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Abstract

Biochar is a soil amendment capable of influencing plant growth and potentially toxic elements (PTEs) bioavailability in soils. At the same time *Eisenia fetida* (Savigny) is able to interact with biochar influencing its performance. As such they could constitute a resource for assisted phytostabilisation of PTE-polluted soils. To this end, a softwood-derived biochar was added at 2 and 5% (w/w) rate, with and without *E. fetida*, to a soil contaminated with Cd, Pb, Zn, As, and Sb, to evaluate the PTE phytostabilisation potentials when combined with *Sorghum vulgare*. The combination of sorghum, 5% biochar, and earthworms reduced the mobility of most PTEs in soil (e.g., up to 65% and

60% for Pb and Zn), while sorghum biomass was greatly increased (i.e., ~ 3- and 2-fold for roots and shoots, respectively).

Biochar addition alone reduced the PTE uptake by plants, while the presence of earthworms slightly increased it. Overall, the joint action of biochar and earthworms increased the PTE removal efficiency by *S. vulgare* compared to control plants (e.g., the amount of root As, Pb and Sb was ~ 5-, 4- and 3-fold higher, respectively). Although 2% biochar didn't affect *E. fetida* fitness, the highest biochar rate (5%) exhibited toxic effects (the survival rate reduced by ~2-fold; weight loss increased by ~3-fold). Taken together, these results indicated that *S. vulgare*, in combination with softwood biochar and *E. fetida*, could be used for the assisted phytostabilisation of PTEs contaminated soils.

Keywords: Gentle remediation options; Organic amendments; Sorghum; Soil macrofauna; Potentially Toxic Elements Bioaccumulation

1. Introduction

Potentially toxic elements (PTEs, e.g. Cd, Cu, Pb, Zn, As, and Sb) are naturally present in soil at low concentrations. However, when certain thresholds are exceeded, they can have remarkable toxic effects towards living organisms and soil biochemical functioning (Abou Jaoude et al., 2019; Antoniadis et al., 2019).

Among gentle remediation options for the recovery of PTE-contaminated soils, phytoremediation represents an interesting cost-effective and low-impact alternative with respect to traditional and high-impact remediation solutions (Ali et al., 2013). This technology is based on the plant's capacity to remove PTEs from soil translocating them to the aboveground part (i.e., phytoextraction), stabilizing them into the soils or roots (i.e., phytostabilisation), or converting the contaminants (mainly organic, but also inorganic such as Se) into gaseous form and releasing them into the atmosphere through leaf stomatal openings (i.e. phytovolatilisation) thus reducing their labile fractions in soil (Ali et al., 2013; Barbosa and Fernando, 2018; Fiorentino et al., 2018).

Among the plant species that could be used for phytoremediation, *Sorghum vulgare* Pers is particularly interesting because it is able to grow in contaminated soils (robustness), and to produce high amounts of biomass in a relatively short time (Al Chami et al., 2015). Jadia and Fulekar (2008) found that low concentrations of PTEs (e.g. Cd, Cu, Ni, Pb and Zn between 5 - 20 mg kg^{-1}) stimulated shoot growth and total plant biomass compared to control plants. Also, Ningyu et al. (2016) reported a high PTE absorption efficiency of *S. vulgare* and showed that Pb, Cd and Zn were particularly accumulated in the roots. Finally, Zand et al. (2020) detected high Sb accumulation in the aboveground parts of *S. vulgare*, supporting its use for Sb phytoextraction in contaminated soils. All this experimental evidence suggests that sorghum plants have promising phytostabilisation and/or phytoextraction potentials which overall depend on soil physico-chemical properties, PTE type, and concentration.

Phytoremediation potential can be further enhanced through the use of selected organic and/or inorganic amendments (e.g. compost, biochar, zeolites, water treatment residuals, red muds, and lime) (Barbosa and Fernando, 2018; Castaldi et al. 2018; Fiorentino et al., 2018; Garau et al., 2022). This kind of approach, which is defined as assisted phytoremediation, was successfully applied in many instances for the remediation of PTE-contaminated soils (e.g., Liu et al 2022; Radziemska et al 2022;

Zeremski et al 2021), but very limited and inconsistent information is available for sorghum.

