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IMPACT OF *EISENIA FETIDA* EARTHWORMS AND BIOCHAR ON POTENTIALLY TOXIC ELEMENT MOBILITY AND HEALTH OF A CONTAMINATED SOIL

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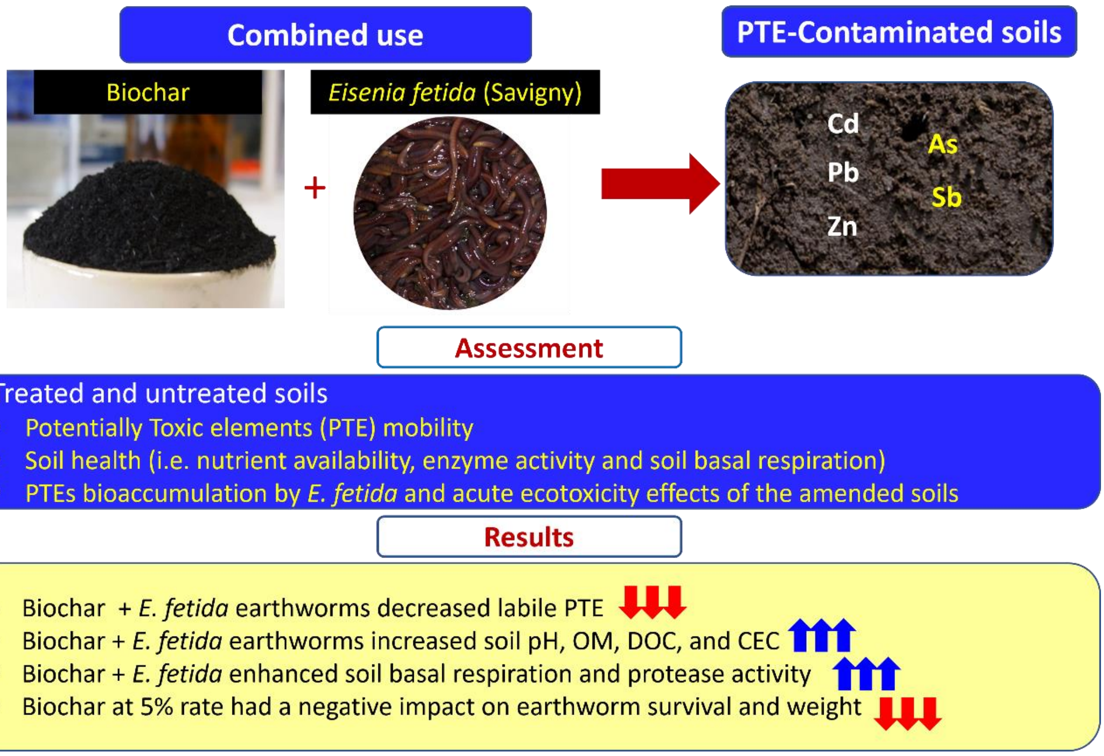
Abstract

This study aimed to evaluate the influence of *Eisenia fetida* (Savigny), added to an acidic soil contaminated with potentially toxic elements (PTEs; As, Sb, Cd, Pb, Zn) and amended with a softwood-derived biochar (2 and 5% w/w), on the mobility of PTEs and soil health (i.e. nutrient availability, enzyme activity and soil basal respiration). The PTEs bioaccumulation by *E. fetida* and the acute ecotoxicity effects of the amended soils were also evaluated. The interaction between earthworms and biochar led to a significant increase in soil pH, organic matter, dissolved organic carbon content, cation exchange capacity, and exchangeable Ca compared to the untreated soil. Moreover, the water-soluble and readily exchangeable PTE fraction decreased (with the exception of Sb) between 1.2- and 3.0-fold in the presence of biochar and earthworms. Earthworms, biochar, and their combination, led to a reduction of phosphomonoesterase activity which in soils amended with biochar and earthworms decreased between 2.2- and 2.5-

fold with respect to the untreated soil. On the other hand, biochar and earthworms also enhanced soil basal respiration and protease activity. Although the survival rate and the weight loss of *E. fetida* did not change significantly with the addition of 2% biochar, adding the highest biochar percentage (5%) resulted in a survival rate that was ~2-fold lower and a weight loss that was 2.5-fold higher than the other treatments. The PTE bioaccumulation factors for *E. fetida*, which were less than 1 for all elements (except Cd), followed the order Cd>As>Zn>Cu>Pb>Sb and were further decreased by biochar addition. Overall, these results highlight that *E. fetida* and biochar, especially at 2% rate, could be used for the restoration of soil functionality in PTE-polluted environments, reducing at the same time the environmental risks posed by PTEs, at least in the short time.

Keywords: Soil Restoration; PTE; Organic Amendment; Enzyme Activities; Soil macrofauna



1. Introduction

Mining activities represent a major source of pollution to the environment, since they cause an anthropogenic alteration to the natural biogeochemical cycling of potentially toxic elements (PTEs; e.g. Cd, Cu, Pb, Zn, As, and Sb) (Gu, 2018). In particular, the closure of mines, which often took place without adequate controls and safety interventions, leaves high quantities of mine wastes and tailings, containing significant concentrations of PTEs (Boussen et al., 2013). PTEs can affect the biochemical functioning of soil and plant growth, alter the composition of waters and sediments, and enter the food chain (Garau et al., 2014, 2017). Therefore, the recovery of abandoned mining sites and neighbouring areas affected by contamination is necessary through appropriate interventions that can limit the possible risks associated with PTE pollution (Trifi et al., 2019). In situ chemical stabilization, a technique that involves the addition to the contaminated soil of inorganic and/or organic materials capable of stabilizing contaminants through reactions of adsorption, immobilization and/or precipitation, could represent an appropriate technique to remediate contaminated mine soils (Manzano et al., 2016). The sorbent materials used for the *in-situ* stabilization of PTE-polluted soils, in order to be economically and environmentally sustainable, should be readily available, contain no substances toxic to the environment, and derive from municipal or industrial waste re-cycling (Yu et al., 2020). Examples include composts, Fe-rich by-products, red muds, and biochar (Garau et al., 2017; Lu et al., 2017; Niroshika et al., 2020).

The use of biochar, a carbonaceous material originating from the pyrolysis of organic wastes in low-oxygen conditions, as a soil amendment to remediate PTEs contaminated soils is of particular interest. However, a limiting factor to its widespread adoption is the high cost of production due to the use of pyrolytic furnaces, whose functioning depend on external energy supply, though pyrolysis units can be used to produce biochar and at the same time to create energy (Peters et al. 2015; Kung and Mu 2019). Moreover, considering also the costs related to transport and distribution into the soil, Aguirre et al. (2021) estimated that biochar application costs approximately 190 euros per ton.

The chemical and physical properties of biochar depend on the feedstock used, the pyrolysis process applied, the temperature, and the heating rate (Cai et al., 2021; Subedi et al., 2017). In particular, high pyrolysis temperatures (>550 °C) may lead to biochars with high pH, high cation exchange capacity (CEC), high specific surface area, high porosity and aromaticity, and low polarity (Sizmur et al., 2017; Subedi et al., 2017). Increasing pyrolysis temperatures also decreases O and H contents, as a consequence of the loss of surface functional groups (Sizmur et al., 2017). Irrespective of the beneficial effects that this amendment can explicate on soil fertility and properties (e.g. increasing organic carbon, available nutrient content and water retention capacity), biochar could effectively reduce the mobility and toxicity of PTEs in soils (Lehmann, 2007; Manzano et al., 2020). The effectiveness of biochar as an immobilizing agent is governed by the type of biochar (i.e. feedstock and pyrolysis conditions), as well as soil properties, PTE type, speciation, and concentration (Shaheen et al., 2019). Several studies showed that biochars applied to alkaline and acidic soils and mine tailings significantly reduced PTEs mobility (Abou Jaoude et al., 2020; Gu et al., 2020; Manzano et al., 2020). This effect could be ascribed to different processes and reactions induced by biochar addition, such as precipitation, specific and non-specific adsorption on biochar surface, and adsorption on soil surfaces as a result of pH changes. Cationic PTEs can be precipitated as metal-phosphates and carbonates and surface complexation can occur due to the presence of amorphous Fe (hydr)oxides (particularly relevant for anionic PTEs) and carboxylic and phenolic functional groups (particularly relevant for anionic PTEs), while the presence of aromatic structures in biochar can lead to physical adsorption of cationic PTEs by cation-π interactions (Abou Jaoude et al., 2020; Lehmann and Joseph, 2017; Lu et al., 2017; Sizmur et al., 2016). In addition, biochar may modify the content of dissolved organic carbon (DOC) in soil, by increasing or decreasing it, and this could influence the formation of soluble PTEs-organic complexes in soil (Abou Jaoude et al., 2020; Manzano et al., 2020).

