spp.; Microbiol, Cagliari, Italy). Colony counts were carried out after 48 h of incubation at 28 °C for heterotrophic bacteria and fungi and after 72 h at 28 °C for actinomycetes and *Pseudomonas* spp. Microbial counts were expressed as Log_{10} colony-forming units (CFU g^{-1} soil).

Soil microbial biomass (SMB) was estimated in each mesocosm using the chloroform-fumigation extraction method, as reported by Nunan et al. [43]. In brief, duplicate soil samples (40 g) from each mesocosm were divided into two 20 g aliquots: one was immediately extracted with 80 mL of a 0.5 M K_2SO_4 solution after shaking (60 min) and filtering with Whatman No. 42 filter paper; the other was incubated for 24 h under vacuum with ethanol-free chloroform as described by ISO 14240-2 [44] and subsequently extracted as described for the unfumigated samples. Afterwards, the increase in UV readings at 280 nm (A_{280}) of the fumigated vs. unfumigated extracts was used to estimate soil microbial biomass C, as previously reported [40]. The values of soil microbial biomass C were expressed as µg C kg^{-1} soil.

2.5. Molecular Analysis of the Soil Bacterial Community through 16S rRNA Gene Amplicon Sequencing

2.5.1. Bioinformatics

After the contact time, the PowerSoil DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA, USA) was used to extract DNA from soil samples (~500 mg) of each mesocosm. DNA extracts were provided to the Integrated Microbiome Resource sequencing center (Dalhousie University, Halifax, NS, Canada), and amplicon sequencing was performed according to their Illumina MiSeq 2x300bp in-house protocol for amplicons generated with the V4-V5 515FB (5′-GTGYCAGCMGCCGCGGTAA-3′)/926R(5′-CCGYCAATTYMTTTRAGTTT-3′) primers [45,46]. The retrieved sequences were subjected to quality assessment and control with the dada2 v1.24.0 [47] pipeline using the R software v4.1.3 [48], and ASV matrices were obtained as follows. Sequence reads were trimmed at the first instance of very low bases (Phred Q values of 2) while screened from the read error-prone end towards the start. The remaining parts were rejected if the expected error rates were at most 2 or if the remaining read parts were shorter than 150 bp. Moreover, read-pairs where the reconstruction of the amplicon of origin via merging (allowing no mismatches) was not possible were removed. Finally, chimeric, non-specific, or off-target amplicons (non-prokaryotic, unclassified, mitochondrial, or chloroplast) were also rejected from downstream analysis. Classification of the ASVs into taxa was performed with the Bayesian Classifier [49] version of dada2 against the Silva v138 database using an 80% bootstrap cutoff value [50]. The retrieved phylogenetic markers were also analyzed for their functional potential with PICRUSt2 [51] using the default parameters.

2.5.2. Biostatistics

The retrieved ASV and predicted microbial function matrices were used for a series of statistical analysis tasks. α-diversity indices representing members or functions of the studied microbial communities of various dominance levels were calculated with the Vegan v2.6-4 [52] and the Entropart v1.6-11 [53] R packages. Specifically, the observed richness (representing all communities), the Shannon index (representing the, at least, low-dominance community members), the Inverse Simpson index (representing the, at least, intermediate-dominance community members), and the Fisher's α index (representing the highly dominant community members) were calculated. Permutational multivariate analysis of variance (PERMANOVA) and canonical analysis were performed with the vegan package of R to assess the effect of the biochar treatment on the microbial communities and their functions. Analysis of variance with the Tukey's post hoc test or their non-parametric equivalents (Kruskal–Wallis and the Wilcoxon rank sum analysis) was used for comparing α-diversity indices, while analysis for differentially abundant taxa between treatments was performed with the Kruskal–Wallis (k test-factor levels, with k > 2) and the Wilcoxon rank sum (pairwise) analysis.

2.6. Soil Enzyme Activities and Community Level Physiological Profile

Dehydrogenase (DHG) and urease (URE) were quantified (after the incubation period) in duplicate soil samples from each mesocosm. Both enzyme activities were determined using the protocols described by Alef and Nannipieri [54]. Briefly, the DHG activity was determined colorimetrically (A_{480}) as triphenyl formazan released after incubation of soil samples (10 g at 30 °C for 24 h) with triphenyl tetrazolium chloride, while URE was determined as ammonia released (A_{690}) after incubation of soil samples (5 g at 37 °C for 2 h) with urea [54].

The Biolog community-level physiological profile (CLPP) was obtained for soil microbial communities extracted from the different mesocosms, as reported by Diquattro et al. [2]. In particular, soil microbial communities from the different mesocosms were inoculated in 96-well Biolog (microtiter) Ecoplates (Biolog Inc., Hayward, CA, USA) containing a total of 31 C sources of environmental relevance (one in each well) and a blank well replicated three times. After recording the A_{590} readings for each well (every 24 h for 5 days), using a Biolog MicroStation™ reader (Biolog Inc., Hayward, CA, USA), the following CLPP indexes were determined, i.e., the Average Well Color Development (AWCD), the Shannon–Weaver index (H'), and the Richness (S) value.

The AWCD, or the potential catabolic activity of the different soil microbial communities, was calculated as in Equation (1):

$$\text{AWCD} = \sum_{i=1}^{31}(Ri - C)/31 \quad (1)$$

where Ri is the absorbance value (A_{590}) of each response well, C is the absorbance value of the control well, and 31 is the number of C substrates in the plate [52].

H', indicating the catabolic functional diversity (substrate use) of the different soil microbial communities, was calculated as in Equation (2):

$$H' = -\sum(pi\ (\text{Log}\ pi)) \quad (2)$$

where pi is the absorbance ratio of each of the 31 substrates to the total absorbance value of the plate [55].

S was calculated as the number of C substrates used ($A_{590} > 0.15$) by the different soil microbial communities [56].