In the context of phytostabilisation, the use of biochar, a carbonaceous material originated from the pyrolysis of organic biomass (Beesley et al., 2011), looks particularly interesting (Simiele et al., 2020). The addition of biochar to degraded and/or contaminated soils may increase the content of stable organic carbon and available nutrients, and at the same time can reduce PTE mobility and their potential phytoavailability (Abou Jaoude et al., 2020; Garau et al., 2022; Lehmann, 2007; Manzano et al., 2020; Sheng and Zhu, 2018). In particular, the addition of biochar generally increases soil pH, favouring the precipitation of PTEs in cationic form as metal-carbonates and metal-hydroxides. Furthermore, the presence in biochar of carboxylic and phenolic functional groups and of amorphous Fe and Al (hydroxy)oxides, could be useful to promote the formation of stable surface complexes with both cationic and anionic PTEs such as Pb, Cd, Cu, Sb and As (Abou Jaoude et al., 2020; Lu et al., 2017; Manzano et al., 2020).

When biochar and *S. vulgare* were tested together, increased plant yields were observed in soils amended with 5, 10, 15 and 20 t ha^{-1} biochar (compared to unamended soil), accompanied by a reduction of Cd, Cu, Pb and Zn in sorghum plants (Oziegbe et al., 2019). Zand et al. (2020) also observed a reduction of Sb accumulation in roots and shoots of *S. vulgare* grown in a soil amended with a wood biochar (at a rate of 2.5 - 5%). On the other hand, Ali et al. (2017) observed that the reduction of PTE uptake in a contaminated soil amended with biochar (1% rate) was not accompanied by an increase in sorghum biomass. These results, although sometimes not fully consistent, suggest that the co-presence of biochar and *S. vulgare* could be an effective combination for the recovery of PTE-contaminated soils that requires more in-depth investigation.

Other relevant soil components, such as earthworms, which are able to interact with both biochar and sorghum roots, can possibly influence the effectiveness of the biochar-*S. vulgare* combination in the remediation of PTE-contaminated soils. Earthworms, recognised as an essential part of soil fauna, are known to increase plant growth irrespective of their feeding habits (detritivorous or geophagous), accelerating the degradation of organic matter and ensuring a better availability of nutrients to plants and microorganisms (Blouin et al., 2013; Van Groenigen et al., 2014). Moreover, the activities of these soil invertebrates can alter soil pH and increase dissolved organic carbon (DOC), accelerating the biogeochemical cycling of PTEs, and increasing their plant uptake (Blouin et al., 2013; Karaca et al., 2010; Sizmur and Richardson, 2020; Udovic and Lestan, 2007). However, biochar may be stressful to soil earthworms due to its high pH and the presence of potentially toxic substances such as ammonia (especially from nitrogen rich biochars) and polycyclic aromatic hydrocarbons (Malev et al., 2015). Given the importance of earthworms to plant growth and PTE mobility, and considering their key role in soil health, it is important to define their possible influence on assisted phytoremediation programmes. To the best of our knowledge, these aspects have not been comprehensively investigated thus far (Wang et al., 2020).

This study evaluated the influence of biochar and *Eisenia fetida* on i) *S. vulgare* growth in a PTE-polluted soil; ii) PTE uptake, bioaccumulation and translocation in *S. vulgare*; and iii) PTE mobility and selected soil fertility parameters [e.g., pH, electrical conductibility (EC), dissolved organic carbon (DOC), available P, cation exchange capacity (CEC) and exchangeable Ca, Na, Mg and K] after *S. vulgare* growth.

Furthermore, the earthworm fitness, PTE concentration in earthworm tissues and PTE bioaccumulation factors were determined for *E. fetida* grown in soils (treated and untreated with biochar) planted with *S. vulgare*.

2. Materials and methods

2.1 Soil origin and sampling

Soil samples (upper 30 cm) were randomly collected from a 2 ha site neighbouring the decommissioned Montevecchio mine (SW Sardinia, Italy, N 39°40′29.71″; E 8°37′17.97″, Montevecchio-Levante), where galena (PbS) and sphalerite (ZnS) were extracted since ancient times (Manzano et al., 2020; Wanty et al., 2013). In this area, mine tailings containing high concentrations of PTEs (i.e. As, Cd, Cu, Pb, Sb and Zn) are the main source of contamination (Garau et al., 2019, 2020; Manzano et al., 2020).