However, carbonaceous amendments such as biochar, could have a detrimental effect on the survival and growth of soil macrofauna (i.e. earthworms) that play a key role in soil functioning (Liesch et al., 2010). Biochar can create a stressful environment to earthworms, due to the presence of toxic substances such as ammonia gas (especially from biochars rich in nitrogen) or polycyclic aromatic hydrocarbons (Liesch et al., 2010). However, some authors have pointed out that the content of bioavailable PAHs in biochar is low (e.g. <200 ng L^{-1}), because of the very strong bonds formed between biochar and PAHs generated during pyrolysis (Godlewska et al. 2021). Li et al. (2011) and Huang et al. (2020) both observed that biochar inhibited earthworm growth and antioxidant enzyme activities when applied to PTEs contaminated soil. However, Sanchez-Hernandez et al. (2019a) showed that, although some earthworms (e.g. *Lumbricus terrestris)* did not appreciate high biochar concentration in soil (5% w/w), they stimulated some physiological mechanisms, such as antioxidant defences, to tolerate biochar.

The importance of earthworms in soil functioning has been widely documented (e.g. Ramadass et al., 2017). Depending on their feeding habits (detritivorous or geophagous), earthworms are considered as essential part of the soil fauna, since they affect soil aeration and degradation of organic matter, ensuring better availability of nutrients to plants and influencing the activity of microorganisms (Ramadass et al., 2017). Earthworms can increase PTE mobility and influence their speciation. For example, earthworms promote the degradation of organic matter, releasing low-molecular weight organic acids that decrease soil pH and mobilise PTEs (Gomez-eyles et al., 2011; Huang et al., 2020; Sizmur et al., 2011a, 2011b). Nevertheless, PTE mobilization by earthworms is less evident in amended soils or in soils with an organic matter content higher than 2% (Sizmur and Richardson, 2020). Beesley and Dickinson (2011) observed that anecic earthworms (i.e. *L. terrestris*) reduced DOC in a PTEs contaminated soil amended with green waste compost, resulting in a reduction of As, Cu, Pb and Zn solubility. Furthermore, the interaction between epigeic (i.e. *E. fetida*) and aneic (i.e. *L. terrestris*) earthworms and biochar (added at $<5\%$ rate) increased soil extracellular enzyme activities, progressing the functional restoration of PTE-contaminated soils (Sanchez-Hernandez et al., 2019a; Xiao et al., 2021).

Therefore, considering the key role of earthworms in the soil ecosystem and given the possible synergistic (and sometimes antagonistic) interactions between biochar and earthworms (Sizmur et al., 2011b), the combined addition of biochar and earthworms could benefit the reclamation of PTEs contaminated soils. In any case, the great variability of biochar properties, the complexity of the interactions occurring between amendments and earthworms, and the contrasting results often found in the literature, suggest a necessity for further studies to better understand the effect of earthworms on biochar-amended PTE-contaminated soil. The aim of this work was therefore to investigate: i) the influence of the earthworm *E. fetida*, softwood biochar, and their combination on the mobility of PTEs (i.e. As, Cd, Pb, Sb and Zn) in a contaminated soil, ii) the influence of *E. fetida*, biochar, and their combination on the health (nutrient availability, enzyme activity and microbial respiration) of the same soil, and iii) whether biochar decreases PTE bioaccumulation by earthworms and PTE-derived toxicity.

2. Materials and methods

2.1. Soil sampling and experimental set-up

Soil samples were collected in proximity to an ex-mining dump located in Southwestern Sardinia (Italy, N 39°40′29.71″; E 8°37′17.97″, Montevecchio-Levante), where galena (PbS) and sphalerite (ZnS) were the main ores extracted (Ciccu et al., 2003; Wanty et al., 2013). The area is characterized by the presence of mine tailings containing high concentrations of PTEs (including As, Cd, Cu, Pb, Sb and Zn; Garau et al., 2019, 2020). Topsoil (upper 30 cm) was randomly collected from an area which extends for about 2 ha, mixed, air-dried, and sieved to < 2 mm. The soil was a sandy clay loam (USDA classification) with a bulk density of 1.32 g cm^{-3}, it had an acidic pH (6.01), a substantial content of organic matter (OM, 3.60%), macro and micro nutrients, P in particular (22.25 mg kg^{-1} of available P), and a high cation exchange capacity (CEC, 22.78 $cmol_{(+)} \cdot kg^{-1}$) (Table 1). The total concentration of As, Cd, Cu, Pb, Sb and Zn exceeded the threshold levels established by the Italian legislation (Dlgs. 152/2006) and Finnish legislation (Government Decree on the Assessment of Soil Contamination and Remediation, Needs 214/2007), which represents a satisfying approximation of the mean values of different European countries (Tóth et al., 2016).

Table 1 Characteristics of the unamended (C) and amended soils (B2 and B5), treated (+E) and untreated (-E) with *Eisenia fetida*.

	C	C+E	B2	B2+E	B5	B5+E
pH	6.01±0.04^{a*}	5.95±0.01	6.35±0.01^{b}	6.33±0.03	6.56±0.02^{c}	6.58±0.01
EC (μS cm^{-1})	386.5±3.54^{c*}	401.5±4.95	303.0±12.73^{b*}	320.5±9.19	267.5±6.36^{a*}	296.5±17.68
Total organic matter (%)	3.60±0.11^{a}	3.71±0.09	5.40±0.08^{b}	5.21±0.16	7.78±0.25^{c}	8.08±0.23
Total N (%)	0.16±0.01^{a}	0.18±0.01	0.16±0.01^{a}	0.16±0.01	0.18±0.00^{a}	0.17±0.01
DOC (mg·g^{-1})	0.23±0.02^{a}	0.21±0.01	0.21±0.03^{a*}	0.60±0.02	0.19±0.02^{a*}	0.27±0.01
Total P (g·kg^{-1})	2.72±0.10^{b}	2.79±0.13	2.24±0.10^{a}	2.33 ±0.16	2.34±0.12^{a}	2.51±0.08
P available (mg·kg^{-1})	22.25±0.04^{a*}	23.46±0.16	21.84±0.45^{a*}	24.60±0.40	25.93±0.04^{b*}	27.07±0.04
CEC (cmol$_{(+)}$·kg^{-1})	22.78±0.10^{a}	22.91±0.24	23.94±0.41^{b}	24.12±0.11	24.15±0.12^{b}	24.80±0.54
Exchangeable Na (cmol$_{(+)}$·kg^{-1})	0.48±0.08^{a}	0.46±0.01	0.43±0.01^{a}	0.44±0.01	0.41±0.01^{a}	0.44±0.01
Exchangeable K (cmol$_{(+)}$·kg^{-1})	1.39±0.05^{a}	1.32±0.19	1.25±0.11^{a}	1.42±0.06	1.33±0.05^{a}	1.42±0.05
Exchangeable Ca (cmol$_{(+)}$·kg^{-1})	19.55±0.67^{a}	19.85±0.31	20.01±0.83^{a}	20.51±0.80	21.17±0.30^{b}	21.81±0.51
Exchangeable Mg (cmol$_{(+)}$·kg^{-1})	1.15±0.01^{a}	1.12±0.05	1.12±0.05^{a}	1.07±0.01	1.10±0.05^{a}	1.10±0.05
Total PTEs (mg·kg^{-1})						
As	27.85±0.23^{a}	28.02±3.19	29.26±2.20^{a}	30.90±5.42	29.45±5.34^{a}	32.50±4.88
Cd	28.62±0.10^{b}	28.40±0.85	28.75±0.66^{b}	28.67±0.84	25.44±0.38^{a}	26.11±1.11
Cu	211.95±2.03^{a}	216.88±12.74	212.65±8.96^{a}	227.92±12.28	209.67±5.69^{a}	227.71±26.51
Pb	10,942±432^{b}	13,134±1785	10,764±527^{b}	12,110±1188	9,807±409	10,529±500
Sb	62.14±5.15^{b*}	71.60±3.43	60.23±3.63^{b*}	80.13±5.42	48.05±3.09^{a*}	60.19±5.02
Zn	2,853±70^{a}	2,881±136	2,911±89^{a}	3,080±154	2,710±92^{a}	2,818±119