Standardized A_{590} values, i.e., [(Ri—C)/AWCD of the plate], were also subject to Principal Component Analysis (PCA) using the variance/covariance matrix [56] to allow for a more straightforward data interpretation of multidimensional data.

2.7. Plant Growth and PTE Uptake

After the incubation time, the soil from each mesocosm was used to fill 2 pots (approx. 2 kg of soil each), which were planted with triticale (x *Triticosecale* Wittm. cv. Trimour) and tomato (*Lycopersicon esculentum* L. cv. Rio Grande) seeds. These species, characterized by different physiologies and taxonomically distant, were chosen as bioindicator organisms to fully evaluate the remediation effectiveness of biochar and not to test the possibility of growing food or feeding crops in the polluted soil. Ten and five plants of triticale and tomato were grown, respectively, in each pot (without fertilization) for 2 months at 20–22 °C. At harvest, plants were removed from pots, and roots and shoots were carefully washed. All plant heights were recorded, shoots and roots were separated, and their dry weight was determined after 10 days in the oven at 55 °C. To quantify PTE uptake, root and shoot tissues were mineralized using microwave (ultraWave, Milestone, Sorisole, Italy) and a digestion solution containing 2 mL of suprapure H_2O_2 and 4 mL of a mixture of HNO_3 and ultrapure H_2O (ratio 1:1). After mineralization, Pb, Cd, and Zn were determined using FAAS for Zn and GFAAS for Pb and Cd. Peach leaves (NIST-SRM 1547) were used as standard reference material for quality assurance.

2.8. Data Analysis

Soil chemical, biochemical, and microbiological data are reported in tables and figures as mean values ± standard errors (SE). Data were analyzed to investigate differences due to the treatments applied (i.e., biochar at two different rates). All traits were evaluated for normality and homoscedasticity using the Shapiro and Bartlett tests, respectively. The variables that passed both tests were analyzed through ANOVA, whereas those that did not were analyzed through the Kruskal–Wallis test ($p < 0.05$). In Table S2, the statistical analysis adopted for each of the investigated traits was reported. All statistical analyses were carried out in R 4.2.1 [48].

3. Results and Discussion

3.1. Influence of Biochar on the Chemical Characteristics of the Polluted Soil

The main physico-chemical characteristics of the polluted soil used in this study are reported in Table 1. This latter soil had a sandy loam texture with an acidic pH and a low content of organic matter, total N, and DOC. However, available P and CEC values were high, but pseudo-total concentrations of Pb, Cd, and Zn were all abundantly exceeding the threshold values established by the Italian law for potentially contaminated soils devoted to commercial and/or industrial use (i.e., 1000, 15, and 1500 mg kg^{-1} for Pb, Cd, and Zn, respectively; [57]) or to agriculture (i.e., 100, 5, and 300 mg kg^{-1} for Pb, Cd, and Zn, respectively; [58]). Overall, these data suggest limited soil fertility, with N being the most limiting factor for agricultural yields [59], and with low pH and high PTE content adding more stress for plant establishment and growth, as well as for the soil microbial community [42,56,60]. The low DOC content also suggests some additional constraints on microbial growth and abundance in the studied soil.

Biochar addition increased soil pH, which approached neutrality in 5.0-Bio (Table 1). This was due to biochar alkalinity (Table S1), and it is expected to have a positive impact on both soil physico-chemical properties, e.g., through the reduction of soluble Al^{3+} and PTE, and soil microbial activities [61]. Also, the increase in available P (especially in 5.0-Bio) and CEC recorded in amended soils is deemed positive, as these latter are important soil fertility parameters. The biochar ability to increase soil CEC was previously reported and attributed to the presence of oxygen-containing functional groups on biochar surfaces (e.g., carboxylic and phenolic) able to retain cations [12]. Moreover, biochar's natural oxidation and/or its incubation with soil can further increase the formation of oxygenated groups [62], likely explaining the CEC values of amended soils. The high amount of available P in biochar (i.e., 85 mg kg^{-1} soil; Table S1) can finally explain its increase in the amended soils (especially in 5.0-Bio; Table 1).

Table 1. Selected physico-chemical characteristics of the contaminated (C soil) and biochar-amended soils (2.5-Bio and 5.0-Bio). Mean values ± SE followed by different letters within a row denote statistically significant differences ($p < 0.05$).

Physico-Chemical Characteristics	C Soil	2.5-Bio	5.0-Bio
Texture	Sandy loam	-	-
pH	5.74 ± 0.02 ^{a}	6.35 ± 0.01 ^{b}	6.58 ± 0.01 ^{c}
CE (μS cm^{-1})	376 ± 9 ^{a}	325 ± 5 ^{b}	333 ± 4 ^{b}
Organic matter (g kg^{-1})	32.67 ± 0.58 ^{a}	34.67 ± 0.58 ^{b}	36.33 ± 0.58 ^{c}
Total N (g kg^{-1})	1.00 ± 0.0 ^{a}	1.00 ± 0.0 ^{a}	0.91 ± 0.0 ^{b}
P Olsen (mg kg^{-1})	31.53 ± 1.22 ^{a}	32.43 ± 0.98 ^{a}	35.73 ± 0.47 ^{b}
CEC (cmol$_{(+)}$ kg^{-1})	24.36 ± 0.02 ^{a}	24.55 ± 0.08 ^{b}	25.15 ± 0.42 ^{c}
DOC (mg kg^{-1})	13.79 ± 0.43 ^{b}	12.51 ± 0.26 ^{a}	12.04 ±0.12 ^{a}
Pb (mg kg^{-1})	10.625 ± 2058 ^{a}	10.238 ± 372 ^{a}	10.064 ± 141 ^{a}
Cd (mg kg^{-1})	28.3 ± 0.4 ^{a}	27.2 ± 1.05 ^{a}	27.1 ± 1.07 ^{a}
Zn (mg kg^{-1})	3407 ± 140 ^{a}	3291 ± 241 ^{a}	3323 ± 120 ^{a}

