



Article Application of Biochar Produced from Crop Residues on Trace Elements Contaminated Soils: Effects on Soil Properties, Enzymatic Activities and *Brassica rapa* Growth

Paloma Campos 🔍, Heike Knicker 🔍, Rafael López 🔍 and José María De la Rosa *🕲

Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS-CSIC), Reina Mercedes Av., 10, 41012 Seville, Spain; pcampos@irnas.csic.es (P.C.); knicker@irnase.csic.es (H.K.); rlnunez@irnase.csic.es (R.L.) * Correspondence: jmrosa@irnase.csic.es

Abstract: Soil pollution by trace elements is a huge problem around the globe. In addition, heavy metal immobilization and primary productivity are two soil ecosystem services of contemporary importance to society. The goal of this study was to evaluate the effects of using olive pit and rice husk biochars as soil amendments for the immobilization of trace elements and on plant development growing in heavy metals-polluted soils under greenhouse conditions. The application of high doses (5% and 10%) of biochar significantly increased pH, water holding capacity and total C content of the soils. Dehydrogenase activity in the moderately acidic soil was greater than in the acidic soil due to the high concentration of metals and high acidity of the latter. The application of biochar reduced the β -glucosidase activity. Furthermore, the concentrations of CaCl₂-extractable heavy metals significantly decreased in biochar amended pots, indicating metal immobilization, which was consistent with the increase in soil pH. Distribution of trace elements in the different fractions was modified after 65 days of incubation, independently of the treatment. The Cu and Zn contents in the oxidizable fraction were reduced with incubation, whereas Cd and Zn in the residual fraction increased. The reduction of bioavailable concentrations and increments in the residual or more stable fractions indicated less risk for the organisms in the environment. All biochars addition significantly increased the root-to-shoot ratio compared to the control soil. Particularly, 10% of amendment increased this ratio in the greatest extent. The application of 10% w/w of rice husk biochar produced at 500 °C was the most effective in restoring soil functionality and reducing the availability of heavy metals in the polluted soils.

Keywords: olive pit; rice husk; soil remediation; incubation experiment; dehydrogenase; bioavailable concentration

1. Introduction

Land degradation is a worldwide concern. Much of this degradation is due to contamination of soils by heavy metals that negatively affect the ecosystem services provided by soils, which are the direct or indirect contributions of the complex soil system to human wellness. In fact, it is estimated that over 37% of degraded soils in the European Union are polluted with trace elements [1], most of which were introduced by anthropogenic activities, such as mining, waste disposal, industrial processing or agriculture. A high concentration of heavy metals inhibits biomass production, decreases plant respiration, limits antioxidant enzymatic activities, induces leaf chlorosis [2,3] and influence processes that represent the system's capacity to provide ecosystem services. Heavy metals in soil are bound, adsorbed or complexed to organic components and/or to minerals, but the total concentration of heavy metals in soils does not necessarily represent the actual risk for soil flora, fauna and microorganisms. This risk depends largely on their bioavailability and mobility within the soil. This is not only affected by their physical and chemical structure, but also by soil conditions altering their solubility, such as soil pH, redox stage or the



Citation: Campos, P.; Knicker, H.; López, R.; De la Rosa, J.M. Application of Biochar Produced from Crop Residues on Trace Elements Contaminated Soils: Effects on Soil Properties, Enzymatic Activities and *Brassica rapa* Growth. *Agronomy* **2021**, *11*, 1394. https:// doi.org/10.3390/agronomy11071394

Academic Editors: Evan A. N. Marks and Xavier Domene

Received: 11 June 2021 Accepted: 9 July 2021 Published: 11 July 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). concentration of competing cations for adsorption sites. The potential availability of heavy metals in soils is often approached by a sequential extraction protocol yielding an acid extractable (F1), a reducible (F2), an oxidizable (F3) and a residual (F4) fraction [4]. The heavy metals in the acid extractable fraction are the most dangerous for the environment, as they are very labile and can be solubilized in water or slightly acidic conditions. The trace elements in the reducible fraction are bound to Fe and Mn oxides. The metals in the oxidizable fraction are bound to organic matter or sulphides. Finally, the residual fraction is composed of the most stable metals, as they are strongly associated with the crystalline structure of the soil minerals.

Heavy metal contamination in soils can be remediated by various physical, chemical or biological methods or a combination of them [5]. "Ex-situ" treatments are more intensive, requiring the transport of soil to a controlled treatment, which has the advantage that leakages in the groundwater can be controlled. Nevertheless, "ex-situ" methods cause the destruction of the soil structure [6] and are very costly.

As an alternative to removal and replacement of the degraded soils, numerous organic and inorganic amendments, such as compost or lime, have been used to immobilize the trace elements in order to reduce their bioavailability [7]. Bearing in mind the higher mobility under acid conditions, organic and inorganic alkaline amendments are traditionally employed to bind the heavy metals in the soil and, therefore, reduce their toxicity. Madejón et al. [7] reported great efficiency of a biosolid compost amendment for the remediation of a trace element-contaminated soil by achieving an increment of pH and the consequent reduction in trace element availability, with a benefit to the primary productivity and microbial development, which led to the recovery of soil ecosystem services. However, these authors indicated that further amendment applications may be required to maintain the effect.

During the last decade, numerous studies have reported the potential of biochar, an emerging highly aromatic, porous material produced by pyrolysis of organic residues, to sequester carbon and mitigate greenhouse gases emissions [8]. Moreover, biochar has shown great biochemical stability [9], which is an advantage over less recalcitrant organic amendments, which require continuous replacement of decomposing organic material to avoid lixiviation of released heavy metals. Besides this, several authors have raised that the alkaline pH, the very porous structure and the great water-holding capacity (WHC) of many biochars support metal adsorption and, thus, the reduction of the availability of heavy metals in the soil [10–14]. Biochar can bind metals by processes such as adsorption, ionic exchange, complexation, precipitation or electro-static interaction [15]. Zhao et al. [16] reported the immobilization of trace elements including Cr³⁺, Cd²⁺, Cu²⁺ and Pb²⁺ under controlled conditions. The application of biochars to acid-polluted soils could therefore be relevant in many fundamental aspects to preserve the ecosystem services of those degraded soils. These services are valued by society, influencing decision-making and soil management. However, here one must bear in mind that these studies consist in batch adsorption experiments in aqueous solutions that were artificially contaminated with trace elements. Thus, their results do not account for the complexity of the soil matrix. Nevertheless, the effectiveness of biochars to immobilize several trace elements simultaneously in multi-polluted soils is still under debate. Kim et al. [11] showed that rice husk biochar amendment induced a decline of more than 80% of the trace elements (Cd, Cu, Pb and Zn) that could be extracted with NH₄NO₃ from the soil. After the application of bamboo biochar to a mine-contaminated soil, Ali et al. [10] observed a reduction of 4 and 8% for Zn and Cd availability, respectively, whereas that of Pb and Cu increased by 65 and 17% for Pb and Cu, respectively, probably by the formation of soluble Pb and Cu complexes with dissolved organic matter. Nevertheless, although biochar application usually diminishes the trace element availability, we still need to improve our understanding of the efficiency of biochar amendment on trace metal sequestration in soils contaminated by various trace elements and of how this treatment affects microbial activity, plant development, nutrient availability and soil health. Thus, it has been hypothesized that the utilization of abundant

agricultural wastes with low or no trace metal contents for biochar production and its subsequent application to polluted soil may be an efficient way for recycling the wastes and, simultaneously, for soil remediation.

In order to test this hypothesis, we studied the effect of the application of biochars from two different and very abundant agricultural residues, rice husk (RH) and olive pit (OP), to acidic Fluvisols contaminated with Cu, Fe, Pb, Sr and Zn, among other trace elements. The biochars tested were produced with contrasting conditions, with 400 and $500 \,^{\circ}\text{C}$ being the pyrolysis temperature, and 1 and 4 h being the pyrolysis time. The soils were collected from an area contaminated by one of the largest mining accidents in Europe. The mine was located in the Iberian Pyritic Belt, where the trace elements background concentration is very high [17]. This study was performed under greenhouse-controlled conditions to reduce the number of variables and to facilitate the understanding of the effects of biochar on the physical, chemical and biological properties of the soil. For that, soil composition, physical properties and water loss were studied. Brassica rapa pekinensis, which has been used previously as an indicator plant for trace element contamination, was sown for determining the effects of biochar application on germination and plant development [18–20], which are also relevant to assess the impact on soil ecosystem services. Brassica rapa, a variety of cabbage widely used as an ingredient in some Asian cuisines, was selected due to its fast growth with broad leaves and its ability to grow in neutral and acidic soils [21]. The focus of using plants in the experiment was to determine the germination and survival, not their potential to accumulate metals. Finally, the availability of trace elements was determined in the incubated soils with and without biochars, as it represented the real risk of those pollutants for soil flora, fauna and microorganisms.

The success of soil remediation is often approached by measuring the enzymatic activities [22]. Therefore, we also included the determination of enzymatic activities. One group of the enzymes that has been proposed as a good indicator for the toxicity of trace elements represents intracellular dehydrogenases. They transfer hydrogen and electrons from organic substrates to catalyze the oxidative degradation of the soil organic matter [23]. Extracellular enzyme activities, such as β -glucosidase, are also considered as important indicators of soil quality [24]. β -glucosidase hydrolyses β -D-glucopyranosides, a monomer which also results from the degradation of cellulose [25], representing the major biopolymer of plant residues. Dehydrogenase and β -glucosidase enzymatic activities measurements were also performed for their value as indicators of general soil health and of the preservation of soil ecosystem services.