Mean values ± SE (n= 3) followed by different letters within a row denote statistically significant differences between soils treated and untreated with biochar (C, B2 and B5), asterisk (*) denote statistically significant differences between earthworm-treated and untreated soils (-E, +E) within each amendment treatment, according to the Fisher's Least Significant Difference (LSD) test ($P < 0.05$).

The biochar, provided by Ronda SpA (Zanè, Italy), was obtained by slow pyrolysis at 700 °C of beech, poplar and elder softwood. The main chemical characteristics of the biochar are reported in Table S1 and described by Manzano et al. (2020). Briefly, the biochar (sieved to <2 mm; the fraction <0.5 mm was 15% w/w) had a alkaline pH (i.e., 9.3), high total organic carbon content (61.32%), high concentrations of extractable P (84.52 mg kg^{-1}) and exchangeable Ca (45.08 $cmol_{(+)}\cdot kg^{-1}$), medium cation exchange capacity (CEC, 18.81 $cmol_{(+)}$ kg^{-1}), and low concentrations of total N (0.3%), dissolved organic carbon (DOC, 0.02 mg kg^{-1}) and PTEs, which were under the detection limit (i.e. <0.2 $\mu g\cdot kg^{-1}$), except for Cu.

Mesocosms, each consisting of approx. 10 kg soil, were set up and subject to the following treatments:

- unamended soil used as a control (C);
- 2% (w/w) amendment with softwood biochar (B2);
- 5% (w/w) amendment with softwood biochar (B5)

All treatments were applied to three replicate mesocosms. The amendment rates (2 and 5% w/w) were selected based on previous experimentation (Manzano et al., 2020) and a preliminary earthworms survival test. Soil samples and biochar were left in contact for 1 month. During this period, they were kept at constant moisture (30% of their water holding capacity, WHC) and turned twice a week to aerate the soil.

After the contact period, the main physico-chemical properties of the replicated mesocosms (e.g. pH, DOC, organic C, CEC) were determined to ascertain possible variability between replicates and avoid the use of mesocosms characterized by extreme values which could have impacted on earthworm growth and development (other than

PTEs). This was particularly relevant especially for biochar treated soils. Since no significant variability, nor extreme values, were recorded between replicated mesocosms (data not shown), soils from these latter were pooled together, carefully mixed and wetted to 60% of their WHC to provide optimum moisture conditions for earthworms. Subsequently, amended and unamended soils were weighed out into plastic boxes, 1 kg of wet soil in each box (5 replicated boxes per treatment), and 8 adult earthworms (*E. fetida*) fully clitellate, with an average weight of 0.5 g, were then placed in each box (+E). *E. fetida* was chosen because it is a good candidate for inoculation to mine soils because of how easily it can be reared in the laboratory and its tolerance to contaminated soils. Soil and earthworms were kept in contact in a dark room at 20 °C for 3 months. No manure or other food source were added to the boxes to be sure that the observed effects were exclusively due to the presence of the earthworms or biochar. Moreover, 3 boxes without earthworms (-E) for each treatment were kept in the same experimental conditions (24 boxes in total).

2.2. Soil samples characterization and analytical determinations

At the end of the incubation period (1 month soil + biochar and 3 months with earthworms) the earthworms that survived were collected and counted, rinsed with deionised water, and depurated for 24 hours (Arnold and Hodson, 2007). After this time, the earthworms were cleaned, weighed, frozen at -18 °C for 48 h, and dried at 55 °C for 72 h. The number and fresh body biomass of *E. fetida* were recorded at the start and the end of the experiment to determine the effects of PTEs and biochar on earthworm fitness. After three months, the presence of eggs and/or juveniles has not been noticed. The survival rate and the biomass change (weight loss) rate were also calculated (Huang et al., 2020).

After the incubation period soil samples were collected, air dried at 25 °C for 72 hours, and stored at room temperature. Soil pH and electric conductivity (EC) were determined in soil samples from each box following the Italian standard guidelines (Gazzetta Ufficiale, 1992, Table 1). Total carbon and nitrogen were determined using a CHN analyzer (Leco CHN628) with Oat meal Leco part n° 502-276 as calibration sample. DOC was quantified after 24 h agitation using a 1:10 ratio (w/v) soil to deionised water suspension. The liquid phase was then filtered and its absorbance at 254 nm was determined (Silvetti et al., 2014). Total P was determined by treating soil samples with H_2SO_4, H_2O_2, and HF and quantified by the ascorbic acid method (Gazzetta Ufficiale, 1992). Available P was quantified following the Olsen method. Cation exchange capacity (CEC) and the concentrations of exchangeable Na, Ca, K and Mg were measured using the $BaCl_2$ and triethanolamine methods (Gazzetta Ufficiale, 1992).

To detect the labile (i.e. water-soluble and readily exchangeable) pool of cationic PTEs (i.e. Cd, Pb and Zn), soil samples (1 g) were shaken with 25 mL of a 0.5 M $Ca(NO_3)_2$ solution for 16 h at 20 °C (Basta and Gradwohl, 2000). The non-specifically sorbed As and Sb (i.e. labile pool) was determined by shaking soil samples (1 g) with 25 mL of a 0.05M $(NH_4)_2SO_4$ solution for 4 h at 20 °C (Wenzel et al., 2001). The PTEs extracted were quantified in the liquid phase using an Inductively Coupled Plasma Optical Emission Spectrometry (Perkin Elmer Optima 7300 DV ICP-OES).

The total concentration of PTEs (i.e. As, Cd, Pb, Sb and Zn) in soils and earthworm tissues were determined by microwave acid digestion (MARS 6). Soil samples were digested with a mixture of HNO_3 + HCl (3:1 v/v ratio), following U.S. EPA Method 3051A and the earthworm samples with 2 mL of H_2O and 8 mL of HNO_3, following U.S. EPA Method 3052. A certified reference material for soil (NIST-SRM 2711) and mussel tissues (ERM CE278) were included for quality assurance. The bioaccumulation factor (BAF) was calculated by dividing the PTEs concentration in earthworm tissues by soil concentration (Table 2).

Table 2 Earthworms fitness, PTEs concentration in earthworms tissues and PTEs bioaccumulation factors (BAF) in earthworms grown in unamended (C+E) and amended soil (B2+E and B5+E) treated with earthworms.