3.2. Influence of Biochar on the Mobility of Pb, Cd and Zn in Soil

Both rates of biochar had a great influence on PTE mobility, significantly reducing the concentration of labile (readily soluble and exchangeable) Pb, Cd, and Zn in the amended soils (Figure 1). For instance, labile Pb [extracted with $Ca(NO_3)_2$] reduced by approx. 76% in 5.0-Bio, while in the same soil, Cd and Zn reduced up to 27 and 37%, respectively (Figure 1). Weakly complexed Pb (extracted with NaOAc) reduced up to 46% in Bio-5.0, while Cd and Zn increased or remained unchanged, respectively (Figure 1). After biochar addition, the surface complexed and precipitated PTE (extracted with Na_2EDTA) reduced in the case of Pb (up to ~5%) and Zn (up to ~26%) but remained unchanged for Cd. Very insoluble and occluded fractions (residual PTE) remained unaffected for Pb and Cd, while significantly increasing for Zn (up to ~45%).

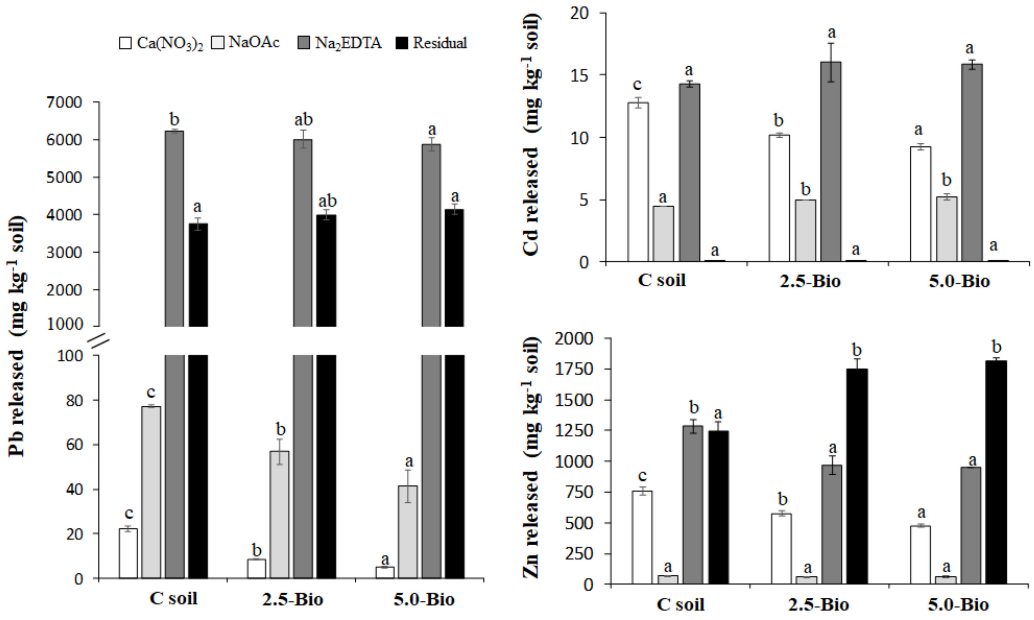

Figure 1. Concentrations of Pb, Cd, and Zn extracted from contaminated control (C soil) and amended soils (2.5-Bio and 5.0-Bio) using the sequential extraction procedure. Color bars refer to the different extraction solutions. For each PTE and within the same extraction solution, different letters indicate significant differences between treatments ($p < 0.05$).

During the three-month contact period, a PTE redistribution clearly occurred in amended soils (especially in 5.0-Bio), leading to a shift from more mobile and potentially bioavailable fractions [i.e., labile PTE extracted with $Ca(NO_3)_2$] to less mobile and poorly bioavailable ones (e.g., those extracted with Na_2EDTA and/or residual). This was previously reported by other studies (e.g., [63]) and is of outmost importance from a remediation perspective since labile PTE are the most impactful on plants and soil (micro)organisms [4,18,40]. Such biochar-driven PTE redistribution towards less bioavailable fractions can be due to a variety of mechanisms, such as: (i) Pb, Cd, and Zn partial precipitation as oxides or hydroxides following the significant pH increase in the amended soils [7]; (ii) the formation of insoluble PTE-phosphates or PTE-carbonates (e.g., the biochar used contained substantial available phosphate, Table 1; [8]); (iii) the formation of strong complexes between PTE and oxygenated functional groups of biochar (e.g., phenolic and carboxylic, Table 1; [64]); (iv) PTE surface adsorption and diffusion within biochar pores [7,14].

3.3. Influence of Biochar on Culturable Microorganisms and Soil Microbial Biomass

The size of the targeted culturable soil microbial communities was mostly unaffected by biochar addition (Figure 2). The number of total heterotrophic bacteria, actinomycetes, and fungi did not change after soil amendment, while that of *Pseudomonas* spp. was reduced by approx. 10-fold (Figure 2). This is interesting as in the very few studies focusing on the effect of biochar on soil culturable microorganisms, increased microbial numbers were commonly reported after amendment (e.g., [65–67]). Our results can be explained by the DOC values recorded in the amended and unamended soils: DOC represents an important source of C for soil microorganisms [68], and its marginal reduction in the amended soils, also reported by Manzano et al. [19] and explained by adsorption phenomena, did not allow an increase of culturable microorganisms, while it reduced the number of *Pseudomonas* spp. [69]. However, PAHs accumulated in biochar during pyrolysis could have contributed to such adverse effects against *Pseudomonas* spp. [24]. Both DOC reduction and PAH accumulation in amended soils could also explain the approx. 60% reduction of SMB recorded in 5.0-Bio (Figure 3). Similar results were reported by Andrés et al. [27] after adding maize biochar to Mediterranean vineyards and by Dempster et al. [30] after using eucalyptus biochar in wheat cultivation. While the impact of the highest amount of soft-wood biochar was clear, at least vs. *Pseudomonas* spp. and SMB, its relevance for soil functioning is hardly predictable, although microbial biomass is recognized to play a relevant role in soil ecosystem functioning and productivity [70].