2.2 Mesocosms set up and biochar treatment

Soil samples were mixed together in the laboratory, air-dried, and sieved to < 2 mm. Previous characterisation measurements on the control soil (C) determined that it was a sandy clay soil (USDA classification) with an acidic pH (6.01), and was characterised by a good content of organic matter (OM, 3.6%), total N (1.6 g kg^{-1}) and available P (P Olsen, ~ 22 mg kg^{-1}), and a high cation exchange capacity (CEC, 22.8 $cmol_{(+)} \cdot kg^{-1}$) (see Supplementary Table S1 and Garau et al., 2022 for more details). The total concentration of PTEs (i.e. As, Cd, Cu, Pb, Sb and Zn) were previously determined to exceed the thresholds established by the Italian legislation for agricultural soils (Ministerial Decree 46, 2019) and/or green (public or private) areas (Legislative Decree 152, 2006). The biochar was provided by Ronda SpA (Zanè, Italy), and was obtained by beech, poplar and elder softwood pyrolyzed at 700 °C (e.g. Mukome et al., 2013). The main chemical characteristics of the biochar were previously described (Manzano et al., 2020) and reported in Supplementary Table S1. Briefly, the biochar had a strongly alkaline pH (9.30), a total carbon content (61.32%) in line with values reported in the literature for biochar obtained from the same matrices (Mukome et al., 2013), and high concentrations of available phosphorus (84.52 g kg^{-1}) and exchangeable calcium Ca (45.08 $cmol_{(+)}$ kg^{-1}). The content of total N (3.03 g kg^{-1}) and DOC (0.020 mg g^{-1}) was lower than in similar biochar or other organic soil amendments such as compost (Manzano et al., 2020). Different mesocosms, each containing 50 kg of mass (soil alone or with biochar), were prepared as follows:

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

The amendment rates (2% and 5% w/w) were selected based on the results obtained in previous studies (Garau et al., 2022; Manzano et al., 2020). Mesocosms were kept at constant moisture (30% of their water holding capacity) for 1 month at 25 °C. During this period, they were turned carefully by hand (about 10 minutes for each mesocosm) twice a week to aerate the soil and encourage the mixing of the soil and biochar.

2.3 Pot experiment set up, E. fetida treatment and plant analysis

After the pre-incubation period, a total of 30 pots (22 cm diameter, 16 cm height) each containing 3 kg of soil derived from the different mesocosms were set up, i.e., 5 replicated pots x 3 biochar-treatments (C; B2 and B5) x 2 earthworm-treatments (+E and -E) x 1 plant species. Ten sorghum seeds (*Sorghum vulgare* L. Moench) were sown in each pot. Seven days after the seeding, 24 adult fully clitellate earthworms (*E. fetida*), with an average weight of 0.5 g each, were placed in half of the sown pots (+E treatment). This number was chosen on the basis of results obtained in our previous work (Garau et al., 2022; Sizmur et al. 2011). The earthworms were supplied by the company Bioss Sardegna (Sassari, Italy), then they were purified for 48 hours (Arnold and Hodson, 2007) before inoculation into the soil. *E. fetida* was chosen because of its tolerance to high PTE concentrations, and the ease with which they can be reared and cultured in the laboratory, making them suitable for deployment in inoculation schemes. No manure or other food source were added to pots to exclude their influence on PTE mobility, plant growth and uptake, and earthworm activity. A wire mesh (1mm x 1mm mesh size) was placed under each pot to prevent earthworms from escaping.

Pots were arranged in a completely randomized design and plants were grown over 4 months in a greenhouse under semi-controlled conditions (20-25 °C temperature, 60-70% relative humidity, and ~ 16,400 kJ m^{-2} mean global radiation) and irrigated every day. At harvest, shoots and roots were separated, washed with deionized water and dried at 55 °C for 72 h to determine the dry weight. At the same time, surviving earthworms were collected, washed with deionised water, and depurated for 24 hours (Arnold and Hodson, 2007). Afterwards, they were counted, weighed, frozen at -18 °C for 48 h, and dried at 55 °C for 72 h. The number and fresh body weight of *E. fetida*, recorded at the beginning and at the end of the experiment, were used to determine earthworms' survival rate and weight change (Huang et al., 2020). After plant growth, juveniles and eggs were searched for, but none were found.