2. Materials and Methods

2.1. Soils

The soils used for this experiment were taken at "Las Doblas" site (37°23'7.152" N, $6^{\circ}13'43.175''$ W), which is located close to the Guadiamar river (Province of Seville, SW Spain). On the 25th of 1998, a mine located in Aznalcóllar, about 10 km north of the sampled area, broke and released over 5.5 million m³ of acidic water and 1.9 million m³ of acid sludge, with a high content of toxic trace elements such as As, Cu, Pb and Zn, to the Guadiamar and Agrio rivers [26]. The composition of the sludge was similar to the pyrite one, as this residue came from a mine of the Iberian Pyrite Belt. Background concentrations of As, Cu, Pb and Zn in the soils of the area are very high [17]. Thus, copper and silver mining activities started in this region since ancient times, and in the mid-19th century, a great number of mines were opened [27]. The soils used for this study consisted of two sandy loam soils (8–10% clay, 27–34% silt and 56–64% sand) classified as Fluvisol [28], with two levels of contamination, hereinafter called acidic polluted soil (APS) and moderately acidic polluted soil (MAPS). The bulk density of APS was 1.09 g mL⁻¹, and of MAPS, it was 1.18 g mL⁻¹. The soils were described in more detail in Campos and De la Rosa [29]. Their pH values were 3.6 ± 0.1 for APS and 6.5 ± 0.1 for MAPS. After sampling, soils were transported to the laboratory in sealed bags, dried at 40 °C, sieved to <2 mm and stored in sealed bags at 4 °C until they were used.

2.2. Biochar Production

The biochars were obtained by the pyrolysis of rice husk (RH) and olive pit (OP), both of which are agricultural residues abundant in SW Spain. The pyrolysis for biochar production was carried out according to De la Rosa et al. [30] in a 0.7 L cylindrical steel batch reactor, with a heating rate of 30 °C min⁻¹ under an N₂ atmosphere at 400 °C, with a residence time of 1 h (samples RHB400_1 and OPB400_1), and at 500 °C, with residence times of 1 h and 4 h (samples RHB500_1, OPB500_1, RHB500_4 and OPB500_4). Their properties have been described in Campos et al. (2020a).

2.3. Soil Parameters

Soil pH was measured using a pH meter with a glass electrode (pH Basic 20, CRI-SON, Barcelona, Spain) in the supernatant of a mixture prepared with the ratio 1:5 (w/v) soil:CaCl₂ after 30 min shaking followed by 30 min of resting. Soil moisture was elucidated by weighting after drying the sample at 40 °C. The water-holding capacity (WHC) of soils was approached by the method of Veihmeyer and Hendrickson [31], as adapted by Campos et al. [32]. It was expressed as the percentage relative to the total dry weight of the sample. Total C and N contents were obtained by dry combustion (1050 °C) (LECO TRUSPEC CHNS MICRO, LECO, St. Joseph, MO, USA).

2.4. Enzymatic Activities

Dehydrogenase and β -glucosidase enzymatic activities were measured after 2, 16 and 65 days of incubation. The dehydrogenase activity (DHA) was determined according to the method developed by Trevors [33]. Therefore, 1 g of moist-soil samples was incubated for 20 h, with 1 mol L⁻¹ TRIS–HCl buffer (pH 7.5) and 2(*p*-iodophenyl)–3-(*p*-nitrophenyl) 5-phenyl tetrazolium chloride (INT). After adding methanol, the iodonitrotetrazolium formazan (INTF) produced was measured spectrophotometrically at 490 nm.

The β -glucosidase activity was determined according to the method of Tabatabai [34]. Briefly, 1 g of moist-soil was incubated for 1 h at 37 °C with *p*-nitrophenyl- β -D-glucopyranoside. The product of the reaction, *p*-nitrophenol (PNP), was determined spectrophotometrically at 400 nm. Results for enzymatic activities were based on the 40 °C-dried weight of soil.

2.5. Quantification of Trace Element Species in Soils

Bioavailable (CaCl₂-extractable) trace element concentrations (As, Ba, Cd, Cr, Cu, Fe, Mn, Ni, Pb, Sr, Zn) in soils were determined after the incubation step, which lasted 65 days, in 0.01 mol L^{-1} CaCl₂ at a ratio of 1:10 soil/solution after shaking for 3 h [35]. Subsequently, the trace element concentrations were measured by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES; ICP 720-ES, Varian, CA, USA).

In addition, the EU sequential extraction procedure [36] was used to determine the speciation between soil fractions of As, Ba, Cd, Cr, Cu, Fe, Mn, Ni, Pb, Sr and Zn at days 5 and 65 of incubation. This procedure separated the following fractions:

- Fraction 1 (Acid extractable): extracted with acetic acid 0.11 mol L⁻¹.
- Fraction 2 (Reducible): extracted with hydroxylammonium chloride 0.5 mol L^{-1} .
- Fraction 3 (Oxidizable): extracted with hydrogen peroxide 8.8 mol L⁻¹ and ammonium acetate 1.0 mol L⁻¹.
- Fraction 4 (Residual): extracted with aqua regia $(1:3 v/v \text{ of HNO}_3:\text{HCl})$.

The contents of Ba, Cd, Cu, Fe, Mn, Ni, Pb, Sr and Zn in the filtered extracts of the four phases were measured using ICP-OES. The reference material ERM-CC141 (loam soil) [37] was used to assess the quality of the analysis, obtaining recoveries from 83 to 116%.

2.6. Pot Trial under Greenhouse Conditions

2.6.1. Incubation Step

An incubation step was performed for 65 days before the plant growth experiment to have a fast estimation of the potential effects of the biochar amendment on soil properties.

For that, soil–biochar mixtures were prepared with low (2%), medium (5%) and high (10% w/w) doses of each biochar, with four replicates (n = 4) per treatment. In addition, for each soil, four control pots were prepared without biochar. All the pots were randomly distributed, and soil moisture was adjusted to 60% WHC and maintained constant by adding the daily-lost water. After the incubation, pH, WHC, elemental analysis (total C and N contents), enzymatic activities, available trace elements and their speciation in soil fractions were measured, as previously explained.

2.6.2. Pot Trial with Plants of Brassica rapa L. ssp. pekinensis

For the pot trial, the pre-incubated soils with and without amendment were homogenized and divided into plastic containers with a volume of 0.5 L. Since, in the pre-incubation, none of the soil properties of the pots amended with 2% of the biochars showed changes in comparison to control soils, the amendment with 2% biochar (w/w) was discarded for the next step. Subsequently, three certified seeds of *Brassica rapa* ssp. *pekinensis* were sown per pot. *Brassica rapa* ssp. *pekinensis* is a plant widely used in Cantonese cuisine, and due to its great leaf size, it is susceptible to metal accumulation. Its short growth cycle allows for the understanding of biochar effects in reclaiming polluted soils by performing short-term greenhouse experiments. The plants grew in a greenhouse under controlled conditions (25 ± 2 °C and 12 h light day⁻¹) for 65 days. Soil moisture was maintained constant at 60% WHC.

The number of germinated and living plants were counted after 4, 8, 16, 25, 31 and 65 days. After the 65th day, plant shoots were cut, dried (72 h at 60 °C) and weighed to evaluate the biomass productivity. The length of plant stems and leaves (mm) were determined at 4, 8, 19, 30 and 65 days to assess the plant development. At the end of the pot trial, the root content per pot was determined. For that, the material contained in each pot was placed in glass plates, then roots were carefully hand-separated by using tweezers and a magnifying glass (10x). Once washed and dried (72 h at 60 °C), the root weight and the root-to-shoot ratios were calculated. Plant development in all pots with APS soil could not be determined, as plants did not germinate or died during the experiment.

2.7. Statistical Analysis

All the results for the incubation and subsequent pot trial are shown as mean values \pm Standard Error (SE) of four and three replicates, respectively. The Shapiro–Wilk test and Levene test were used for testing normality and homoscedasticity of data. The effects of biochar application on soils were analyzed by a one-way analysis of variance (ANOVA), followed by Tukey's Honestly Significant Difference test. When response variables were not-normal, the Kruskal–Wallis test was conducted. Pearson correlations were obtained between soluble-CaCl₂ trace elements and pH. To test the effects of the soils and amendments conditions on soluble-CaCl₂ trace elements, a multifactor ANOVA (MANOVA) was performed to obtain F values and the significance of each studied variable. Pillai's trace test and mean differences were performed. A significant level of *p* = 0.05 was used throughout the study. Statistical analyses were carried out using IBM SPSS Statistics 26.0 (SPSS, Chicago, IL, USA).