3.4. Influence of Biochar on the Structure of Soil Bacterial Community

ASV matrices were generated with dada2 as described in the materials and methods. Out of a total of 261,009 read pairs, a final amount of 47,618 high-quality sequences passed the quality control process and were used in the analysis (Table S3).

Significant differences between the control soil and 5.0-Bio were observed in α-diversity indices, i.e., the observed richness S, the Shannon, and the inverse Simpson (Figure 4A). In the case of the Fisher's α index, which is more representative of the highly dominant ASVs, no significant differences resulted from the tests performed. Twelve phyla dominated the samples, with Acidobacteriae, α-Proteobacteria, Bacteroidia, γ-Proteobacteria, Gemmatimonadetes, and Vicinamibacteria being the most dominant among those (Figure 4B). Principal coordinates analysis (PCoA) showed a partial separation of the treatments, mostly due to the 5% biochar treatment (Figure 4C). Differential abundance analysis showed that 5 ASVs were mainly responsible for these structural differences, belonging to Bacteroidota, Proteobacteria, and Acidobacteriota (Figure 4D).

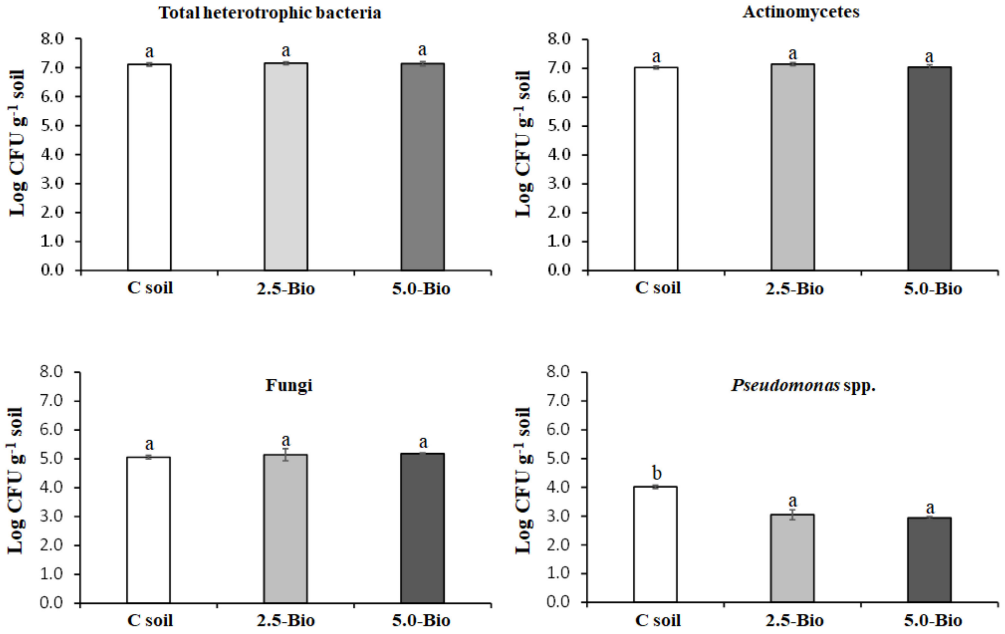

Figure 2. Number of culturable microorganisms in contaminated control (C soil) and amended soils (2.5-Bio and 5.0-Bio). For each microbial group, different letters indicate significant differences between treatments ($p < 0.05$).

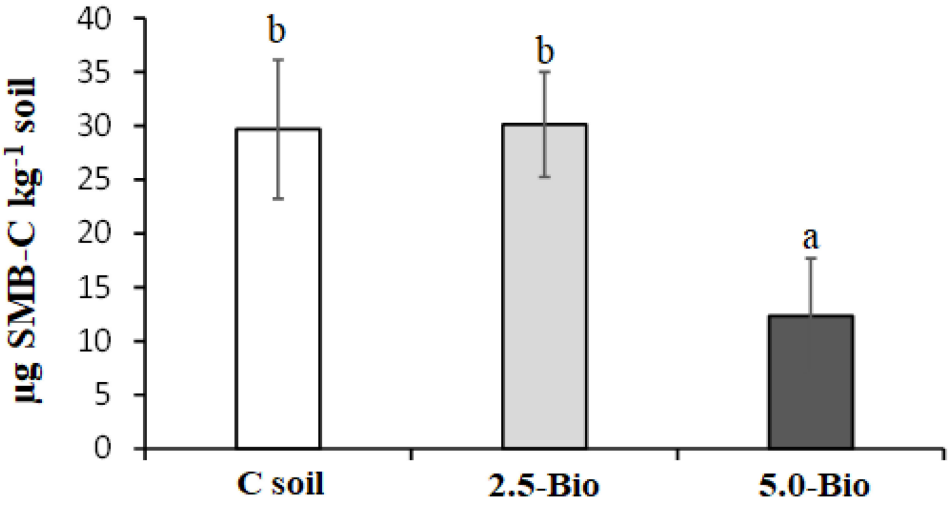

Figure 3. Soil microbial biomass C (SMB-C) in contaminated (C soil) and amended soils (2.5-Bio or 5.0-Bio). Different letters indicate significant differences between treatments ($p < 0.05$).

These data indicated a positive influence of biochar, when used at the highest rate, on soil bacterial diversity. This was previously reported by other authors (e.g., [71,72]) and can be attributed to the highest reduction of labile PTE and the highest increase of soil pH, which occurred in 5.0-Bio. Both factors likely contributed to reducing the environmental pressure faced by microbial communities in the polluted soil, allowing for the appearance (and/or increase) of rare or intermediate-dominant bacterial taxa [4,71,73]. The significant abundance of *Lysobacter* in 5.0-Bio could also be relevant from an environmental perspective, as members of this genus produce antibiotics and can be useful in the control of plant diseases [74].