	C+E	B2+E	B5+E
Survival rate (%)	86.02±1.57^{b}	88.98±1.58^{b}	43.06±0.51^{a}
Weight loss rate (%)	8.01±0.78^{b}	6.50±0.76^{b}	18.03±0.2^{a}
PTEs in earthworms (mg·kg^{-1} d.w.)			
As	20.79±0.05^{b}	20.87±0.03^{b}	14.40±0.08^{a}
Sb	1.37±0.03^{a}	1.92±0.05^{b}	2.77±0.06^{c}
Cd	107.88±3.57^{b}	115.00±3.60^{b}	72.57±1.15^{a}
Cu	98.31±^{c}	82.22±^{b}	53.32±^{a}
Pb	4743.58±^{c}	3800.30±^{b}	2000.80±^{a}
Zn	1457.65±^{c}	1136.65±^{b}	572.71±^{a}
Earthworms BAF			
As	0.742±0.00^{c}	0.675±0.00^{b}	0.443±0.00^{a}
Sb	0.019±0.00^{a}	0.024±0.00^{b}	0.046±0.00^{c}
Cd	3.798±0.11^{b}	4.011±0.13^{b}	2.779±0.08^{a}
Cu	0.453±0.00^{c}	0.361±0.00^{b}	0.234±0.00^{a}
Pb	0.361±0.00^{c}	0.314±0.00^{b}	0.190±0.00^{a}
Zn	0.506±0.00^{c}	0.369±0.00^{b}	0.203±0.00^{a}

Mean values ± SE (n= 3) followed by different letters within a row denote statistically significant differences, according to the Fisher's Least Significant Difference (LSD) test ($P < 0.05$).

2.3. Enzyme activities and soil basal respiration

Selected enzyme activities were determined in all soil samples. Protease activity was determined by quantifying the amino acids released after the incubation of soil samples with sodium caseinate for 2 h at 50 °C using Folin-Ciocalteu reagent as described by Alef and Nannipieri (1995). Phosphatase activities were determined as acid and alkaline phosphomonoesterase (PHA and PHB) by quantifying the p-nitrophenol released in soil samples incubated for 1 h at 37 °C with p-nitrophenyl phosphate at pH 6.5 and 11.0 respectively. Pyrophosphatase activity (PHY) was determined by quantifying the orthophosphate (inorganic phosphorus) released in soil samples incubated for 5 h at 37 °C with buffered pyrophosphate solution (Alef and Nannipieri, 1995).

In order to determine soil basal respiration, soil samples (20 g) at 60% of WHC were placed in plastic containers inside stoppered glass jars together with 4 mL of 1 N NaOH which served to trap the evolved CO_2. Each jar was then incubated in the dark at 25 °C for 7 days. Four jars were set up for each treatment, while six jars without soil were used as control. After incubation, the NaOH was removed from the jar and calcium carbonate precipitated by adding 8 mL of 0.75 N $BaCl_2$. The NaOH excess was finally titrated with 0.1 N HC1 until pH 8.8 and the CO_2 produced was then reported as μg C–CO_2 (Marabottini et al., 2013).

2.4. Data analysis

All chemical, biochemical and microbial analyses were performed at least in triplicate for each mesocosm (24 in total) and mean values ± standard errors (SE) are reported in tables and figures. One-way analysis of variance (ANOVA) was carried out to compare mean values between -E soils amended and unamended with biochar (indicated by different lowercase letters), and to evaluate a possible influence of earthworms within each amendment treatment, i.e. C, B2 and B5 (-E and +E soil; differences indicated by asterisks). Two-way ANOVA was also performed to evaluate the influence of biochar (at 2 and 5% rates) and earthworms on soil chemical features, PTE labile fraction, soil respiration and enzyme activities. When significant *P*-values ($P < 0.05$) were obtained, differences between individual means were compared using the post-hoc Fisher's least significant difference test (LSD, $P < 0.05$). Statistical analyses were carried out using the NCSS 2007 Data Analysis software (v. 07.1.21; Kaysville, Utah).

3. Results and discussion

3.1 Influence of E. fetida, biochar and their combination on the chemical properties of contaminated soil

The main soil chemical characteristics of differently treated soils are reported in Table 1. The biochar addition led to an increase of soil pH (+ ~0.34 and ~0.55 units in B2 and B5 respectively, Table 1). This agreed with the results of other researches (Abou Jaoude et al., 2020; Gu et al., 2020; Manzano et al., 2020) which reported that biochar alkalinity may increase the soil pH, alleviating acidity. Earthworm activities resulted in a significant pH decrease only in the unamended soil (C+E), which could be attributed to the ability of earthworms to lower pH through the release of organic acids (Huang et al., 2020; Sizmur et al., 2011b; Wang et al. 2019). The buffering capacity of biochar, and the likely adsorption of the low molecular weight organic acids on its surface, may have masked the acidifying effect of *E. fetida* in B+E soils (Gomez-eyles et al., 2011), despite the significant interaction between earthworms and biochar on soil pH (Table S2).

The addition of biochar resulted in a decrease in total P (~1.21- and ~1.16-fold lower in B2 and B5 respectively, with respect to C soil; Table 1) and EC (~1.27- and ~1.44-fold lower in B2 and B5 respectively; Table 1). This can be ascribed to the dilution effect induced by the biochar addition or to its adsorption capacity, as reported by Manzano et al. (2020). In all the earthworm-worked soils the EC increased by 1.04-, 1.06- and 1.11-fold in C+E, B2+E and B5+E, respectively, compared to soils without earthworms (Table 1). Some researchers (e.g. Chaudhuri et al., 2012; Wang et al., 2019) reported similar results, and attributed them to the increased soluble salts released by the activity of epigeic, epi-aneic and endogeic earthworm species. The substantial content of exchangeable or available ionic species (i.e. Ca and P) in biochar likely determined their increase in amended soils (Table 1). In addition, the available P in earthworm-worked soils increased significantly, probably as a result of P mineralization during soil transit in the earthworm's gut. This finding was in agreement with Chaudhuri et al. (2012) which reported that different earthworm species (e.g. *Pontoscolex corethrurus*, *Drawida assamensis*, *Drawida papillife*, *Eutyphoeus comillahnus* and *Metaphire houlleti*) are able to increase the available P. An increase of ~1.05- and ~1.06-fold of CEC, ~1.50- and ~2.16-fold of organic matter was observed in B2 and B5 respectively, compared to the control soil (Table 1). Despite biochar significantly increasing the total C, it did not increase the DOC content, suggesting that carbon in biochar was mostly recalcitrant and insoluble. By contrast, the interaction between earthworms and biochar (Tables 1 and S2) favoured a significant DOC increase of 2.86- and 1.42-fold in B2+E and B5+E respectively, compared to soils without earthworms. This could be due to an accelerated degradation of biochar organic matter by the earthworms, and/or to the poor capacity of biochar to adsorb the DOC mobilized by worms. Generally, the biochar addition either did not affect or slightly decreased the total concentrations of PTEs.

Overall, earthworms and biochar addition, alone or in combination, improved the soil fertility and nutrient status. In particular earthworms increased the EC, extractable P, DOC, and CEC, while biochar addition increased soil pH, total OM, CEC and exchangeable Ca. The interaction between earthworms and biochar determined and increase of soil pH, OM, DOC, CEC and exchangeable Ca (Table S2), and this could be of substantial help for the functional recovery of contaminated soils.

3.2 *Influence of E. fetida, biochar and their combination on PTE mobility in contaminated soils*