3. Results and Discussion

3.1. Effect of Biochar Addition on Soil Physical Properties and Elemental Analysis

The application of 5% and 10% biochar RHB, as soil amendment, significantly increased soil pH for MAPS (p < 0.05) (Figure 1). In contrast, the addition of the lowest dose (2%) of both biochars did not alter soil pH. In general, the application of RHB achieved greater soil pH values than OPB, which was due to the strong alkalinity of RHB (Table 1) derived from its high ash content (27–36%) in comparison with other feedstock [32]. In the APS soil, the changes in the soil pH were of a lower magnitude than in the MAPS (Table 1). Nevertheless, the control and amended APS soils showed the same trend as the MAPS soils.



Figure 1. Soil pH in control and amended pots after 65 days of incubation for (**a**) moderately acidic polluted soils (MAPS) and (**b**) acidic polluted soils (APS). Different letters indicate differences among treatments in each soil (p < 0.05).

The application of RHB at doses of 5% and 10% significantly increased WHC in comparison with control pots after 65 days of incubation (Figure 2). The application of 10% RHB_500 biochar to the MAPS increased, by over 80%, the WHC as compared to the un-amended pots. In contrast, the use of OPB biochars did not increase WHC significantly under any circumstances. The latter is best explained with the WHC of the pure biochars, which was higher for RHB chars, ranging from 121% for 400 °C-1 h to 437–450% for biochars produced at 500 °C, than for OPB biochars (ranging from 28% for 400 °C-1 h to 30–60% for biochars produced at 500 °C; Table 1). These differences were likely related to differences in the porous structure of biochars as it can be deduced from FESEM images of the surface structure published earlier [32].

-

_	Elemental Analysis					Total (and CaCl ₂ -Extractable) Contents of Trace Elements (mg kg ⁻¹)										
_	TC (g kg ⁻¹)	TN (g kg ⁻¹)	C/N	Ash (%)	pН	WHC (%)	As	Ва	Cd	Cr	Cu	Fe	Ni	Pb	Sr	Zn
MAPS_o	17 ± 1	2.0 ± 0.7	8	94.9 ± 0.9	6.5 ± 0.1	51.5 ± 1.2	115.4 (<loq)< td=""><td>93.3 (2.6)</td><td>1.56 (0.1)</td><td>36.1 (<loq)< td=""><td>215.5 (0.2)</td><td>36,945.7 (0.6)</td><td>15.6 (0.1)</td><td>156.5 (0.3)</td><td>38.6 (2.5)</td><td>293.5 (1.3)</td></loq)<></td></loq)<>	93.3 (2.6)	1.56 (0.1)	36.1 (<loq)< td=""><td>215.5 (0.2)</td><td>36,945.7 (0.6)</td><td>15.6 (0.1)</td><td>156.5 (0.3)</td><td>38.6 (2.5)</td><td>293.5 (1.3)</td></loq)<>	215.5 (0.2)	36,945.7 (0.6)	15.6 (0.1)	156.5 (0.3)	38.6 (2.5)	293.5 (1.3)
APS_o	8 ± 0	0.9 ± 0.4	8	94.7 ± 0.7	3.6 ± 0.1	32.7 ± 2.4	367.0 (<loq)< td=""><td>47.1 (0.1)</td><td>1.28 (0.1)</td><td>48.0 (<loq)< td=""><td>240.6 (5.1)</td><td>53,023.3 (8.9)</td><td>15.6 (0.6)</td><td>569.0 (<loq)< td=""><td>53.7 (0.2)</td><td>249.3 (20.2)</td></loq)<></td></loq)<></td></loq)<>	47.1 (0.1)	1.28 (0.1)	48.0 (<loq)< td=""><td>240.6 (5.1)</td><td>53,023.3 (8.9)</td><td>15.6 (0.6)</td><td>569.0 (<loq)< td=""><td>53.7 (0.2)</td><td>249.3 (20.2)</td></loq)<></td></loq)<>	240.6 (5.1)	53,023.3 (8.9)	15.6 (0.6)	569.0 (<loq)< td=""><td>53.7 (0.2)</td><td>249.3 (20.2)</td></loq)<>	53.7 (0.2)	249.3 (20.2)
RHB400_1 RHB500_1 RHB500_4 OPB400_1 OPB500_1 OPB500_4	$501 \pm 3 \\ 518 \pm 1 \\ 522 \pm 3 \\ 670 \pm 2 \\ 605 \pm 9 \\ 587 \pm 2$	$5.2 \pm 0.3 \\ 6.2 \pm 0.1 \\ 5.7 \pm 0.1 \\ 12.9 \pm 0.3 \\ 15.7 \pm 0.2 \\ 13.7 \pm 0.0$	96 83 91 52 38 43	$\begin{array}{c} 27.9 \pm 0.5 \\ 33.1 \pm 0.8 \\ 35.7 \pm 0.9 \\ 1.4 \pm 0.2 \\ 0.8 \pm 0.3 \\ 0.9 \pm 0.7 \end{array}$	$\begin{array}{c} 9.1 \pm 0.0 \\ 10.5 \pm 0.0 \\ 10.3 \pm 0.0 \\ 7.2 \pm 0.0 \\ 8.5 \pm 0.1 \\ 9.1 \pm 0.2 \end{array}$	$\begin{array}{c} 121.0 \pm 22.3 \\ 437.9 \pm 4.4 \\ 449.8 \pm 9.7 \\ 28.0 \pm 2.0 \\ 30.0 \pm 7.4 \\ 60.2 \pm 9.2 \end{array}$	1.3 7.5 12.7 5.0 2.4 6.1	2.5 3.4 3.3 0.8 0.8 <loq< td=""><td><loq 0.14 0.07 <loq 0.08 <loq< td=""><td>0.4 1.5 2.3 0.4 0.3 0.5</td><td>6.6 9.2 7.5 6.2 5.9 3.7</td><td>150.0 307.4 309.3 40.6 42.8 <loq< td=""><td><loq <loq <loq <loq <loq <loq< td=""><td><loq <loq 0.3 <loq <loq 3.2</loq </loq </loq </loq </td><td>10.1 14.8 13.4 6.6 10.9 3.6</td><td>24.3 38.9 34.4 <loq <loq <loq< td=""></loq<></loq </loq </td></loq<></loq </loq </loq </loq </loq </td></loq<></td></loq<></loq </loq </td></loq<>	<loq 0.14 0.07 <loq 0.08 <loq< td=""><td>0.4 1.5 2.3 0.4 0.3 0.5</td><td>6.6 9.2 7.5 6.2 5.9 3.7</td><td>150.0 307.4 309.3 40.6 42.8 <loq< td=""><td><loq <loq <loq <loq <loq <loq< td=""><td><loq <loq 0.3 <loq <loq 3.2</loq </loq </loq </loq </td><td>10.1 14.8 13.4 6.6 10.9 3.6</td><td>24.3 38.9 34.4 <loq <loq <loq< td=""></loq<></loq </loq </td></loq<></loq </loq </loq </loq </loq </td></loq<></td></loq<></loq </loq 	0.4 1.5 2.3 0.4 0.3 0.5	6.6 9.2 7.5 6.2 5.9 3.7	150.0 307.4 309.3 40.6 42.8 <loq< td=""><td><loq <loq <loq <loq <loq <loq< td=""><td><loq <loq 0.3 <loq <loq 3.2</loq </loq </loq </loq </td><td>10.1 14.8 13.4 6.6 10.9 3.6</td><td>24.3 38.9 34.4 <loq <loq <loq< td=""></loq<></loq </loq </td></loq<></loq </loq </loq </loq </loq </td></loq<>	<loq <loq <loq <loq <loq <loq< td=""><td><loq <loq 0.3 <loq <loq 3.2</loq </loq </loq </loq </td><td>10.1 14.8 13.4 6.6 10.9 3.6</td><td>24.3 38.9 34.4 <loq <loq <loq< td=""></loq<></loq </loq </td></loq<></loq </loq </loq </loq </loq 	<loq <loq 0.3 <loq <loq 3.2</loq </loq </loq </loq 	10.1 14.8 13.4 6.6 10.9 3.6	24.3 38.9 34.4 <loq <loq <loq< td=""></loq<></loq </loq

Table 1. Elemental analysis, physical and chemical properties and contents in trace elements of the two studied soils and of the six biochars used for the incubation experiment.

MAPS_o and APS_o: original soils before incubation. <LOQ = below limit of quantification. Data are presented as mean values ± standard error.



Figure 2. Water-holding capacity in control and amended soils after 65 days of incubation in (**a**) moderately acidic polluted soils (MAPS) and in (**b**) acidic polluted soils (APS). Different letters indicate differences among treatments in each soil (p < 0.05).

As expected, the soil of the control pots revealed the lowest total carbon (TC) values (16.6 and 7.8 g kg⁻¹ for MAPS and APS, respectively) at the end of the incubation step (Table 2). After 65 days of incubation, biochar addition significantly increased the TC contents of soils proportionally to the amount of C applied with the respective biochar, whereas the low N content of the biochars is in line with their negligible impact on total nitrogen content (TN) contents (Table 2).

Table 2. Elemental analysis of control and biochar-amended soils after 65 days of incubation.