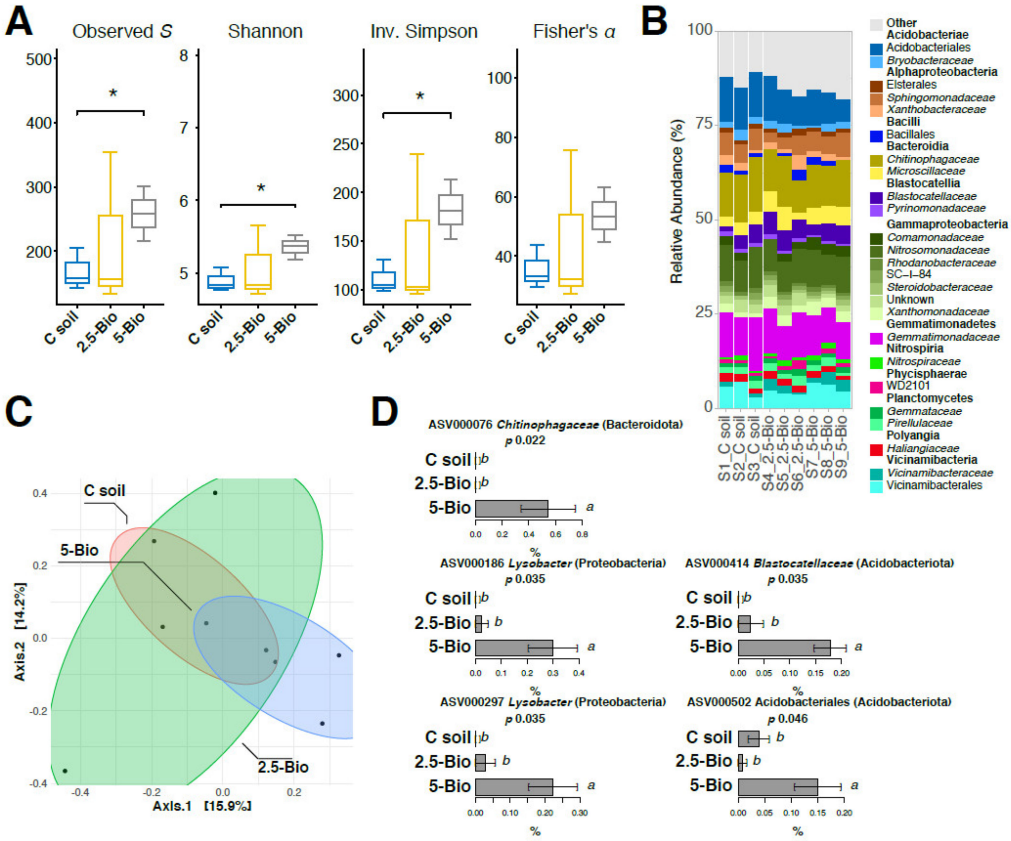

Figure 4. Microbial community data analysis outputs for contaminated (C soil) and amended soils (2.5-Bio or 5.0-Bio). (**A**) α-diversity boxplots with ANOVA results and post hoc pairwise Tukey's analysis (α 0.05). (**B**) Barplots of the dominant taxa with taxonomy resolution as low as family level. (**C**) Principal coordinates analysis (PCoA) scatter plot generated using the Bray–Curtis dissimilarity, with the explained variance provided at each axis. (**D**) Barplots of the five ASVs showing differential abundance between the treatments (different letters indicate significant differences according to Kruskal and Wilcoxon rank sum tests for α of 0.05). * $p < 0.05$.

Statistical analysis of the inferred functions according to Picrust2 output was also performed (Figure 5). The results showed no significant differences in the α-diversity of the functions (Figure 5A). Major identified functional classes included Biosynthesis, Degradation/Utilization/Assimilation, Generation of Precursor Metabolite and Energy, and Macromolecule Modification (Figure 5B). PCoA showed a separation between the control soil and 2.5- and 5.0-Bio, with this latter treatment being more distant (Figure 5C). Pathways showing significant differences were those of the TCA cycle (Helicobacter type) and L-methionine, thiazole, and thiamine diphosphate biosynthesis, with all of them being reduced at an increasing biochar application rate. These data suggest that the observed changes in the bacterial community structure were likely paralleled by functional changes, which could have a role in adapting to changed environmental conditions (e.g., lower labile PTE, increased pH, reduced N and DOC content; Table 1).

3.5. Influence of Biochar on Soil Enzyme Activities and Community Level Physiological Profile

DHG activity in soil is generally reduced according to the amount of biochar added, while the opposite was found for URE. In particular, DHG reduced by approx. 60 and 75% in 2.5-Bio and 5.0-Bio, respectively, compared to control soil, while in the same soils, URE increased by 48 and 78% (Figure 6). DHG data seemed to indicate a negative biochar effect on soil microbial activity, and this was not obvious since a reduction of labile PTE (which occurred in amended soils; Figure 1) is commonly expected to increase DHG (e.g., [4,17,56]). As mentioned for culturable *Pseudomonas* spp., this could be due to a

reduction of readily usable C sources in DOC (which occurred in amended soils; Table 1) and/or to a direct toxic effect of biochar on soil microorganisms [24,25,32]. PAHs, but also other biotoxic compounds adsorbed and/or accumulated on biochar surfaces, e.g., environmentally persistent free radicals and/or catechol, can be responsible for microbial toxicity phenomena and the consequent reduction of DHG and SMB [32]. Interestingly, the DHG decrease recorded in amended soils could be seen as a confirmation of the reduction of the TCA pathway highlighted by Picrust2 (Figure 5C).