The water-soluble and readily exchangeable (labile) fraction of Cd and Zn in all soil samples represented a considerable portion of their total concentration in soil (i.e. between 27.0 and 45.8% of total Cd, 10.2 and 18.5% of total Zn; Fig. 1). In contrast, the labile fraction of Pb was very low compared to its total concentration (i.e. between 0.38 and 1.19% of total Pb), while that of Cu was below the detection limit in all the soil samples (data not shown). This difference between different PTEs is attributed to the higher mobility of Cd and Zn and to their lower affinity towards soil colloidal components, which is mainly due to their higher hydrated ionic radius compared to Cu and Pb (Kabata-Pendias and Pendias, 2001). Biochar addition reduced the water-soluble and readily exchangeable fractions of the selected PTEs; a decrease of 1.25- and ~1.50-fold of Cd, ~1.48- and 2.13-fold of Pb, ~1.24- and 1.57-fold of Zn was observed in B2 and B5 respectively, compared to control (C) soil (Fig. 1). Such a decrease of labile (and potentially bio-available) PTEs could be ascribed to the capacity of biochar to retain Pb, Cd and Zn through non-specific and specific adsorption mechanisms, i.e. inner-sphere complexes with carboxylic and phenolic functional groups (Zhu et al., 2017). Moreover, the liming effect of biochar likely favoured the formation of PTE precipitates as metal (hydr)oxides (Kabata-Pendias and Pendias, 2001; Lu et al., 2017; Zhu et al., 2017). Inorganic anions within the biochar could have played a significant role in the precipitation of Pb above all (e.g. the formation of lead carbonate [$PbCO_3$ and $Pb_3(CO_3)_2(OH)_2$] and lead phosphate [$Pb_5(PO_4)_3(OH,Cl)$ and $Pb_9(PO_4)_6$], favouring its immobilization (Cao et al., 2009). Earthworm addition did not change the mobility of the selected PTEs in the control soil, except for Pb, which increased by 1.56-fold in C+E compared with C-E (Fig. 1). The pH decrease and the OM degradation due to earthworm activity could have led to an increase in Pb mobility in soil, in agreement with the results reported by some researchers (Huang et al., 2020; Sizmur et al., 2011a). However, in biochar amended soils, the earthworm activity reduced or did not change Cd, Pb and Zn mobility, showing that the immobilisation effect of biochar was amplified by earthworms and prevailed with respect to possible mobilising effects (seen for lead in unamended soil). Two-way ANOVA showed that the mobility of Cd and Zn was not affected by the biochar + earthworms treatment (compared to the control) and there was no interaction between biochar and earthworms. However, Pb was significantly influenced by all treatments (Table S2). Similar results have been highlighted by Huang et al. (2020), which reported that the combination of biochar and earthworms did not influence the PTE mobility in soil.

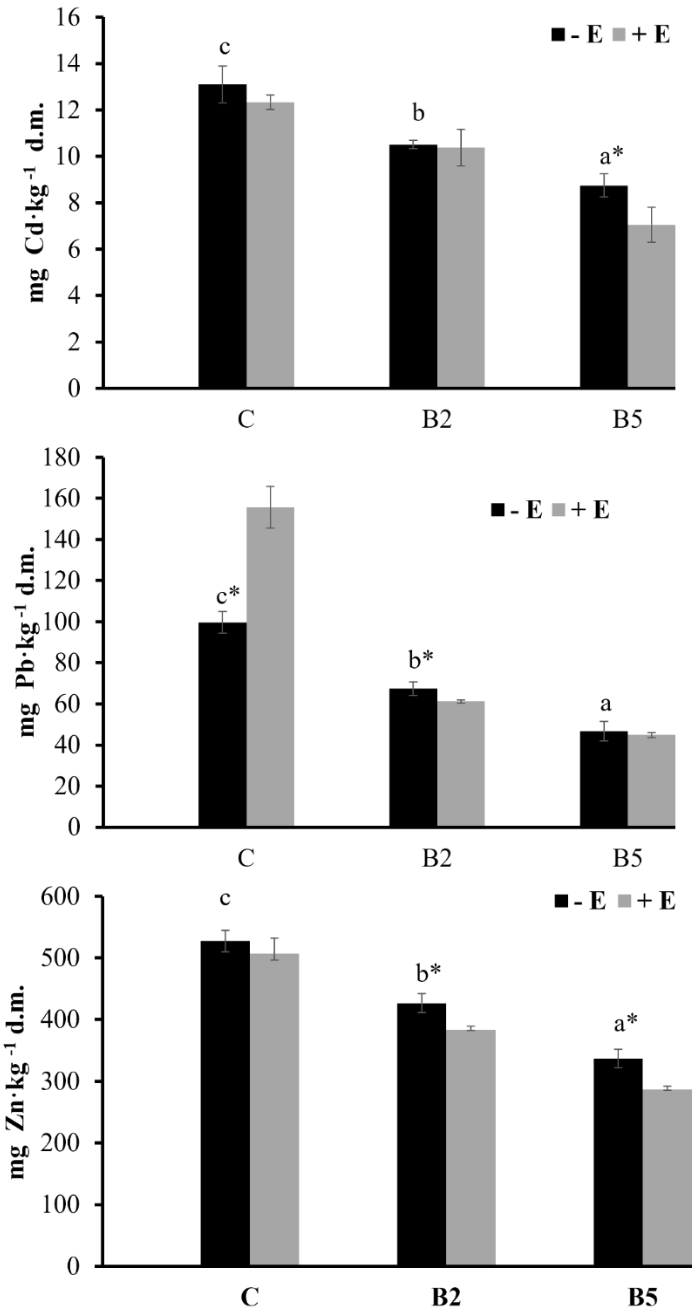


Fig. 1. Labile fraction of Cd, Pb and Zn. For each PTEs, mean values ± SE (n = 3) followed by different letters denote statistically significant differences between soils treated and untreated with biochar (i.e., C, B2 and B5); within each amendment treatment, asterisks (*) denote statistically significant differences between earthworm-treated and untreated soils (i.e., +E and -E) within each amendment treatment, according to the Fisher's Least Significant Difference (LSD) test ($P < 0.05$).

Despite the labile fraction of As being relatively low (<0.2%), the biochar and earthworm addition decreased it further (i.e. <66 and 43% in C+E and B2 soils), compared to control soil, while in B2+E, B5-E and B5+E soils the labile As was below the detection limit. This decrease in labile As was probably due to the affinity of Fe (hydr)oxides in the biochar towards arsenate. In contrast, the very low concentration of water-soluble and exchangeable Sb in C-soil (i.e. 0.2 and 0.3% of total Sb in -E and +E) increased by ~1.41-fold in B5-E. This may be due to the highest pH increase in the soil amended with 5% biochar, which increased the electrostatic repulsion between Sb oxyanion (i.e. antimonate, the most stable form of Sb in the soil) and biochar surfaces (Gu et al., 2020; Igalavithana et al., 2017). Similarly, Gu et al. (2020) reported that *Arundo donax* L. stem-derived biochar increased the mobility of Sb in mine tailings. The water-soluble and readily exchangeable fraction of Sb did not significantly change in C+E with respect to C-E, while it significantly increased by 1.15-fold in B2+E compared to B2-E. The simultaneous increase of DOC and available P occurred in B+E soil (B2+E in particular) could have favoured an increase of Sb mobility as a consequence of competition phenomena for the same adsorption sites between the organic anions and phosphate with Sb oxyanions (Wang et al., 2020).