	TC (g kg ⁻¹)	TN (g kg ⁻¹)	C/N
MAPS_Control	16.6 ± 1.3 a	2.0 ± 0.7 a	8
MAPS 5% RHB400_1	34.4 ± 3.5 ^b	1.1 ± 0.4 a	32
MAPS 5% RHB500_1	41.5 ± 2.6 ^{c,d}	1.4 ± 0.1 a	29
MAPS 5% RHB500_4	34.4 ± 1.2 ^b	0.6 ± 0.2 a	53
MAPS 5% OPB400_1	45.4 ± 1.1 ^{d,e}	1.0 ± 0.4 ^a	45
MAPS 5% OPB500_1	$49.8\pm1.1~^{ m e}$	0.9 ± 0.3 a	54
MAPS 5% OPB500_4	36.9 ± 0.1 ^{b,c}	0.7 ± 0.1 a	52
MAPS 10% RHB500_1	50.3 ± 0.3 $^{ m e}$	1.8 ± 1.2 a	28
MAPS 10% RHB500_4	$67.5\pm2.7~{ m f}$	1.5 ± 0.1 a	45
MAPS 10% OPB500_4	$74.0\pm0.6~^{g}$	1.2 ± 0.0 a	62
APS_Control	7.8 ± 0.0 ^a	0.9 ± 0.4 a	8
APS 5% RHB400_1	$20.6\pm0.1~^{\rm c}$	0.8 ± 0.0 a	25

Table 2. Cont.

				_
	TC (g kg $^{-1}$)	TN (g kg $^{-1}$)	C/N	
APS 5% RHB500_1	17.3 ± 0.6 ^b	0.5 ± 0.6 a	37	
APS 5% RHB500_4	30.0 ± 2.6 ^d	1.0 ± 0.4 a	31	
APS 5% OPB400_1	41.1 ± 0.6 ^h	0.7 ± 0.3 $^{ m a}$	59	
APS 5% OPB500_1	44.9 ± 0.9 g	1.2 ± 0.1 a	36	
APS 5% OPB500_4	$34.0\pm0.4~^{\rm e}$	0.8 ± 0.1 a	41	
APS 10% RHB500_1	39.1 ± 0.2 f	0.9 ± 0.3 $^{ m a}$	43	
APS 10% RHB500_4	45.4 ± 1.2 g	0.6 ± 0.1 a	71	
APS 10% OPB500_4	$65.9\pm0.6~^{ m i}$	0.7 ± 0.2 a	94	

Data are presented as mean values \pm standard error. For each soil, values in the same column followed by the same letter are not statistically different (p < 0.05).

3.2. Effects of Biochar Addition on Enzymatic Activities

In this study, DHA activity ranged from 0 up to 8 µg INTF g⁻¹ dry soil h⁻¹, and β -glucosidase activity took values from 0 to 0.5 µmol PNP g⁻¹ dry soil h⁻¹ (Figure 3). The enzymatic activities in the studied soils were very low in comparison with no polluted soils [24]. Pérez de Mora et al. [38] and Burgos et al. [39] found similar values for DHA and higher values of β -glucosidase activity in soils polluted by the same mining accident.



Figure 3. Enzymatic activity in control and 5%-biochar-amended soils during incubation: (**a**) dehydrogenase activity in MAPS, (**b**) dehydrogenase activity in APS, (**c**) β -glucosidase activity in MAPS and (**d**) β -glucosidase activity in APS.

The DHA values obtained were 10 times higher in MAPS than in APS, which is best explained by the higher acidity and heavy metal concentration in the latter (Figures 1 and 3 and Table 1). Comparable observations were described by Frankenberger and Johanson [40], Shuler and Kargi [41], Wolińska and Stępniewska [23] and Campos et al. [42]. Irrespective of the kind and amount of added biochar, DHA increased in all MAPS after 65 days (from 4.1 up to 5.9–7.4 μ g INTF g⁻¹ dry soil h⁻¹), which may be related to declining metal availability (CaCl₂-extractable contents of Cd, Pb, Zn, Ba and Mn; Table 3). This reduction

was caused due to the amendment addition as it was also suggested by Pérez de Mora et al. [38] and Moore et al. [43]. In APS, the application of 5% of RHB500_4, RHB500_1 and OPB500_1, increased DHA at 65 days. As this parameter is only applicable for living cells [44], its increment after biochar application can be related to an improvement of the microbiological biomass of soil [45].

Table 3. Concentrations (mg kg⁻¹) of CaCl₂-extractable trace elements in soils after 65 days of incubation.

	Ba	Cđ	Cu	Mn	Ni	Pb	Zn
MAPS_Control	2.6 ± 0.5 $^{\rm c}$	0.05 ± 0.02 $^{\rm a}$	0.2 ± 0.0 ^a	$20.5\pm4.3~^{d}$	0.14 ± 0.04 ^{a,c}	0.3 ± 0.0	1.6 ± 0.4 a
MAPS 5% RHB400_1	$2.5 \pm 0.1 \ ^{ m b,c}$	$0.04 \pm 0.00^{\text{ a,b}}$	0.2 ± 0.1 a	26.9 ± 1.0 ^e	$0.20 \pm 0.05 \ ^{\rm c}$	<loq< td=""><td>1.5 ± 0.2 a</td></loq<>	1.5 ± 0.2 a
MAPS 5% RHB500_1	2.0 ± 0.2 b	$0.03 \pm 0.02 \ ^{\mathrm{a,b}}$	0.1 ± 0.1 a	13.7 ± 1.6 ^c	0.11 ± 0.01 ^{a,b}	<loq< td=""><td>0.6 ± 0.0 b,c</td></loq<>	0.6 ± 0.0 b,c
MAPS 5% RHB500_4	2.0 ± 0.1 ^b	$0.02 \pm 0.00^{\text{ a,b}}$	0.2 ± 0.0 a	$10.1 \pm 0.7 \ ^{ m b,c}$	0.10 ± 0.03 a,c	<loq< td=""><td>0.3 ± 0.1 b,c,d</td></loq<>	0.3 ± 0.1 b,c,d
MAPS 5% OPB400_1	2.2 ± 0.0 b,c	0.03 ± 0.00 ^{a,b}	0.1 ± 0.1 a	21.5 ± 0.5 ^d	$0.14 \pm 0.02 \ ^{\rm a,c}$	<loq< td=""><td>1.2 ± 0.2 a</td></loq<>	1.2 ± 0.2 a
MAPS 5% OPB500_1	1.5 ± 0.0 a	0.03 ± 0.01 ^{a,b}	0.2 ± 0.0 $^{\mathrm{a}}$	$10.4 \pm 0.1 \ ^{ m b,c}$	<loq< td=""><td><loq< td=""><td>0.5 ± 0.0 ^{b,c,d}</td></loq<></td></loq<>	<loq< td=""><td>0.5 ± 0.0 ^{b,c,d}</td></loq<>	0.5 ± 0.0 ^{b,c,d}
MAPS 5% OPB500_4	2.1 ± 0.2 b,c	0.03 ± 0.01 ^{a,b}	0.1 ± 0.1 a	12.2 ± 1.3 ^c	$0.16 \pm 0.08 \ ^{ m b,c}$	<loq< td=""><td>0.8 ± 0.1 ^b</td></loq<>	0.8 ± 0.1 ^b
MAPS 10% RHB500_1	$1.2\pm0.0~^{a}$	$0.02\pm0.01~^{\text{a,b}}$	$0.2\pm0.0~^{a}$	7.2 ± 0.1 ^{a,b}	<loq< td=""><td><loq< td=""><td>$0.2\pm0.0~^{c,d}$</td></loq<></td></loq<>	<loq< td=""><td>$0.2\pm0.0~^{c,d}$</td></loq<>	$0.2\pm0.0~^{c,d}$
MAPS 10% RHB500_4	$1.1\pm0.0~^{\rm a}$	0.01 ± 0.01 $^{\rm b}$	$0.2\pm0.0~^{a}$	3.8 ± 0.0 a	$0.06\pm0.01~^{\rm a,c}$	<loq< td=""><td>$0.1\pm0.1~^{\rm d}$</td></loq<>	$0.1\pm0.1~^{\rm d}$
MAPS 10% OPB500_4	1.4 ± 0.1 a	0.01 ± 0.01 ^b	0.1 ± 0.0 a	4.0 ± 0.2 a	$0.03 \pm 0.02^{\mathrm{~a,b}}$	<loq< td=""><td><loq< td=""></loq<></td></loq<>	<loq< td=""></loq<>
Pearson Coefficient	-0.62	-0.79	-0.18	-0.59	-0.20	n.c.	-0.63
APS_Control	0.09 ± 0.07 $^{\rm a}$	0.11 ± 0.03 $^{\rm a}$	5.1 ± 0.2 ^b	$33.9\pm0.1~^{a}$	0.63 ± 0.01 $^{\rm a}$	<loq< td=""><td>$20.2\pm0.3~^{g}$</td></loq<>	$20.2\pm0.3~^{g}$
APS 5% RHB400_1	<loq< td=""><td>0.09 ± 0.01 ^a</td><td>4.1 ± 0.1 ^{a,b}</td><td>36.2 ± 0.5 ^a</td><td>0.69 ± 0.03 ^a</td><td><loq< td=""><td>19.3 ± 0.3 $^{\mathrm{f,g}}$</td></loq<></td></loq<>	0.09 ± 0.01 ^a	4.1 ± 0.1 ^{a,b}	36.2 ± 0.5 ^a	0.69 ± 0.03 ^a	<loq< td=""><td>19.3 ± 0.3 $^{\mathrm{f,g}}$</td></loq<>	19.3 ± 0.3 $^{\mathrm{f,g}}$
APS 5% RHB500_1	<loq< td=""><td>0.09 ± 0.01 ^a</td><td>4.2 ± 0.0 ^{a,b}</td><td>32.8 ± 0.2 $^{\mathrm{a}}$</td><td>0.57 ± 0.06 ^a</td><td>0.3 ± 0.1</td><td>$17.3\pm0.1~^{\mathrm{e}}$</td></loq<>	0.09 ± 0.01 ^a	4.2 ± 0.0 ^{a,b}	32.8 ± 0.2 $^{\mathrm{a}}$	0.57 ± 0.06 ^a	0.3 ± 0.1	$17.3\pm0.1~^{\mathrm{e}}$
APS 5% RHB500_4	<loq< td=""><td>0.08 ± 0.01 a</td><td>2.6 ± 0.1 ^{a,b}</td><td>26.2 ± 0.0 a</td><td>0.54 ± 0.01 a</td><td>0.3 ± 0.1</td><td>13.3 ± 0.3 c</td></loq<>	0.08 ± 0.01 a	2.6 ± 0.1 ^{a,b}	26.2 ± 0.0 a	0.54 ± 0.01 a	0.3 ± 0.1	13.3 ± 0.3 c
APS 5% OPB400_1	<loq< td=""><td>0.06 ± 0.01 ^a</td><td>3.4 ± 0.1 ^{a,b}</td><td>27.1 ± 1.2 a</td><td>0.59 ± 0.07 $^{\mathrm{a}}$</td><td><loq< td=""><td>15.3 ± 0.3 ^d</td></loq<></td></loq<>	0.06 ± 0.01 ^a	3.4 ± 0.1 ^{a,b}	27.1 ± 1.2 a	0.59 ± 0.07 $^{\mathrm{a}}$	<loq< td=""><td>15.3 ± 0.3 ^d</td></loq<>	15.3 ± 0.3 ^d
APS 5% OPB500_1	0.06 ± 0.00 ^a	0.06 ± 0.00 ^a	3.0 ± 0.1 ^{a,b}	26.6 ± 0.3 a	0.38 ± 0.05 $^{\mathrm{a}}$	<loq< td=""><td>15.6 ± 0.3 ^d</td></loq<>	15.6 ± 0.3 ^d
APS 5% OPB500_4	<loq< td=""><td>0.08 ± 0.02 a</td><td>3.6 ± 0.0 ^{a,b}</td><td>34.6 ± 0.5 $^{\mathrm{a}}$</td><td>0.73 ± 0.06 ^a</td><td><loq< td=""><td>18.8 ± 0.3 f</td></loq<></td></loq<>	0.08 ± 0.02 a	3.6 ± 0.0 ^{a,b}	34.6 ± 0.5 $^{\mathrm{a}}$	0.73 ± 0.06 ^a	<loq< td=""><td>18.8 ± 0.3 f</td></loq<>	18.8 ± 0.3 f
APS 10% RHB500_1	<loq< td=""><td>0.08 ± 0.00 $^{\mathrm{a}}$</td><td>1.4 ± 0.1 a</td><td>33.5 ± 1.8 $^{\mathrm{a}}$</td><td>0.38 ± 0.01 $^{\mathrm{a}}$</td><td><loq< td=""><td>$12.5\pm0.6\ ^{\rm c}$</td></loq<></td></loq<>	0.08 ± 0.00 $^{\mathrm{a}}$	1.4 ± 0.1 a	33.5 ± 1.8 $^{\mathrm{a}}$	0.38 ± 0.01 $^{\mathrm{a}}$	<loq< td=""><td>$12.5\pm0.6\ ^{\rm c}$</td></loq<>	$12.5\pm0.6\ ^{\rm c}$
APS 10% RHB500_4	0.01 ± 0.00 ^a	0.05 ± 0.00 $^{\mathrm{a}}$	1.1 ± 0.0 a	26.0 ± 0.6 ^a	0.29 ± 0.04 ^a	<loq< td=""><td>9.8 ± 0.2 $^{\mathrm{a}}$</td></loq<>	9.8 ± 0.2 $^{\mathrm{a}}$
APS 10% OPB500_4	0.04 ± 0.00 ^a	0.05 ± 0.00 $^{\mathrm{a}}$	1.1 ± 0.2 a	21.2 ± 0.8 ^a	0.29 ± 0.02 ^a	<loq< td=""><td>11.4 ± 0.4 ^b</td></loq<>	11.4 ± 0.4 ^b
Pearson Coefficient	n.c.	-0.80	-0.84	-0.64	-0.53	n.c.	-0.82