At harvest (and for each pot), the concentration of PTEs (i.e. Pb, Cd, Zn, Cu, As and Sb) in plant tissues (roots and aboveground part) and earthworms was determined, after microwave-assisted (MARS 6) acid digestion in nitric acid (U.S. EPA Method 3052), with an Perkin Elmer Optima 7300 DV Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES). Peach leaves (NIST-SRM 1547, for plants) and mussel tissues (ERM CE278, for earthworms) were used as standard reference materials for quality assurance. For peach leaves, the measured values of As, Cd, Pb, Sb and Zn were between 87-106% of the certified values, while for mussel tissue, the measured values of all PTEs were between 89-101% of the certified values.

PTE bioaccumulation factors (BAF) and translocation factors (TF), along with mineralomasses (MM), were calculated for plants and/or earthworms as follows (Bonanno and Vymazal, 2017; Lebrun et al., 2018; Moameri and Khalaki, 2019):

- BAF_E: ratio between PTE concentration in earthworm tissues and concentration initially present in soil.
- BAF_R: ratio between PTE concentration in *S. vulgare* roots and concentration initially present in soil.
- BAF_S: ratio between PTE concentration in *S. vulgare* shoots and concentration initially present in soil.
- TF: ratio between PTE concentration in shoots and concentration present in roots.
- MM_R: *S. vulgare* root biomass x PTE concentration in roots.
- MM_S: *S. vulgare* shoot biomass x PTE concentration in shoots.

2.4 Soil properties and PTEs mobility after plant growth

After plant growth, root-adhering soil collected from plants of each pot was bulked together, sieved to < 2mm, and triplicate samples analysed to determine soil pH (ISO 10390 2005) and electric conductivity (EC; ISO 11265 1994, Gazzetta Ufficiale, 1992). Moreover, total C and N were quantified using a CHN analyzer Leco CHN 628 with an oat meal Leco part n° 502–276 as calibration sample. Dissolved organic carbon (DOC) was quantified by UV absorbance (254 nm) in filtered (0.45 μm) soil suspensions as previously described (Brandstetter et al., 1996). Available P was determined following the Olsen P method (Olsen, 1954), while exchangeable Na, Ca, K and Mg and CEC were measured using the $BaCl_2$ and triethanolamine methods (Gazzetta Ufficiale, 1992).

The same soil samples were analysed to quantify As and Sb mobility (i.e. the non-specifically sorbed labile or mobile fraction) by treating 1 g soil aliquots with 25 mL of a 0.05 M $(NH_4)_2SO_4$ solution for 4 h at 20 °C (Wenzel et al., 2001); and the labile fraction of cationic PTEs (i.e. Cd, Pb and Zn) by treating 1 g soil aliquots with 25 mL of a 0.5 M $Ca(NO_3)_2$ solution for 16 h at 20 °C (Basta and Gradwohl, 2000). The extracted PTEs were quantified as previously described (ICP-OES). A soil certified reference material (NIST-SRM 2711) was included for quality assurance.

2.5 Data analysis

Unless otherwise stated, all the analyses were performed in triplicate from each pot and reported as mean values ± standard errors (SE) in tables and figures. One-way analysis of variance (ANOVA) was carried out to investigate the effects of biochar addition (i.e., -E treatments; C, B2 and B5) on plant growth and PTEs uptake, and soil chemical features, as well as to evaluate the influence of earthworms (i.e., +E treatments vs -E ones) on the above mentioned parameters. A one way ANOVA was carried out to assess the effect of biochar on earthworms fitness (i.e. survival rate and weight loss) and PTE bioaccumulation. Two-way ANOVA was also conducted to evaluate the influence of biochar (at 2% and 5% rates) and earthworms on plant growth, PTE uptake and soil chemical features. When significant *P*-values ($P < 0.05$) were obtained for a factor, 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 Chemical properties of soil after S. vulgare growth

According to LSD test, greater soil pH was observed in biochar amended soils (~0.31 and ~0.56 units in B2 and B5 respectively, Table 1), and a further pH increase was observed in the presence of earthworms (~0.14, ~0.06 and ~0.08 units in C+E, B2+E and B5+E respectively, Table 1). These results demonstrate the alleviation of soil acidity due to biochar alkalinity (Abou Jaoude et al., 2020; Garau et al., 2022; Gu et al., 2020; Manzano et al., 2020) and earthworms cutaneous mucus secretion (Desie et al., 2020; Sizmur et al., 2009). This was the main effect of biochar and earthworms on soil characteristics.