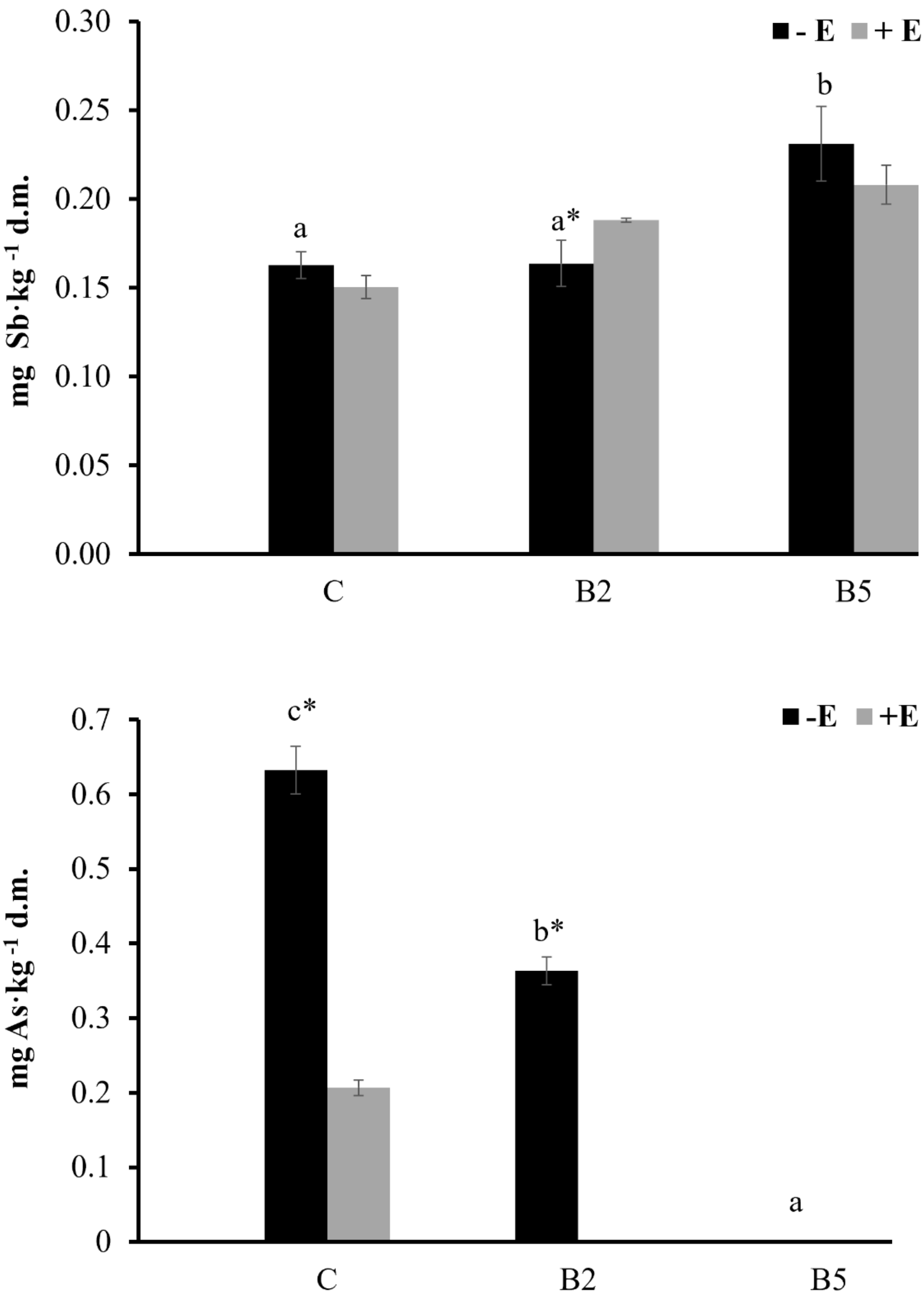


Fig. 2. Labile fraction of As and Sb. For each PTEs mean values ± SE (n= 3) followed by different letters denote statistically significant differences between soils treated and untreated with biochar (i.e., C, B2 and B5); within each amendment treatment, asterisks (*) denote statistically significant differences between earthworm-treated and untreated soils (i.e., +E and -E) within each amendment treatment, according to the Fisher's Least Significant Difference (LSD) test (*P*< 0.05).

The interaction earthworms+biochar significantly reduced the labile fraction of As and Pb (Table S2). Probably earthworms mixed soil and biochar particles, through feeding and burrowing, resulting in a more effective contact between soil and biochar and a breakdown of the biochar particles into smaller fractions. Indeed, *E. Fetida* exhibit geophagous behaviour when added to soils with no litter layer, as do most epigeic species. All of this is likely to have resulted in an increase of the specific surface area of biochar, enhancing its effectiveness as immobilising material. The reduction in the water-soluble and readily exchangeable PTE fraction (with the exception of Sb) due to the combination of earthworms and biochar, was deemed as noteworthy from an environmental point of view. This fraction represents the most mobile pool, that may be bioavailable to plants and microorganisms (Garau et al., 2017, 2014; Garau M. et al., 2019; Manzano et al., 2020).

3.3 Effect of E. fetida, biochar and their combination on enzyme activities and basal respiration in contaminated soils

To assess the biological impact of earthworms on amended and unamended soils, basal respiration and a range of enzyme activities involved in nutrient cycling were determined. Biochar addition increased the soil basal respiration by 2.40-fold in B2 and 3.54-fold in B5, compared to C-soil (Fig. 3). The beneficial effect of biochar on soil microbial activity, could be due to the increase of soil pH, nutrient and water content, and alleviation of PTE toxicity (Paz-Ferreiro et al., 2015; Xu et al., 2018). The earthworm addition further increased the soil basal respiration by +1.90-, +1.25- and +1.37-fold in C+E, B2+E and B5+E respectively, compared to -E soils (Fig. 3). This was in contrast with the observations of some authors (e.g. Bamminger et al., 2014;

Paz-Ferreiro et al., 2015) who did not find a significant influence of earthworms (i.e. endogeic *Aporrectodea caliginosa* and *P. corethrurus*) on CO_2 emissions in soils. Altogether, the influence of the biochar+earthworms (e.g. *L. terrestris* and *A. icterica*) treatment on soil properties, such as, pH, OM, DOC, CEC and exchangeable Ca, and PTE lability probably led to the increased soil microbial population and CO_2 emissions, compared to the control soil (Fig. 3; Table 2S) (Augustenborg et al., 2012, Beesley and Dickinson, 2011).

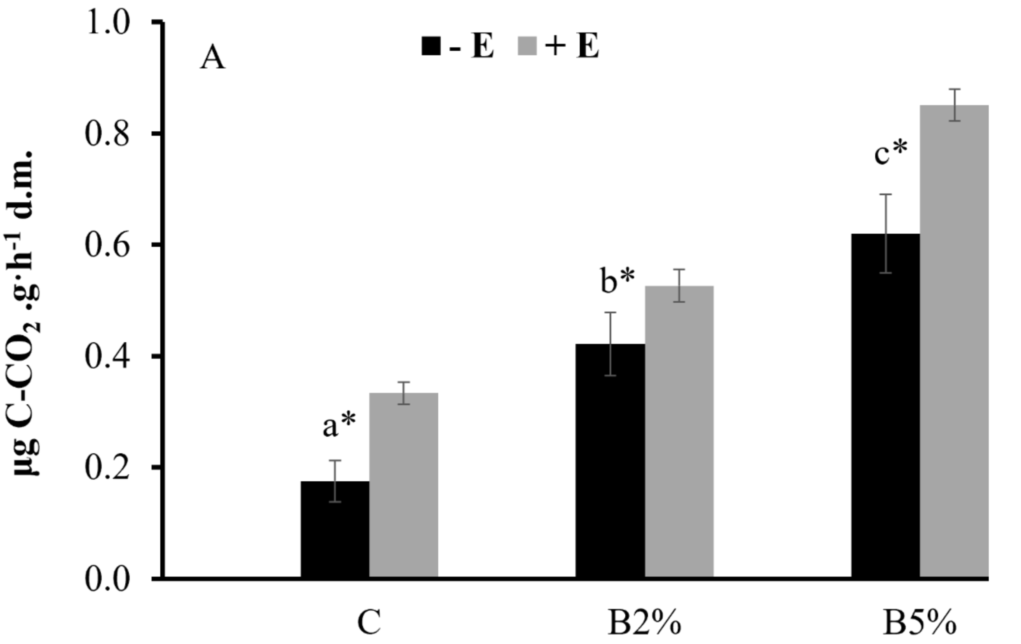


Fig. 3. Soil basal respiration, expressed as μg C-CO_2 g h^{-1} d.m. Mean values ± SE (n = 5) followed by different letters denote statistically significant differences between soils treated and untreated with biochar (i.e., C, B2 and B5); within each amendment treatment, asterisks (*) denote statistically significant differences between earthworm-treated and untreated soils (i.e., +E and -E) within each amendment treatment, according to the Fisher's Least Significant Difference (LSD) test (P< 0.05).