Data are presented as mean values \pm standard error. Pearson coefficient between pH values and extractable trace elements. Coefficients in bold are significant (p < 0.05). <LOQ = below limit of quantification; n.c. = correlation was not calculated. For each soil, values in the same column followed by the same letter are not statistically different (p < 0.05).

After 5 days of incubation, β -glucosidase activity in control MAPS was higher than in the MAPS amended with 5% of OPB500_1, RHB400_1, OPB500_4 and OPB400_1. During incubation, β -glucosidase activity was stabilized in control MAPS. In the APS soil after 65 days of incubation, relative to the control, the β -glucosidase activity was reduced by the amendment of biochar. Similarly, Campos et al. [42] reported significant decreases in β -glucosidase activity in an acidic polluted soil after 1 month of the application of biochar amendment in a field experiment. Jain et al. [46], Günal et al. [47] and Netherway et al. [48] showed decreases, whereas Ali et al. [10] observed increments in this enzymatic activity by the application of biochar in soils. Thus, the effects of biochar application on β -glucosidase activity are still not well understood. A possible explanation of this reduction in the β glucosidase activity may be the adsorption of substrates on the biochar surface, making them less available, or even the extracellular enzyme could be adsorbed on biochar [49,50].

*3.3. Effects of Biochar Addition on the Bioavailability of Trace Elements (CaCl*₂-Extractable *Trace Elements)*

Before incubation, the Cu, Fe and Zn CaCl₂-extractable contents were greater in APS than in MAPS, whereas Ba and Sr CaCl₂-extractable contents were higher in MAPS (Table 1). The biochars produced at 500 °C were more efficient in the immobilization of Ba, Mn and Zn in MAPS and of Zn in APS at the end of the incubation experiment (Table 3). When the application dose was 10%, the biochars produced at 500 °C were also effective in the reduction of the bioavailable Cu content in APS. Finally, the bioavailable content of the toxic Pb decreased in MAPS with the amendment of all biochar samples. The CaCl₂ extractable concentrations of As and Cr were below the quantification limits (0.1 mg kg⁻¹) in all treatments. The bioavailable trace elements, here extracted with 0.01 M CaCl₂, are the most dangerous fraction for the environment, as they are likely to affect soil functions and

to interact with biological targets [51]. Their immobilization may reduce the ecological risk in the polluted areas [52,53].

Applying MANOVA indicated an overall significant effect of the biochar properties on the bioavailability of trace elements depending on soil (MAPS vs. APS), biochar feedstock, biochar dose, temperature of pyrolysis, time of pyrolysis and their interactions (Tables 4 and 5). In contrast, the interactions of Soil:Biochar feedstock, Dose:Temperature and Temperature:Time caused no significant effect. Relating these properties to the CaCl₂extractable trace element concentrations demonstrated the following:

- Soil: APS control soil showed the greatest concentrations of available Cd, Cu, Mn, Ni and Zn, whereas MAPS control soil displayed the highest concentration of available Ba (Table 5).
- Feedstock: The amount of bioavailable Zn was lower for soils amended with RHB than with OPB biochars.
- Biochar dose: The bioavailability of Cd, Cu and Zn was greater for control soils than for that amended with 5% of biochars, whereas it was greater for all elements except for Ni for control soils than for that amended with 10% of biochars. In addition, 10% of biochar amendment significantly reduced the contents of all bioavailable trace elements in comparison with 5% of amendment.
- Biochar pyrolysis temperature: Bioavailable contents of Cd, Cu and Zn were greater for control soils than for soils amended with biochars produced at 400 °C, whereas all contents, except for Ni, were greater than for soils amended with biochars produced at 500 °C. Moreover, soils amended with biochars produced at 500 °C showed lower bioavailable contents of Ba, Mn and Zn than biochars produced at 400 °C, evidencing the importance of the selection of pyrolysis conditions when the biochars are produced.
- Biochar pyrolysis residence time: Soils amended with biochars pyrolyzed for 4 h showed lower bioavailable contents of Mn and Zn than those heated for 1 h.