Total organic matter and DOC content increased after biochar addition, by 1.29- and 2.35-fold for total organic matter, and 1.17- and 1.33-fold for DOC, in B2 and B5 soils, respectively, compared to control (LSD, $P < 0.05$; Table 1). This increase was due to the organic nature of the amendment added, and likely due to an increased metabolic activity in amended soils (e.g., due to enhanced root exudation of low molecular weight organic acids and/or higher microbial activity; Lebrun et al., 2018; Pinto et al., 2008). The addition of earthworms further increased the DOC content (i.e., by 1.58-, 5.50- and 1.87-fold in C+E, B2+E and B5+E treatments compared to the respective -E ones, LSD, $P <$ 0.05), likely due to a positive impact of earthworms on soil microbial activity and accelerated organic matter turnover (Sizmur et al., 2011).

The biochar addition (B2 and B5) led to an increase in available P (e.g., 1.08-fold in B5 compared to C), CEC (e.g., 1.06-fold in B5 compared to C) and exchangeable Na, Ca and Mg (LSD, $P < 0.05$; Table 1), as a result of the high specific area and the high content of these elements in available form in biochar (Table S1). However, exchangeable K decreased in biochar amended soils, and further decreased in the presence of earthworms. This could be due to the low concentration of exchangeable K in biochar, and its high affinity for this soil amendment (Manzano et al., 2020), as well as to increased K requirements of sorghum plants in the presence of biochar and earthworms (i.e., due a better plant growth) which resulted in reduced exchangeable K. This interpretation is supported by the reduction of the available P in +E treatments compared to the respective -E treatments (Table 1).

Biochar, earthworms and their interaction influenced soil chemical properties (e.g. pH, EC, DOC, exchangeable Na, K, Mg; Supplementary Table S2) after sorghum growth.

Biochar proved to be the most important treatment in conditioning soil properties (i.e. total organic carbon, total P, and CEC; Supplementary Table S2).

3.2 PTE mobility in root-adhering soil after S. vulgare growth

The PTE mobility in *S. vulgare* root-adhering soil was assessed to evaluate the potential effectiveness of sorghum, biochar, and *E. fetida* in assisted phytostabilisation interventions. Ideally, effective treatments should be able to stabilise PTEs (i.e., reduce the concentration of mobile/labile contaminants) as well as promoting plant growth and stimulating PTE storage in roots. The first point is of upmost importance since the labile fraction of PTEs is most critical in terms of environmental and human health risks (Aminiyan et al., 2021).

The extraction of mobile PTE fractions (i.e., water-soluble and readily exchangeable fractions) represented a relatively low content of As, Pb and Sb in all treatments, compared to their total concentrations (i.e., 0.00 - 1.14% of total As, 0.19 - 0.48% of total Pb, and 0.24 - 0.28% of total Sb; Fig. 1 and Fig. 2). In contrast, labile Cd and Zn represented a considerable portion of their total concentration in soil (i.e., 20.49 - 31.74% of total Cd, and 5.89 - 14.22% of total Zn; Fig. 2). Labile Cu in all the treatments (data not shown), and labile As in B2 and B5+E were under the detection limit (i.e. <0.2 $\mu g \cdot kg^{-1}$; Fig. 2).

Biochar addition reduced labile PTE concentrations (i.e., labile As, Sb, Cd, Pb and Zn decreased by ~ 4.0-, 1.0-, 1.6-, 2.8- and 2.2-fold, respectively, in B5 soil compared to the control; Fig. 1 and Fig. 2). These results could be ascribed to the biochar’s capacity to immobilize PTEs through specific adsorption mechanisms, such as complexation with carboxylic and phenolic functional groups (Supplementary Table S1; Pinna et al., 2022) and/or Fe (hydr)oxides in the biochar, or and non-specific adsorption to aromatic functional groups due to cation-π interactions (Zhu et al., 2017; Garau et al., 2022). Moreover, the pH increase recorded in biochar treated soils, and the presence of substantial carbonate and phosphate in biochar (Supplementary Table S1), likely favoured the precipitation of PTEs, reducing their mobile fractions (Kabata-Pendias and Pendias, 2000; Cao et al., 2009; Lu et al., 2017; Zhu et al., 2017).