Enzyme activities are useful indicators of soil functionality and can provide information on the impact of environmental stresses (e.g. PTEs) on soil nutrient cycles (i.e C, N and P) (Garau G. et al., 2019; Garau M. et al., 2019; Oliveira and Pampulha, 2006). Soil organisms, to satisfy their P requirements, produce phosphatases, which are extracellular enzymes that catalyse the hydrolysis of phosphate esters and anhydrides (Paz-Ferreiro et al., 2014). Phosphomonoesterase activities were significantly reduced in biochar amended soil; in particular the PHA decreased by ~21 and 50% in B2 and B5 soils respectively, compared to control soil, while the PHB decreased by ~35% in B5 soil, compared to the control (Fig. 4A and 4B). In the in presence of earthworms, phosphomonoesterase activities further decreased (i.e. between 20 and 53%) compared to soils without earthworms (Fig. 4A and 4B). The increase of available P recorded in biochar amended soils and in presence of earthworms could have resulted in a reduction in microbial P demand and a subsequent decrease in the synthesis of phosphomonoesterase by microbial communities. Moreover, biochar application might inhibit these activities through surface adsorption processes (Huang et al., 2017; Tang et al., 2020). These results are in accordance with those reported by Xiao et al. (2021), which observed a reduction of the alkaline phosphatase activity in Cd-spiked soil in presence of earthworms (i.e. *P. corethrurus*) and biochar, and Paz-Ferreiro et al. (2014), who showed a decrease of PHA activities in tropical soils (i.e. Acrisol and Ferralsol) amended with sewage sludge biochar. Pyrophosphatase activity (PHY) was not influenced by biochar or by earthworm addition (Fig. 4C).

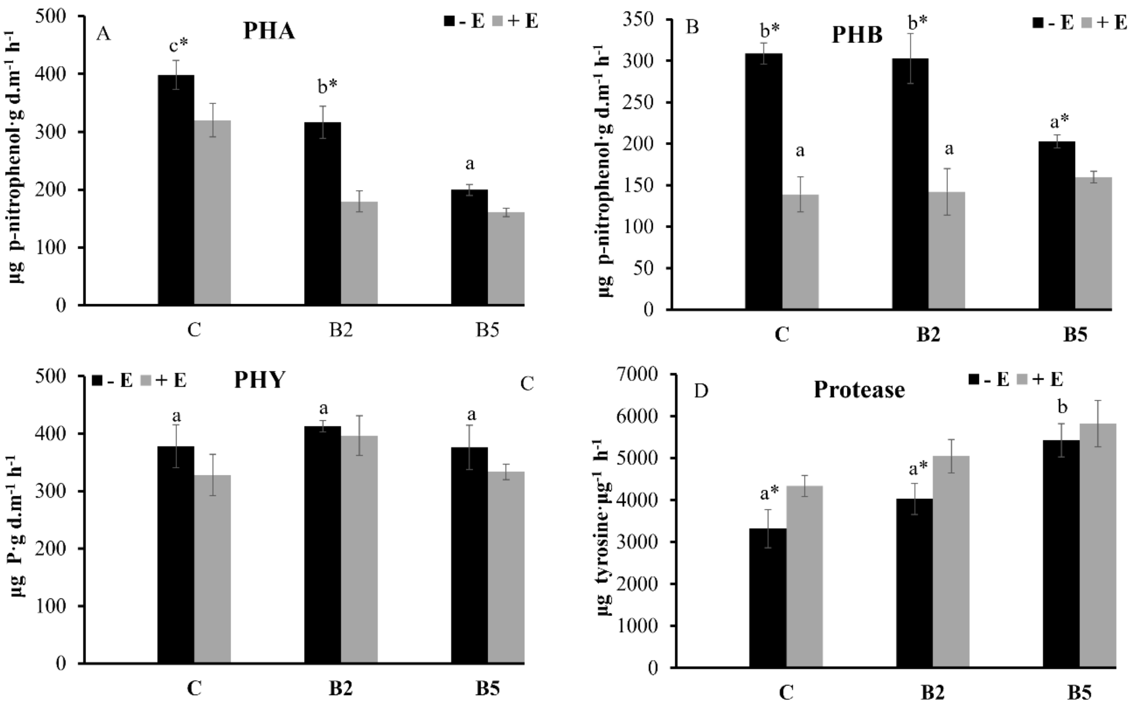


Fig. 4. Selected enzyme activities, acid phosphomonoesterase (PHA) activity (A), alkaline phosphomonoesterase (PHB) activity (B), pyrophosphatase (PHY) activity (C) and protease activity (D). For each enzyme activities, mean values ± SE (n = 3) followed by different letters denote statistically significant differences between soils treated and untreated with biochar (i.e., C, B2 and B5); within each amendment treatment, asterisks (*) denote statistically significant differences between earthworm-treated and untreated soils (i.e., +E and -E) within each amendment treatment, according to the Fisher's Least Significant Difference (LSD) test (P< 0.05).

The biochar addition resulted in a 1.21- and 1.63-fold increase in protease activity in B2 and B5 soils with respect to control (Fig. 4D). This can be explained by the increase of Ca availability in biochar amended soils, especially at 5% rate, because calcium can favour proteases activation (Tchounwou et al., 2012). Earthworm addition increased protease activity by 1.30- and 1.25-fold in C+E and B2+E, compared to soils without earthworms, whereas in B5+E the increase detected was not significant. Earthworms and their gut-associated microflora are able to secrete into the soil solution several digestive enzymes; in particular hydrolases like proteases, which catalyse the degradation of various organic components (Sanchez-Hernandez et al., 2019b). This phenomenon could explain the higher protease activity values detected in the presence of earthworms. Also, the interaction between biochar and earthworms significantly affected this enzyme activity with respect to the control treatment (Table S2).

Taken together these results highlight that the biochar, earthworms, and their combination significantly influenced soil basal respiration and enzymatic activities (Table S2).

3.4 PTEs concentration and bioaccumulation in E. fetida and acute ecotoxicity effects of soils amended and unamended with biochar

In addition to the direct influence that earthworms and biochar have on soil properties, biochar itself may affect the health of earthworms (i.e., their survival rate and weight loss), and affect the bioaccumulation of PTEs into their tissues (Wang et al., 2019). After the three months of incubation with biochar, the survival rate of earthworms was 86.0, 88.9 and 43.1%, while the weight loss was 8.0, 6.5 and 18.0% in C+E, B2+E and B5+E treatments, respectively (Table 2). In addition, no eggs or juveniles were noticed in the different treated soils. All this could be attributed to the non-ideal conditions for the growth and reproduction of *E. fetida* (e.g., low content of C and nutrients available and substantial labile PTEs in soil). The survival rate and the weight loss of earthworms was not significantly different between C+E and B2+E treatments, while in B5+E the survival rate was ~2.0-fold lower and the weight loss was ~2.5-fold higher than the other treatments (Table 2). These results indicate that *E. fetida* may survive in PTE contaminated soils, but biochar addition at higher rates (i.e. 5%) can have toxic effects. These effects could be ascribed to the ingestion of biochar particles; particularly those containing contaminants such as polycyclic aromatic hydrocarbons, which could be formed during biochar pyrolysis (Barbosa et al., 2006). Our results are in agreement with those reported by Huang et al. (2020), who showed that the addition of 2% biochar to PTE polluted soil did not affect the survival rate (~86%) of earthworms in comparison with the unamended soil. On the other hand, Liesch et al. (2010) and Wang et al. (2019) reported very low survival rates (<37%) of earthworms in PTE polluted soils amended with different biochars (i.e. sludge biochar, rice husk biochar and poultry litter biochar) at rates between 2.5 and 9.5%.

PTEs ingested by earthworms can be excreted in the soil within earthworm casts or can be accumulated into earthworm tissues by binding to phosphate-rich granules, O-or S-donating (and other) organic ligands (Sizmur et al., 2011b; Sizmur and Hodson, 2009) in the earthworm chloragogenous tissues. The lowest Cu, Pb, Zn, As and Cd concentrations were recorded in earthworms incubated in soils amended with biochar (although Cd and As concentrations in *E. fetida* grown in B2+E were not statistically different to the control; Table 2). On the contrary, Sb concentrations in earthworms were 1.40- and 2.02-fold higher in earthworms grown in B2+E and B5+E soils, respectively, compared to those grown in C+E soil (Table 2). The BAF values were always less than 1 for all elements (except Cd), and they followed the trend: Cd>As>Zn>Cu>Pb>Sb. Relatively low BAF values, as also observed by Liu et al. (2017), can be explained by the fact that the earthworms are able to regulate the levels of some PTEs in their tissues due to an equilibrium between PTEs absorption and excretion; maintaining a relatively constant body concentration over a range of PTEs concentrations (Ruiz et al., 2009). The observed trend can be justified by the fact that Zn and Cu are essential elements, which differ in terms of accumulation or excretion by earthworms compared to Sb and Pb, which are never deficient (Ruiz et al., 2009). On the other hand, the highest BAF values of Cd and As can be explained by the fact that these PTEs bind in chloragogenous tissues of earthworms with organic ligands (i.e, O-donating, phosphate-rich granules and S-donating ligands), with which they form stable complexes resulting in high BAFs (Sizmur et al., 2011b; Sizmur and Hodson, 2009).