Ind Var	(I) Ind. Var.	(J) Ind. Var	Mean Difference (I-J)						
inu. var.			Ba	Cd	Cu	Mn	Ni	Zn	
Soil	MAPS	APS	1.7896 *	-0.0488 *	-2.6025 *	-15.2353 *	-0.3997 *	-14.6951 *	
Biochar	Control	RHB	0.4058 *	0.0375 *	1.4042 *	7.1037 *	0.0592	3.2481 *	
		OPB	0.4446 *	0.0431 *	1.2009 *	7.4839 *	0.0227	2.8114 *	
	RHB	OPB	0.0388	0.0056	-0.2033	0.3802	-0.0365	-0.4367*	
Dose	0	5	0.2640	0.0334 *	0.9925 *	5.2879	-0.0063	2.0402 *	
		10	0.7410 *	0.0530 *	1.9564 *	11.2422 *	0.1416	5.0816 *	
	5	10	0.4770 *	0.0196 *	0.9639 *	5.9543 *	0.1479 *	3.0414 *	
Temperature	0	400	0.0074	0.0322 *	1.1535 *	3.1692	-0.0100	1.4329 *	
_		500	0.5418 *	0.0421 *	1.3596 *	8.4451 *	0.0581	3.5172 *	
	400	500	0.5344 *	0.0099	0.2061	5.2759 *	0.0681	2.0843 *	
Time	0	1	0.3374 *	0.0350 *	1.1447 *	5.1480	0.0449	2.3486 *	
		4	0.5301 *	0.0461 *	1.5252 *	9.9285 *	0.0406	3.9357 *	
	1	4	0.1927	0.0111	0.3805	4.7805 *	-0.0043	1.5871 *	

Table 4. Effects of independent variables on CaCl₂-extractable trace elements (differences between means) after MANOVA analysis.

(*) p < 0.05.

It is well known that the modification of soil properties by the addition of organic amendments may affect the bioavailability of trace elements in soils [11]. For example, soil pH affects the availability of trace elements in soils. In line with the results obtained in this article, Madejón et al. [7] showed a reduction of the CaCl₂-extractable Cd, Cu and Zn due to the application of compost to severely polluted soils from the same area. Mench et al. [54] reported a significant increase in soil pH due to biochar addition, allowing the precipitation of cationic trace elements and the formation of complexes and secondary minerals. For biochars, Kim et al. [11] explained immobilization of trace elements by their high surface area offering the respective adsorption sites for available trace elements. The strong negative Pearson's coefficients relating pH with CaCl₂-extracted Ba, Cd and Zn in MAPS and Cd, Cu, Mn and Zn in APS suggested that the increase of soil pH due to biochar addition may be a fundamental reason for the decrease of the availability of metals (Table 3). A diminishing of the available Ni and Cu for MAPS and Ni for APS was not observed, possibly because the values were close to the LOD for all the treatments. Contrary to the homogeneous behavior for all the metals considered in this work, some authors have found disparity in trends in the availability of different elements. For example, Beesley et al. [55] and Ali et al. [10] observed decreases in available Zn and Cd, but increases in As, Cu and Pb. These contradictions confirm the necessity of a complete characterization of biochars, as the selected pyrolysis conditions and feedstock may affect trace elements adsorption capacities.

	Pillai's Trace	F	p
Soil	1.000	11823.219	0.000
Biochar	0.619	4.057	0.013
Dose	0.995	484.564	0.000
Temperature	0.921	29.270	0.000
Time	0.758	7.822	0.001
Soil:Biochar	0.194	0.603	0.724
Soil:Dose	0.997	795.507	0.000
Soil:Temp	0.952	49.618	0.000
Soil:Time	0.811	10.754	0.000
Biochar:Dose	0.823	11.656	0.000
Biochar:Temp	0.616	4.007	0.014
Biochar:Time	0.963	64.786	0.000
Dose:Temp	0.000	n.c.	n.c.
Dose:Time	0.904	23.458	0.000
Temp:Time	0.000	n.c.	n.c.

Table 5. MANOVA results for variables affecting CaCl₂-extractable trace elements.

Bold text indicates significant factors (p < 0.05). n.c. = no calculated.

3.4. Impact of Biochar Addition on the Concentrations of Trace Element Species in Soils

In order to assess the effectiveness of the amendments in the soil restoration, the sequential extraction procedure was used, as it indicates the fractionation of trace elements in soils. Thus, the reduction of the labile content of the toxic elements that have major risk is pretended by biochar application, trying to make them more stable in the soil matrix. The distribution of trace elements in the different fractions varied between the studied soils (Figure 4). At day 5, approximately 10% of Ba was found in the acid extractable fraction (F1) in all MAPS, irrespective of the treatments, whereas this element was below detection limit in the acid extractable (F1) and oxidizable (F3) fractions in all APS treatments. The acid extractable fraction (F1) is the most dangerous, indicating that MAPS had a higher risk, with the mobile Ba content, than APS. Nevertheless, at day 5, the Cu content in the acid extractable fraction (F1) in APS was higher than in MAPS, leading to a major risk.

Five days after the beginning of the incubation, the application of biochar effectively reduced the Ni content in the F1 and F2 fractions in APS concomitantly with the increase of this element in F3. After 65 days of incubation, Cd content in the residual fraction (F4) increased with the application of 10% biochars, with the corresponding reduction in the less stable fractions. In these cases, biochar applications enhanced the reduction of risk in polluted soil by the decreases in trace elements mobility.

Distribution of trace elements in both soils was modified during the incubation period. For example, at day 5, 70% of Cu was presented in the residual phase (F4) and 25 and 20% in the oxidizable phase (F3) for MAPS and APS, respectively. The F3 fraction was related to metals associated to the soil organic matter. After 65 days of incubation, 65–70% of Cu was still located in the residual fraction (F4), and the rest was more closely related to F2 and F1. The total amount of trace elements in the studied soils were extremely high, as they were polluted by the toxic spill from the mine located in the Iberian Pyrite Belt. Although



biochar can adsorb and immobilize the available trace elements after a short time, a mobile fraction of these trace elements will appear again.

Figure 4. Fractions of heavy metals extracted from soils in each step of sequential extraction: (**a**) Ba after 5 days of incubation, (**b**) Cd after 5 days of incubation, (**c**) Cu after 5 days of incubation, (**d**) Ni after 5 days of incubation, (**e**) Zn after 5 days of incubation, (**f**) Ba after 65 days of incubation, (**g**) Cd after 65 days of incubation, (**h**) Cu after 65 days of incubation, (**i**) Ni after 65 days of incubation and (**j**) Zn after 65 days of incubation.

3.5. Effects of Biochar Addition on the Germination and Development of Brassica rapa pekinensis

Brassica rapa ssp. *pekinensis* plants were not able to germinate in the APS soil (Table S1). The control APS was very acidic and had a high content of trace elements, which did not allow plant germination on its own. The application of RHB500_4 (5 and 10%), RHB500_1 (10%) and OPB500_1 (2%) enabled plant germination in the acidic APS soil. The amendment of 10% of RHB400_4 allowed the survival of plants until the end of the experiment. Nevertheless, in the rest of the treatments, plants did not survive. Germination rates of *Brassica rapa* ssp. *pekinensis* plants were not affected by biochar application in MAPS pots.

The productivity of *Brassica rapa* plants was assessed as the dry weight of aerial biomass (stem + leaves) and roots for MAPS pots (Figure 5). Plant biomass productivity was enhanced with the application of 5% and 10% of RHB500_1. Similarly, root development significantly increased after the addition of 5% of RHB400_1, 5% of RHB500_4, 10% of RHB500_1 and 10% of RHB500_4 for MAPS soils. In this study, the root-to-shoot ratio of Brassica rapa increased by biochar application (from 0.26 to 0.33–1.13). This ratio has been used previously for determining the enhancement of plant growth in polluted soil, as the ratio is related to the availability of nutrients [56]. Paneque et al. [57] also reported an increase of this ratio after field application of biochars at rates of 5 and 25 t ha⁻¹. Taking into account the key role of plant roots in nutrient uptake, its development would allow a greater absorption of nutrients and water. Similar to germination rates, biochar application did not modify the shoot height (Figure S1).



Figure 5. Effects of biochar amendment on dry weight of aerial biomass, dry weight of roots and root-to-shoot ratio at day 65. R/S = Root-to-shoot ratio.

The improved germination rates, plant survival and plant growth by biochar amendment may be explained by several factors. Firstly, biochar amendment improved properties of the degraded soils, as it increased pH and WHC (Figures 1 and 2). Secondly, the application of biochar effectively reduced the bioavailable concentrations of toxic trace elements (Table 3). Some elements, such as Cu, are essential for plant growth in low concentrations, but in the ranges found in these contaminated soils, they are phytotoxic [58]. Finally, avoiding water loss in soil by biochar application may enhance plant survival in arid and semiarid regions. In general, APS soils showed higher water losses than MAPS (Table S2), which was best explained by the high bulk density (1.18 g mL⁻¹ for MAPS and 1.09 g mL⁻¹

15 of 18

for APS) and soil organic matter content of the former. Biochar addition considerably increased the soil water retention of APS soils proportionally to the applied dose. This was greater with RHB than with OPB, which is in accordance with their corresponding WHC (Table 1). In the case of MAPS, no significant effect on water loss was attributable to biochar addition.