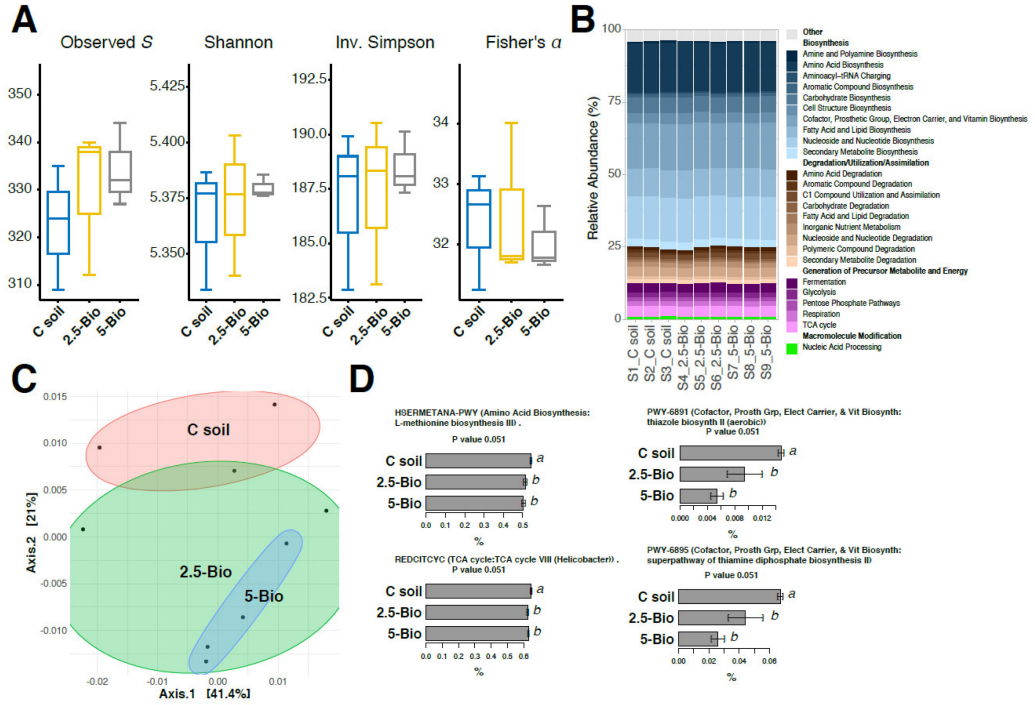

Figure 5. Microbial function analysis outputs according to Picrust 2 for contaminated (C soil) and amended soils (2.5-Bio or 5.0-Bio). (**A**) α-diversity boxplots of functions with ANOVA results and post hoc pairwise Tukey's analysis ($\alpha > 0.05$; no statistically significant differences were identified). (**B**) Barplots of the dominant functions. (**C**) Principal coordinates analysis (PCoA) scatter plot generated using the Bray-Curtis dissimilarity, with the explained variance provided at each axis. (**D**) Barplots of the four differentially enriched pathways showing differences between the treatments (different letters indicate significant differences according to Kruskal and Wilcoxon rank sum tests for α of 0.05).

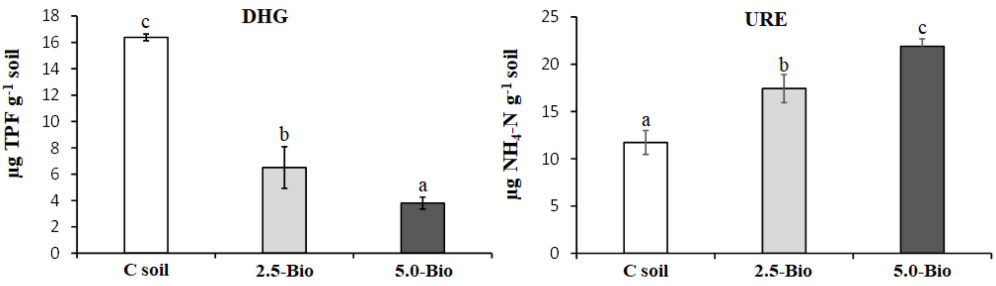

Figure 6. Dehydrogenase (DHG) and urease (URE) activities in contaminated (C soil) and amended soils (2.5-Bio or 5.0-Bio). For each enzyme activity, different letters indicate significant differences between treatments ($p < 0.05$).

The increased URE activity observed in the amended soils, together with the reduction of total N content (Table 1), was likely indicative of a stimulation of urea hydrolysis due

to biochar rather than an increased microbial content in amended soils (i.e., SMB reduced in 2.5- and 5.0-Bio; Figure 3). Such accelerated rate of URE activity in the presence of biochar was recently reported by Zhao et al. [75] and could be partly responsible for the N reduction observed in the amended soils (especially 5.0-Bio; Table 1). The increased URE observed in the amended soils can also be due to an increased microbial synthesis of the enzyme stimulated by a more limited N availability in these soils [76], which in turn can be explained by NO_3-N and NH_4-N adsorption by biochar, as previously observed [19].

The Biolog CLPP did not show significant differences between control and amended soils according to the AWCD, H', and Richness values (Figure S1). However, when C source consumption was analyzed by PCA, clear differences appeared. PCA, which accounted for approx. 80% of the total variance (in PC1 and PC2), highlighted substantial differences in the potential catabolic activity of the microbial communities (Figure 7). PC1 (approx. 55% of the total variance) mainly separated the different microbial communities and was correlated with the catabolism of the following substrates: β-methyl-D-glucoside (r = 0.76), D-xylose (r = −0.99), 2-hydroxy benzoic acid (r = 0.78), L-arginine (r = −0.79), and L-threonine (r = 0.77); while PC2 (approx. 24% of the total variance) was mainly correlated with the usage of α-cyclodextrin (r = 0.76), 4-hydroxy benzoic acid (r = 0.78) and α-ketobutyric acid (r = 0.79). These results support a relevant impact of biochar (and of the rate added) on the structure of the soil microbial community, as also highlighted by the molecular analysis (Figures 4 and 5) and by recent studies (e.g., [23,24,28]). Overall, this kind of impact was somewhat expected given the profound changes that biochar exerted on soil physico-chemical properties and nutrient dynamics (e.g., this study and Li et al. [7]). The reduction of labile PTE in the amended soils could also have been co-responsible for the observed changes, e.g., by decreasing the abundance of PTE-resistant strains in treated soils and favoring the appearance of new ones with different catabolic capacities, as previously reported [4,5].