Earthworms addition to B soils did not affect the mobility of Pb which remained only influenced (i.e., reduced) by biochar (Fig. 1); the addition of earthworms to biochar amended soil did not further reduce Pb mobility to a statistically meaningful extent. On the contrary, according to LSD test, *E. fetida* reduced Zn and Cd mobility in biochar amended soils, i.e. by 1.08- and 1.10-fold (Zn) and by 1.19- and 1.14-fold (Cd) in B2+E and B5+E, respectively compared to -E soils. Likewise, earthworms decreased labile As and Sb between 1.14- and 5.26-fold in +E treatments respectively, compared to the respective -E soils (Fig. 2). Overall, these results showed that adding *E. fetida* reduced labile PTE concentrations in biochar treated soils. This phenomenon could be due to PTE bioaccumulation by earthworms (Xiao et al., 2022) and *S. vulgare* (Vamerali et al., 2010). Indeed, *S. vulgare* biomass and PTE uptake increased when the plant was grown in biochar-treated soils and in the presence of *E. fetida* (Table 1).

Altogether, biochar, earthworms, and their interaction affected As, Pb and Zn mobility in soil (Supplementary Table S2), although biochar treatment was the most effective at influencing PTEs lability (with the exception of Sb).

3.3. S. vulgare growth in PTE-contaminated soil: influence of biochar and E. fetida

As previously mentioned, an assessment of plant growth is another necessary step in the evaluation of assisted phytostabilisation interventions (Castaldi et al. 2018; Garau et al., 2020). Independently of biochar and earthworm presence, sorghum was able to grow in the PTE-contaminated soil (Fig. 3) and no phytotoxicity symptoms were detected. The plant biomass, particularly that recorded in C soil, was similar to that observed by Ali et al. (2017) for *S. vulgare* grown in a comparable PTE-contaminated soil.

The amendment rate apparently affected plant growth, since the highest root and shoot biomass was recorded in B5 soil (Fig. 3). Root biomass increased by 1.43- and 1.71-fold in plants grown in B2 and B5 soils (-E), respectively, compared to control plants; while shoot biomass increased by 1.22- and 1.65-fold, respectively (Fig. 3). Similar findings were reported by Zand et al. (2020), i.e. significant increases in sorghum biomass were observed in a Sb-contaminated soil with increasing biochar rates (e.g., 0, 2.5 and 5%). Essentially the same finding was highlighted by Oziegbe et al. (2019) for sorghum grown in landfill soils (contaminated by multiple PTEs) amended with up to 10 t ha^{-1} biochar. These results are most likely due to a reduction of PTEs mobility, and consequent phytoavailability, in biochar-amended soils (Fig. 1 and Fig. 2) as well as due to the greater fertility of biochar-amended soils (Table 1; Garau et al. 2022).

Earthworm addition led to a further increase in plant growth, since root biomass increased up to 1.70-fold in plants grown in B5+E, compared to plants grown in B5 (Fig. 3). More subtle (yet significant) increases were also detected for shoot biomass (Fig. 3). The earthworm-driven biomass effect (which was seen in the presence and absence of biochar) could be due to a further improvement of soil fertility, as supported by soil chemical analyses after plant growth (e.g. increases in pH values and DOC when earthworms were present; Table 1), also recognised by several other authors (Yong-Li et al., 2009; Chaudhuri et al. 2012; Wang et al., 2019; Huang et al., 2020; Garau et al., 2022).

Biochar, earthworms, and their interaction influenced *S. vulgare* biomass (particularly root biomass), although biochar was the most significant treatment (Supplementary Table S2).

The results obtained highlight a clear positive interaction between biochar and earthworms which, together, effectively increased sorghum biomass in the PTE-contaminated soil.

3.4. PTE uptake by S. vulgare

With the only exception of Pb in roots, biochar addition reduced the PTE uptake by sorghum roots and shoots, and this was mostly evident for the highest biochar rate (Fig. 4 and Fig. 5). For instance, in accordance with LSD test, in B5 soil, Cd and Zn concentrations in roots were 26% and 45% lower compared to those recorded in C. Moreover, in the same soil, 43% and 56% lower concentrations were observed for Cd and Zn in shoots (Fig. 4). Lower magnitude reductions in PTE uptake were noticed for metalloids (i.e., As and Sb; Fig. 5). However, in this case shoot As and Sb were under the detection limit (i.e. $<0.2\ \mu g \cdot kg^{-1}$) when 5% biochar was added.

Interestingly, earthworm addition overall increased PTE uptake by the roots (not always and with the exception of Cd) and this was especially true for the highest biochar rate (Fig. 4 and Fig. 5). For instance, Pb, As and Sb uptake in B5+E roots was greater by 1.6-, 2.0 and 2.6-fold, respectively, compared to B5 soil, while Cd in roots reduced by ~

20% in the presence of *E. fetida* (Fig. 4 and Fig. 5). The same trend was found for sorghum shoots; e.g. Pb and Zn uptake in B5+E shoots was 1.6- and 1.2-fold greater, respectively, than B5 soil (Fig. 4).