4. Conclusions

This study showed that the application of high doses (5–10%) of biochar produced from agricultural residues on contaminated soils increased pH, water holding capacity and organic carbon content and reduced trace elements bioavailability. Biochar dose affected the bioavailability of trace elements. In addition, feedstock and pyrolysis conditions were strongly determinant for the effectiveness in the remediation of polluted soils. The application of biochar to acid-polluted soils may be the first step of the remediation process, as in soils with such a high content of trace elements, there constantly will be an input to the bioavailability from the total concentrations. Biochar application enhanced the germination of *Brassica rapa pekinensis* in APS, where the germination in un-amended soil was not possible. Thus, after the application of biochar, the highly polluted soil may have the required conditions for allowing the plant germination and growth to start the phytoremediation process. In light of the positive effects observed on soil properties and on *Brassica rapa pekinensis* growth, the application of 10% of RHB produced at 500 °C to contaminated acid soils has been shown to be a useful tool for the recovery of the functionality of these soils and for the protection of ecosystem services.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10 .3390/agronomy11071394/s1, Figure S1. Shoot height measured for un-amended, (a) RHB-amended MAPS soils and (b) OPB-amended MAPS soils after 4, 8, 19, 30 and 65 days; Table S1: Germination and plant survival (%) in control and biochar-amended soils during the pot experiment; Table S2: Water loss (%) in control and biochar-amended pots determined every three days.

Author Contributions: Conceptualization, J.M.D.I.R. and P.C.; methodology, P.C., R.L. and J.M.D.I.R.; formal analysis, P.C.; investigation, P.C.; resources, J.M.D.I.R. and R.L.; data curation, P.C.; writing—original draft preparation, P.C.; writing—review and editing, H.K., R.L. and J.M.D.I.R.; visualization, J.M.D.I.R. and P.C; supervision, J.M.D.I.R. and H.K.; project administration, J.M.D.I.R.; funding acquisition, J.M.D.I.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by The Spanish Ministry of Economy, Industry and Competitiveness (MINEICO) and AEI/FEDER [project CGL2016-76498-R]; MINEICO is also thanked for funding the "Ramón y Cajal" post-doctoral contract of José M. De la Rosa (ref: RYC2014-16338). P. Campos thanks the "Fundación Tatiana Pérez de Guzmán el Bueno" for funding her PhD. CSIC is thanked for funding the project 2020AEP095.

Institutional Review Board Statement: Not Applicable.

Informed Consent Statement: Not Applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: Orivarzea S.A. and Cooperativa Nuestra Señora de los Ángeles are thanked for providing the feedstock used for biochars production. Desiré Monis and Alba Carmona are thanked for technical support with elemental analysis.

Conflicts of Interest: The authors declare no conflict of interest.

References

- 1. EEA. Progress in Management of Contaminated Sites; CSI 015; European Environmental Agency: Copenhagen, Denmark, 2007.
- Hmid, A.; Chami, Z.A.; Sillen, W.; Vocht, A.D.; Vangronsveld, J. Olive mill waste biochar: A promising soil amendment for metal immobilization in contaminated soils. *Environ. Sci. Pollut. Res.* 2015, 22, 1444–1456. [CrossRef]
- Xian, Y.; Wang, M.; Chen, W. Quantitative assessment on soil enzyme activities of heavy metal contaminated soils with various soil properties. *Chemosphere* 2015, 139, 604–608. [CrossRef]

- Sungur, A.; Soylak, M.; Ozcan, H. Investigation of Heavy Metal Mobility and Availability by the BCR Sequential Extraction Procedure: Relationship between Soil Properties and Heavy Metals Availability. *Chem. Speciat. Bioavailab.* 2014, 26, 219–230. [CrossRef]
- Sharma, S.; Tiwari, S.; Hasan, A.; Saxena, V.; Pandey, L.M. Recent advances in conventional and contemporary methods for remediation of heavy metal-contaminated soils. *Biotechnology* 2018, 8, 1–18. [CrossRef] [PubMed]
- 6. Sales da Silva, I.G.; Gomes de Almeida, F.C.; Padilha da Rocha e Silva, N.M.; Casazza, A.A.; Converti, A.; Asfora Sarubbo, L. Soil Bioremediation: Overview of Technologies and Trends. *Energies* **2020**, *13*, 4664. [CrossRef]
- Madejón, P.; Domínguez, M.T.; Gil-Martínez, M.; Navarro-Fernández, C.M.; Montiel-Rozas, M.M.; Madejón, E.; Murillo, J.M.; Cabrera, F.; Marañón, T. Evaluation of amendment addition and tree planting as measures to remediate contaminated soils: The Guadiamar case study (SW Spain). *Catena* 2018, *166*, 34–43. [CrossRef]
- 8. Woolf, D.; Amonette, J.; Street-Perrott, F.; Street-Perrott, F.A.; Lehmann, J.; Joseph, S. Sustainable biochar to mitigate global climate change. *Nat. Commun.* **2010**, *1*, 1–9. [CrossRef] [PubMed]
- 9. Spokas, K.A. Review of the stability of biochar in soils: Predictability of O:C molar ratios. *Carbon Manag.* **2010**, *1*, 289–303. [CrossRef]
- Ali, A.; Guo, D.; Zhang, Y.; Sun, X.; Jiang, S.; Guo, Z.; Huang, H.; Liang, W.; Li, R.; Zhang, Z. Using bamboo biochar with compost for the stabilization and phytotoxicity reduction of heavy metals in mine-contaminated soils of China. *Sci. Rep.* 2017, 7, 1–12. [CrossRef] [PubMed]
- 11. Kim, H.-S.; Kim, K.R.; Kim, H.-J.; Yoon, J.-H.; Yang, J.E.; Ok, Y.S.; Owens, G.; Kim, K.-H. Effect of biochar on heavy metal immobilization and uptake by lettuce (*Lactuca sativa L.*) in agricultural soil. *Environ. Earth Sci.* 2015, 74, 1249–1259. [CrossRef]
- Yu, H.; Zou, W.; Chen, J.; Chen, H.; Yu, Z.; Huang, J.; Tang, H.; Wei, X.; Gao, B. Biochar amendment improves crop production in problem soils: A review. J. Environ. Manag. 2019, 232, 8–21. [CrossRef] [PubMed]
- Shaaban, M.; Van Zwieten, L.; Bashir, S.; Younas, A.; Núñez-Delgado, A.; Chhajro, M.A.; Kubar, K.A.; Ali, U.; Rana, M.S.; Mehmood, M.A.; et al. A concise review of biochar application to agricultural soils to improve soil conditions and fight pollution. *J. Environ. Manag.* 2018, 228, 429–440. [CrossRef] [PubMed]
- 14. Conte, P.; Bertani, R.; Sgarbossa, P.; Bambina, P.; Schmidt, H.-P.; Raga, R.; Lo Papa, G.; Chillura Martino, D.F.; Lo Meo, P. Recent Developments in Understanding Biochar's Physical–Chemistry. *Agronomy* **2021**, *11*, 615. [CrossRef]
- 15. Li, H.; Dong, X.; da Silva, E.B.; de Oliveira, L.M.; Chen, Y.; Ma, L.Q. Mechanisms of metal sorption by biochars: Biochars characteristics and modifications. *Chemosphere* **2017**, *178*, 466–478. [CrossRef]
- 16. Zhao, J.; Shen, X.-J.; Domene, X.; Alcañiz, J.-M.; Liao, X.; Palet, C. Comparison of biochars derived from different types of feedstock and their potential for heavy metal removal in multiple-metal solutions. *Sci. Rep.* **2019**, *9*, 1–12. [CrossRef]
- 17. González, I.; Galán, E.; Romero, A. Assessing Soil Quality in Areas Affected by Sulphide Mining. Application to Soils in the Iberian Pyrite Belt (SW Spain). *Minerals* **2011**, *1*, 73–108. [CrossRef]
- Xiong, Z.-T.; Li, Y.-H.; Xu, B. Nutrition Influence on Copper Accumulation by *Brassica pekinensis* Rupr. *Ecotoxicol. Environ. Saf.* 2002, 53, 200–205. [CrossRef]
- Zhang, S.; Song, J.; Gao, H.; Zhang, Q.; Lv, M.-C.; Wang, S.; Liu, G.; Pan, Y.-Y.; Christie, P.; Sun, W. Improving prediction of metal uptake by Chinese cabbage (*Brassica pekinensis L.*) based on a soil-plant stepwise analysis. *Sci. Total Environ.* 2016, 569–570, 1595–1605. [CrossRef]
- 20. Ou, J.; Li, H.; Yan, Z.; Zhou, Y.; Bai, L.; Zhang, C.; Wang, X.; Chen, G. In situ immobilisation of toxic metals in soil using Maifan stone and illite/smectite clay. *Sci. Rep.* **2018**, *8*, 1–9. [CrossRef] [PubMed]
- 21. Larkcom, J. Oriental Vegetables: The Complete Guide for Garden and Kitchen; John Murray Ltd.: London, UK, 1991.
- 22. Tang, J.; Zhang, J.; Ren, L.; Zhou, Y.; Gao, J.; Luo, L.; Yang, Y.; Peng, Q.; Huang, H.; Chen, A. Diagnosis of soil contamination using microbiological indices: A review on heavy metal pollution. *J. Environ. Manag.* 2019, 242, 121–130. [CrossRef] [PubMed]
- 23. Wolińska, A.; Stępniewska, Z. *Dehydrogenase Activity in the Soil Environment*; Dehydrogenases, Chapter 8; Canuto, R.A., Ed.; Intechopen Limited: London, UK, 2012. [CrossRef]
- Panettieri, M.; Knicker, H.; Murillo, J.M.; Madejón, E.; Hatcher, P.G. Soil organic matter degradation in an agricultural chronosequence under different tillage regimes evaluated by organic matter pools, enzymatic activities and CPMAS ¹³C NMR. Soil Biol. Biochem. 2014, 78, 170–181. [CrossRef]
- Acosta-Martínez, V.; Klose, S. Soil Enzymes. In *Encyclopedia of Soil Science*, 2nd ed.; Taylor and Francis: New York, NY, USA, 2008; pp. 1–5. [CrossRef]
- 26. CMA. Memorias de Actuaciones. Report by the Consejería de Medio Ambiente, Government of Andalucía, Spain. 1999. Available online: http://www.cma.junta-andalucia.es/guadiamar/indguadiamar.html (accessed on 10 March 2020).
- Van Geen, A.; Adkins, J.F.; Boyle, E.A.; Nelson, C.H.; Palanques, A. A 120-yr record of widespread contamination from mining of the Iberian pyrite belt. *Geology* 1997, 25, 291–294. [CrossRef]
- IUSS Working Group WRB. World Reference Base for Soil Resources 2014, Update 2015. In International Soil Classification System for Naming Soils and Creating Legends for Soil Maps; World Soil Resources Reports, 106; FAO: Rome, Italy, 2015; pp. 1–203.
- 29. Campos, P.; De la Rosa, J.M. Assessing the Effects of Biochar on the Immobilization of Trace Elements and Plant Development in a Naturally Contaminated Soil. *Sustainability* **2020**, *12*, 6025. [CrossRef]