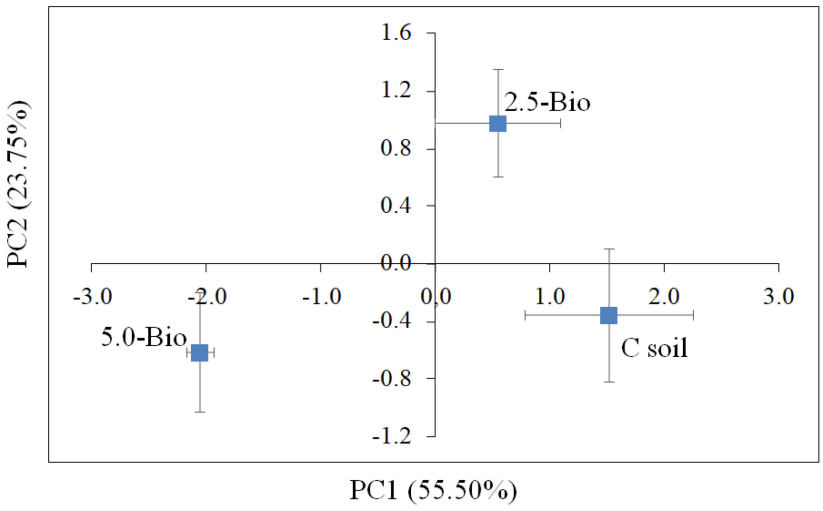

Figure 7. PCA plot of standardized C source utilization data of microbial communities extracted from contaminated (C soil) and amended soils (2.5-Bio or 5.0-Bio).

3.6. Influence of Biochar on Plant Growth and PTE Uptake

Plant growth was tested to gain a wider view of the role of biochar in restoring soil fertility in PTE-polluted soils. Interestingly, biochar had a different impact on the growth of triticale and tomato. The height of the former species, together with the respective shoot dry weight, were unaffected by biochar, while substantial increases were recorded for tomato (Figures S2 and 8). The height of tomato plants increased by approx. 2.5- and 2.1-fold for 2.5-Bio and 5.0-Bio, respectively (Figure S2), while shoot dry weight increased by approx. 8.0- and 4.5-fold in the same soils (Figure 8). Moreover, the higher biochar rate

had a negative effect on triticale root dry weight, while both rates had a positive effect on the weight of tomato roots, with 2.5-Bio revealing the most effective treatment (Figure 8).

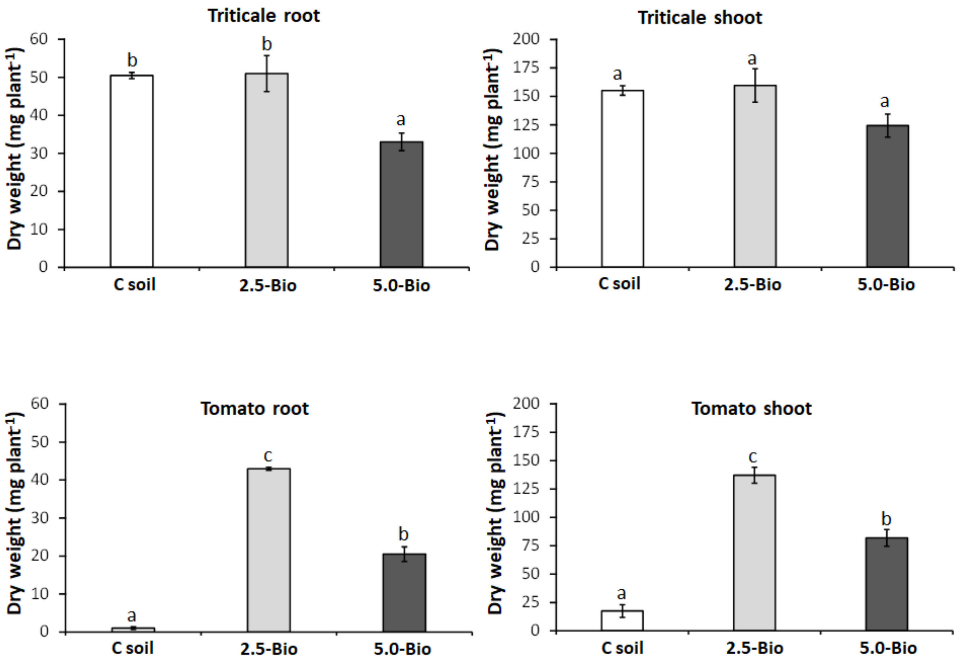

Figure 8. Root and shoot dry weight of triticale and tomato plants grown in contaminated (C soil) and amended soils (2.5-Bio or 5.0-Bio). For each parameter, different letters indicate significant differences between treatments ($p < 0.05$).

Overall, these data highlight the importance of using different plant species to understand the biochar potentials in the recovery of soil fertility in PTE-polluted soils. Our results suggest a quite different tolerance/sensitivity of the two plants towards labile PTE (as evident from the comparison of plant growth in control soil) and a different adaptation to soil chemical characteristics, e.g., pH and total N. Tomato yield increased dramatically when labile PTE was reduced in the amended soils, while triticale did not (Figure 8), likely suggesting a higher PTE tolerance of the latter species that allowed substantial plant growth in the control soil. This is probably why triticale, likewise other grass species, has been used in different phytoremediation studies (e.g., [77–80]). Furthermore, *S. lycopersicum* is very sensitive to soil acidity (and soluble Al^{3+}), and the pH increase recorded in 2.5- and 5.0-Bio could have contributed to improving its growth in the amended soils [81]. Finally, the yield reduction observed for both plant species in 5.0-Bio compared to 2.5-Bio was likely due to excessive nutrient adsorption by biochar, which reduced plant growth, and/or to other biochar toxicity effects previously reported [24,25]. In the first case, combining biochar with fertilizers and/or using biochar enriched with nutrients could mitigate the nutrient depletion effects arising from excessive biochar rates [24]; in the second case, phytotoxic effects can be avoided using lower biochar amounts, e.g., $\leq$2.5% [25].