The results obtained showed that sorghum can take up considerable quantities of PTEs and accumulate them mainly in the root system (although this may vary in relation to the PTE considered), showing good phytostabilisation capabilities (Faruruwa et al., 2013; Al Chami et al., 2015). Overall, softwood biochar reduced the uptake of PTEs by sorghum, likely as a result of immobilization of labile PTEs by the amendment (Figs. 1-2; Garau et al., 2022; Manzano et al., 2020; Oziegbe et al., 2019; Ali et al., 2020).

In contrast to the effect of the biochar, in the majority of cases (i.e., with the exception of Cd) earthworms activities led to increased PTE uptake by *S. vulgare*. Given that labile PTEs were reduced (or unaffected) in the presence of *E. fetida* (Fig. 1 and Fig. 2), the observed phenomenon could be attributed to a general improvement of soil fertility due to earthworms, which eventually promoted plant growth, root activity and PTE uptake. The lower Cd concentration in *S. vulgare* roots grown in B+E soils, compared to B-E soils, may be due to the significant reduction of bioavailable Cd pool induced by earthworms (i.e. Cd could be specifically bioaccumulated by the earthworms). This interpretation is supported by previous findings showing that *E. fetida* can accumulate Cd in the chloragogenous tissues where it is fixed into phosphate-rich granules, and/or O- or S-donating (Cd has a high affinity with sulfhydryl groups) organic ligands (Sizmur and Hodson, 2009).

Overall, biochar, earthworms, and their interaction affected the PTEs uptake by sorghum, with biochar being the main significant factor (Supplementary Table S2).

3.5 PTEs bioaccumulation, translocation and mineralomasses in S. vulgare

In order to evaluate the effect of softwood biochar, *E. fetida* and their combination on the phytoremediation capabilities of *S. vulgare*, PTEs bioaccumulation (i.e. BAF_R and BAF_S), and translocation factors (i.e. TF) were calculated along with mineralomasses (i.e. MM_R and MM_S) for plants grown on biochar amended and unamended soils, with and without earthworms.

In general, BAFs were quite low in aboveground and belowground organs, i.e. between 0.04-0.67 (Table 2). The only exception was recorded for Cd in roots ($BAF_R \geq 1$), that indicated higher concentration of this PTE in roots than initially recorded in soil. Biochar addition decreased or did not affect PTE-BAF by *S. vulgare*, in particular BAF_R values were in the order: C ≥ B2 ≥ B5 (LSD, $P < 0.05$; Table 2). The PTE-BAFs followed the same trend (Table 2). Generally, earthworm addition increased BAF_R values (with the exception of Cd-BAF_R and As- and Cu-BAF_R in C soil), with an increase between 1.02- and 2.12-fold. The BAFs did not vary between soils with or without earthworms (with the exception of Sb-BAFs in C soil). The lower PTE-BAF in plants grown in amended soils confirm a strong immobilisation of the PTEs by biochar, while the addition of earthworms led to an increase in the bioavailability of PTEs and uptake in roots.

Based on the ability of plants to accumulate and/or translocate PTEs from roots to shoots, plant species can be selected for phytostabilisation or phytoextraction programs. For this reason, the translocation factor (TF), an index that quantifies the ability of plants to transfer PTEs from roots to shoots (Bonanno and Vymazal, 2017), was calculated. Irrespective of the treatment applied, sorghum plants showed TF < 1 (i.e. between 0.00 -

0.45) for all the PTE considered, which followed the order: Cd > Zn > Sb > Cu >Pb > As (LSD, $P < 0.05$; Table 2). This indicates that PTEs were mainly accumulated in belowground organs and poorly translocated in aboveground parts. These data are in agreement with the results reported by other researchers, which showed low TFs for As, Cd, Co, Cu, Pb and Zn in sudan gass (Marchiol et al., 2007; Wei et al., 2008). Biochar addition at 5% rate consistently decreased TF for Sb, Cd, Pb and Zn, while 2% rate had less of an effect (Table 2). Moreover, it should be noted that Pb and Zn TF recorded in plants grown in control soil decreased in the presence of earthworms (C+E) whereas, in B+E soils, the effect of earthworms on PTE translocation was more limited. The effect of biochar (and to some extent the combination with the earthworms) on soil fertility and the reduction of PTE mobility may have favoured the development of adaptive characteristics in sorghum, such as the reduction of PTE translocation in the aboveground part of the plant (Noguera et al., 2012; Soudek et al., 2015, 2017; Razaq et al., 2017).