Earthworms grown in control soil showed higher BAFs (except for Sb) compared to those grown in amended soils, especially at 5% rate (Table 2). The earthworms probably ingested biochar particles containing PTEs. However, these contaminants were strongly retained by biochar and not bioaccessible, with the consequent lower PTEs bioaccumulation observed in earthworms grown in the amended soils. These results are also in agreement with those reported by other authors, who showed lower bioaccessibility (in vitro and on *Eisenia* spp.) of PTEs in contaminated soils amended with biochar (Huang et al., 2020; Manzano et al., 2020; Wang et al., 2020). For example, the As-BAF values detected in control soil were 1.10- and 1.68-fold higher than those in B2+E and B5+E respectively. The Cd-BAF was higher than 1 also in the treated soils, even if a significant decrease it was observed in B5+E (i.e. <27%), compared to the other treatments. Similar results are reported by other authors (Gomez-eyles et al., 2011; Huang et al., 2020; Sizmur et al., 2011a), which highlighted that the PTEs concentrations in earthworm tissues and BAF values were lower for *E. fetida* grown in soils amended with biochar. These results highlight that the use of biochar for soil restoration may reduce the chance of PTEs entering the terrestrial food web through earthworms, if they are predated by higher animals (i.e. mammals, birds, reptiles and amphibians) (Elliston and Oliver, 2020). Only Sb-BAF increased for earthworms grown in the amended soils (i.e. +1.25- and 2.40-fold in B2+E and B5+E compared to C+E), although Sb concentration in earthworm tissues and Sb-BAF were very low. This result is in accordance with the known low affinity between biochar and Sb, which resulted in a high bioaccessibility of this PTE, with its consequent high bioaccumulation by earthworms in the amended soils.

The results obtained highlighted that earthworms were able to survive in a heavily PTEs contaminated soil probably by adopting several detoxification strategies (i.e. PTEs excretion or accumulation in chloragogenous tissues, and/or induction of metallothioneins by PTEs that can contribute to their sequestration in earthworm tissues). Although biochar addition at 5% rate significantly decreased PTE concentration and bioaccumulation by earthworms, it negatively affected the earthworms' survival and weight, probably due to the presence of substances such as polycyclic aromatic hydrocarbons (Barbosa et al., 2006), capable of exhibiting toxic effects when biochar is added at the highest concentrations. Therefore, the biochar addition at a rate of 2%, which led to a significant decrease of PTEs bioaccumulation without reducing the health of earthworms, could represent an optimal environmental remediation treatment as part of an ecological restoration strategy.

4. Conclusions

Biochar, earthworms, and their combination, improved soil fertility and nutrient status. It is likely that earthworms mixed soil and biochar particles, through feeding and burrowing, resulting in effective soil/biochar interaction, soil aeration and degradation of biochar into smaller fractions. All of this is likely to have resulted in an increase in the biochar reactivity and nutrient availability in soil.

Biochar addition reduced the water-soluble and readily exchangeable PTEs fraction (with the exception of Sb) and reduced the PTE bioaccumulation by earthworms.

Earthworms were able to survive in a multi-PTE contaminated soil, but the high concentration of PTEs in earthworm tissues, especially Cd, may facilitate their entry into the food chain. The biochar addition decreased the bioaccumulation of PTEs by *E. fetida* when applied at 2% and 5%, but the treatment at 5% rate had a negative impact on the earthworm survival and weight.

Taken together, these results highlight a favourable influence of earthworms on PTEs mobility in soils amended with biochar, especially at 2% rate. As a result, the combined action biochar and earthworms was the best solution for the restoration of soil ecological functions, while reducing the environmental risks. However, further studies are needed to investigate the long-term impact of biochar, especially at high rates, on PTE speciation and earthworm populations in field experiments. Finally, a possible drawback of using biochar on a large scale could be its high production cost. In this respect, Kon-tiki kilns technology (and other similar) could be an attractive solution for generating low-cost, fast and cleaner biochar.

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Table S1

Chemical characteristics of biochar. Values represent mean ± SE (n = 3). n.d.: under detection limit (i.e. <0.2 $\mu g \cdot kg^{-1}$).

Chemical analyses	Biochar
pH	9.30 ± 0.01
EC ($\mu S \cdot cm^{-1}$)	9.91 ± 2.79
Ash (%)	2.44±0.10
CEC ($cmol_{(+)}\ kg^{-1}$)	18.81±0.30
Total C (%)	61.32±0.06
Total N (%)	0.30±0.02
Total carbonate (%)	1.52±0.02
DOC ($mg \cdot g^{-1}$)	0.020±0.003
Available P ($\mu g \cdot g^{-1}$)	84.52±3.01
Exchangeable K ($cmol_{(+)}\ kg^{-1}$)	0.62±0.02
Exchangeable Ca ($cmol_{(+)}\ kg^{-1}$)	45.08±0.95
Exchangeable Mg ($cmol_{(+)}\ kg^{-1}$)	3.28±0.03
COOH groups($meq \cdot g^{-1}$ d.wt)	0.14±0.02
Phenolic groups ($meq \cdot g^{-1}$)	2.10±0.32
Total PTE concentration ($mg \cdot kg^{-1}$)	
Total Sb	n.d.
Total As	n.d.
Total Cd	n.d.
Total Fe	524.8±12.7
Total Mn	358.1±5.1
Total Pb	n.d.
Total Cu	207.1±2.9
Total Zn	n.d.

Table S2

Results of the two-way ANOVA (i.e. F values) showing the influence of earthworms, biochar and earthworms x biochar on soil chemical features, PTE labile fraction, soil respiration and enzyme activities. * P < 0.05; ** P < 0.01; *** P < 0.001.

Parameter	Earthworm	Biochar	Earthworm x Biochar
pH	6.85^{*}	2405.72^{***}	10.92^{**}
EC	35.65^{***}	380.72^{***}	1.58
Total organic matter	0.22	992.84^{***}	4.84^{*}
Total N	0.01	0.81	1.65
DOC	1012.50^{***}	649.50^{***}	685.50^{***}
Total P	2.45	14.04^{***}	0.17
P available	37.88^{***}	74.15^{***}	3.75
CEC	29.08^{***}	32.25^{***}	9.42^{**}
Exchangeable Na	0.10	3.17	1.42
Exchangeable K	1.8	0.25	2.06
Exchangeable Ca	2.34	19.59^{***}	4.23^{*}
Exchangeable Mg	2.25	2.25	0.75
Labile As	185.10^{***}	157.94^{***}	147.14^{***}
Labile Sb	10.34^{**}	21.12^{***}	47.74^{***}
Labile Cd	3.63	88.56^{***}	2.90
Labile Pb	40.85^{***}	395.37^{***}	64.79^{***}
Labile Zn	26.47^{***}	258.32^{***}	1.44
Soil basal respiration	0.37	238.60^{***}	45.77^{***}
Acid Phosphomonoesterase	79.86^{***}	121.70^{***}	9.03^{***}
Basic Phosphomonoesterase	344.43^{***}	17.41^{***}	36.93^{***}
Pyrophosphatase	0.17	5.24^{*}	5.37^{*}
Protease	0.12	5.86^{*}	5.20^{*}