Also, biochar influence on PTE uptake differed depending on the plant species: the concentration of Pb and Cd in triticale roots increased in the amended soils (up to 36 and 100%, respectively, vs. control), while that of Zn was reduced (up to 76% vs. control; Table 2). These results could be explained by a higher and/or altered root activity in the amended soils (e.g., increased secretion of siderophore, organic acids, and other root exudates), which led to enhanced Pb and Cd mobilization from the soil and their subsequent uptake [79], as well as a reduced Zn uptake (which was also correlated with the reduction of labile Zn in the amended soils; Figure 1).

Table 2. PTE uptake (mg kg^{-1}, mean ± SE) by triticale and tomato plants grown in contaminated (C soil) and biochar-amended soils (2.5-Bio or 5-Bio). Mean values ± SE, followed by different letters within each column, denote statistically significant differences between treatments ($p < 0.05$). ND was not detected because of the limited availability of root biomass.

	Triticale					
	Pb Uptake (mg kg^{-1})		Cd Uptake (mg kg^{-1})		Zn Uptake (mg kg^{-1})	
	Shoots	Roots	Shoots	Roots	Shoots	Roots
C soil	39.2 ± 0.3 ^{b}	434.8 ± 21.5 ^{a}	5.7 ± 0.1 ^{b}	30.3 ± 1.1 ^{a}	631.1 ± 25.8 ^{a}	1596.3 ± 8.3 ^{c}
2.5-Bio	38.3 ± 0.5 ^{a}	593.7 ± 2.9 ^{b}	5.1 ± 0.1 ^{a}	61.6 ± 1.1 ^{c}	733.2 ± 5.0 ^{c}	1307.4 ± 19.7 ^{b}
5.0-Bio	53.3 ± 0.2 ^{c}	456.8 ± 25.3 ^{ab}	7.9 ± 0.1 ^{c}	52.6 ± 0.7 ^{b}	683.4 ± 10.1 ^{b}	928.0 ± 6.7 ^{a}
	Tomato					
	Pb uptake (mg kg^{-1})		Cd uptake (mg kg^{-1})		Zn uptake (mg kg^{-1})	
	Shoots	Roots	Shoots	Roots	Shoots	Roots
C soil	514.7 ± 22.8 ^{c}	ND	17.7 ± 0.6 ^{b}	ND	2843.1 ± 22.3 ^{c}	ND
2.5-Bio	89.2 ± 7.4 ^{a}	695.4 ± 20.3 ^{b}	15.3 ± 1.1 ^{a}	133.5 ± 2.1 ^{b}	879.1 ± 86.5 ^{b}	4859.2 ± 28.3 ^{b}
5.0-Bio	115.5 ± 9.2 ^{b}	616.8 ± 17.0 ^{a}	24.9 ±0.2 ^{c}	104.2 ± 1.7 ^{a}	680.3 ± 9.5 ^{a}	3101.2 ± 28.4 ^{a}

With regards to tomato, PTE uptake by roots was reduced in 5.0-Bio vs. 2.5-Bio (Table 2) in agreement with labile PTE in these soils (Figure 1; control root yield was not enough to quantify PTE uptake). Differently from triticale, these data support a clear positive influence of biochar on the fertility recovery of PTE-polluted soils, as highlighted elsewhere [5,18].

In both triticale and tomato plants, PTE was largely accumulated in the roots rather than the shoots (Table 2). Overall, biochar impact on PTE uptake by shoots was more limited in the case of triticale and, for both plants, confirmed a better effectiveness of 2.5-Bio rather than 5.0-Bio in reducing Cd and Pb concentrations in the aerial part.

4. Conclusions

The results from this study showed that softwood biochar added at 2.5 and 5.0% rates was able to significantly reduce labile (and potentially bioavailable) Pb, Cd, and Zn in a PTE-polluted mining soil and to increase selected fertility parameters (e.g., soil pH, available P, and CEC). This is relevant from a practical viewpoint since it suggests reduced ecotoxicological effects in amended soils as well as increased functionality. However, soil microbiological and biochemical data did not support this view, with the exception of bacterial α-diversity (which increased in 5.0-Bio vs. control) and urease activity (which increased in both 2.5- and 5.0-Bio vs. control). These results raise some questions about the overall biochar impact on soil functionality, or at least the ideal amount that should be added to any soil. In this regard, 2.5-Bio appeared to be the most effective treatment able to combine soil chemical restoration with a limited impact on soil microorganisms (e.g., on *Pseudomonas* ssp.) and biochemical activity (DHG was repressed but URE was stimulated by 2.5-Bio). This was supported by plant growth data, which showed reduced tomato and triticale yields for 5.0-Bio vs. 2.5-Bio, likely due to excessive nutrient adsorption by biochar. Overall, our results showed that chemical data alone cannot be sufficient to predict the effect of biochar on soil functionality, while the measurement of several (micro)biological proxies and the use of different bioindicators, such as different plant species, can be helpful. Given the significant role of plants in shaping rhizosphere microbial communities and their activities (e.g., through their root exudates), further studies should focus on the impact of endemic plants on the microbial abundance and diversity in biochar-amended polluted soils.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/soilsystems7040096/s1, Figure S1: AWCD, H', and Richness values of contaminated (C soil) and amended soils (2.5-Bio or 5.0-Bio); Figure S2: height of triticale and tomato plants grown in contaminated (C soil) and amended soils (2.5-Bio or 5.0-Bio); Table S1:

selected chemical properties of the biochar used in this study; Table S2: statistical analysis adopted for each of the investigated traits; Table S3: quality control (QC) of the received sequence data.

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