- De la Rosa, J.M.; Sánchez-Martín, A.M.; Campos, P.; Miller, A.Z. Effect of pyrolysis conditions on the total contents of polycyclic aromatic hydrocarbons in biochars produced from organic residues: Assessment of their hazard potential. *Sci. Total Environ.* 2019, 667, 578–585. [CrossRef]
- 31. Veihmeyer, F.J.; Hendrickson, A.H. The moisture equivalent as a measure of the field capacity of soils. *Soil Sci.* **1931**, *32*, 181–193. [CrossRef]
- 32. Campos, P.; Miller, A.Z.; Knicker, H.; Costa-Pereira, M.F.; Merino, A.; De la Rosa, J.M. Chemical, physical and morphological properties of biochars produced from agricultural residues: Implications for their use as soil amendment. *J. Waste Manag.* **2020**, 105, 256–267. [CrossRef] [PubMed]
- Trevors, J.T. Dehydrogenase activity in soil: A comparison between the INT and TTC assay. Soil Biol. Biochem. 1984, 16, 673–674.
 [CrossRef]
- Tabatabai, M.A. Soil enzymes. In Method of Soil Analysis, Part 2. Chemical and Microbiological Properties; Page, A.L., Miller, R.H., Keeney, D.R., Eds.; American Society of Agronomy: Madison, WI, USA, 1982; pp. 903–948.
- 35. Houba, V.J.G.; Temminghoff, E.J.M.; Gaikhorst, G.A.; van Vark, W. Soil analysis procedures using 0.01 M calcium chloride as extraction reagent. *Commun. Soil Sci. Plant Anal.* 2000, *31*, 1299–1396. [CrossRef]
- 36. Pueyo, M.; Rauret, G.; Lück, D.; Yli-Halla, M.; Muntau, H.; Quevauviller, P.; López-Sánchez, J.F. The certification of the extractable contents (mass fractions) of Cd, Cr, Cu, Ni, Pb and Zn in freshwater sediment following a sequential extraction procedure. *J. Environ. Monit.* **2001**, *3*, 243–250. [CrossRef]
- 37. Joint Research Centre; European Commission. Available online: https://ec.europa.eu/jrc/en (accessed on 25 April 2020).
- Pérez de Mora, A.; Burgos, P.; Madejón, E.; Cabrera, F.; Jaeckel, P.; Schloter, M. Microbial community structure and function in a soil contaminated by heavy metals: Effects of plant growth and different amendments. *Soil Biol. Biochem.* 2006, *38*, 327–341. [CrossRef]
- Burgos, P.; Madejón, P.; Cabrera, F.; Madejón, E. By-products as amendment to improve biochemical properties of trace element contaminated soils: Effects in time. *Int. Biodeterior. Biodegrad.* 2010, 64, 481–488. [CrossRef]
- 40. Frankenberger, W.; Johanson, J. Effect of pH On Enzyme Stability in Soils. Soil Biol. Biochem. 1982, 14, 433–437. [CrossRef]
- 41. Shuler, M.; Kargi, F. Bioprocess Engineering Basic Concepts; Prentice-Hall Incorporation: Englewood Cliffs, NY, USA, 2010.
- 42. Campos, P.; Miller, A.Z.; Prats, S.A.; Knicker, H.; Hagemann, N.; De la Rosa, J.M. Biochar amendment increases bacterial diversity and vegetation cover in trace element-polluted soils: A long-term field experiment. *Soil Biol. Biochem.* 2020, 150, 1–11. [CrossRef]
- Moore, F.; González, M.-E.; Khan, N.; Curaqueo, G.; Sanchez-Monedero, M.; Rilling, J.; Morales, E.; Panichini, M.; Mutis, A.; Jorquera, M.; et al. Copper immobilization by biochar and microbial community abundance in metal-contaminated soils. *Sci. Total Environ.* 2018, 616–617, 960–969. [CrossRef] [PubMed]
- 44. Furtak, K.; Gałązka, A. Enzymatic activity as a popular parameter used to determine the quality of the soil environment. *Pol. J. Agron.* **2019**, *37*, 22–30. [CrossRef]
- 45. Pérez de Mora, A.; Ortega-Calvo, J.J.; Cabrera, F.; Madejón, E. Changes in enzyme activities and microbial biomass after "in situ" remediation of a heavy metal-contaminated soil. *Appl. Soil Ecol.* **2005**, *28*, 125–137. [CrossRef]
- 46. Jain, S.; Mishra, D.; Khare, P.; Yadav, V.; Deshmukh, Y.; Meena, A. Impact of biochar amendment on enzymatic resilience properties of mine spoils. *Sci. Total Environ.* **2016**, *544*, 410–421. [CrossRef]
- Günal, E.; Erdem, H.; Demirbaş, A. Effects of three biochar types on activity of β-glucosidase enzyme in two agricultural soils of different textures. *Arch. Agron. Soil Sci.* 2018, 64, 1963–1974. [CrossRef]
- 48. Netherway, P.; Gascó, G.; Méndez, A.; Surapaneni, A.; Reichman, S.; Shah, K.; Paz-Ferreiro, J. Using Phosphorus-Rich Biochars to Remediate Lead-Contaminated Soil: Influence on Soil Enzymes and Extractable P. *Agronomy* **2020**, *10*, 454. [CrossRef]
- Foster, E.J.; Fogle, E.J.; Cotrufo, M.F. Sorption to Biochar Impacts β-Glucosidase and Phosphatase Enzyme Activities. *Agriculture* 2018, *8*, 158. [CrossRef]
- 50. Lammirato, C.; Miltner, A.; Kaestner, M. Effects of wood char and activated carbon on the hydrolysis of cellobiose by β-glucosidase from *Aspergillus niger*. *Soil Biol. Biochem.* **2011**, *43*, 1936–1942. [CrossRef]
- Vangronsveld, J.; Cunningham, S.D. Introduction to the concepts. In Metal Contaminated Soils: In-situ Inactivation and Phytorestoration; Vangronsveld, J., Cunningham, S.D., Eds.; Springer: Berlin, Germany, 1998; pp. 219–225.
- 52. Adriano, D.C. Trace Elements in Terrestrial Environments: Biogeochemistry, Bioavailability and Risks of Metals, 2nd ed.; Springer: New York, NY, USA, 2001; p. 867.
- 53. Kabata-Pendias, A. Soil-plant transfer of heavy metals—An environmental issue. Geoderma 2004, 122, 143–149. [CrossRef]
- 54. Mench, M.; Vangronsveld, J.; Didier, V.; Clijsters, H. Evaluation of metal mobility, plant availability and immobilization by chemical agents in a limed-silty soil. *Environ. Pollut.* **1994**, *86*, 279–286. [CrossRef]
- Beesley, L.; Moreno-Jiménez, E.; Gomez-Eyles, J.L. Effects of biochar and greenwaste compost amendments on mobility, bioavailability and toxicity of inorganic and organic contaminants in a multi-element polluted soil. *Environ. Pollut.* 2010, 158, 2282–2287. [CrossRef] [PubMed]
- 56. Mašková, T.; Herben, T. Root:shoot ratio in developing seedlings: How seedlings change their allocation in response to seed mass and ambient nutrient supply. *Ecol. Evol.* **2018**, *8*, 7143–7150. [CrossRef] [PubMed]

- 57. Paneque, M.; Knicker, H.; Kern, J.; De la Rosa, J.M. Hydrothermal Carbonization and Pyrolysis of Sewage Sludge: Effects on *Lolium perenne* Germination and Growth. *Agronomy* **2019**, *9*, 363. [CrossRef]
- 58. Borkert, C.M.; Cox, F.R.; Tucker, M.R. Zinc and copper toxicity in peanut, soybean, rice, and corn in soil mixtures. *Commun. Soil Sci. Plant Anal.* **1998**, *29*, 2991–3005. [CrossRef]