PTE mineralomasses (MM_R and MM_S) are useful to estimate the contaminant removal by the plant, since they quantify the actual amounts of PTEs accumulated and stored in plant tissues (Lebrun et al., 2018). MM_R values in all treatments were higher (between 1.85- and 34.02-fold) than those of MM_S, confirming that all the PTEs considered were preferentially stored in roots, as opposed to shoots (Table 2). For all the PTEs considered (except Zn) MM_R were always higher in sorghum grown on biochar amended soils compared with control plants (Table 2). This supports the view that biochar decreased PTE mobility (Fig. 1 and Fig. 2), but at the same time stimulated plant growth (Fig. 3), which eventually led to a higher PTE removal efficiency by roots. However, a very limited influence of biochar was noted on MM_S (Table 2). Earthworm addition increased the MM_R of all the PTEs considered, especially in biochar amended soil, where increases between 1.06- and 3.50-fold were observed (Table 2). An influence, albeit reduced, of earthworms was observed also in MM_S, where the increases were smaller than in MM_R, and ranged from 1.00- to 2.45-fold compared to -E plants.

Taken together, these results make it possible to state that *S. vulgare* could be effectively used in combination with softwood biochar and earthworms for the assisted phytostabilisation of PTE-contaminated soils.

3.6 PTE concentration and bioaccumulation in E. fetida and acute ecotoxicity effects

It was shown in previous studies that biochar and plants can influence the health status of earthworms (i.e., their survival rate and weight loss), as well as the bioaccumulation of PTEs into their bodies (Wang et al., 2019). Eggs or juveniles were not found in any of the soils, likely because the presence of multiple PTEs impaired *E. fetida* reproduction. The survival rate and the weight loss of earthworms in C+E and B2+E were not statistically different, whereas survival rate decreased by ~2-fold and average weight loss increased by ~ 2.3-fold in B5+E, compared to C+E (Table 3). This finding suggests that *E. fetida* was able to survive in PTE contaminated soils, though the addition of softwood biochar at the higher rate (i.e., 5%) showed toxic effects. This was in agreement with Garau et al. (2022) and Shi et al. (2021), who showed that the addition of different biochars (e.g., cow dung, corncob and sewage sludge) at 5.0 and 7.5% rates induced mortality and weight losses in *E. fetida*. Earthworms probably ingested biochar particles, which may contain toxic contaminants (i.e. polycyclic aromatic hydrocarbons; Malev et al., 2015) as well as high concentrations of PTEs, thus explaining the decline of the survial rate and the increased weight loss in the presence of 5% biochar (Sizmur and Hodson, 2009).

The highest As, Sb, Cu, Pb and Zn concentrations were recorded for earthworms incubated in B2+E soil followed by C+E and B5+E (LSD, $P < 0.05$; Table 3). With regards to Cd concentration in earthworms tissues, it followed the order: B5+E ≥ B2+E ≥ C+E (Table 3).

The PTEs BAF values were generally lower than 1 for all PTE considered, with the exception of Cd, and followed the trend: Cd>As>Cu≥Zn>Pb>Sb (LSD, $P < 0.05$; Table 3). A similar order and relatively low BAF values were recorded by Ruiz et al. (2009), Liu et al. (2017) and Garau et al. (2022). Given the higher mobility of Cd, compared to other PTEs, it is not surprising that the Cd BAF values were higher than 1 in all the samples, reaching the highest values in B5+E soil. The BAF values of all PTEs (except Cd) increased for earthworms grown in B2+E compared to C+E and overall followed the order B2+E > C+E > B5+E, while BAF for Cd followed the trend B5+E > B2+E > C+E (LSD, $P < 0.05$; Table 3).

The *E. fetida* PTE concentrations and BAFs seem to contrast with those reported by other authors (Garau et al., 2022; Huang et al., 2020; Wang et al., 2020), who showed lower PTEs-BAF and concentration in *Eisenia* spp. in contaminated bare soils amended with biochar. Greater secretion of exudates by sorghum roots in biochar-amended soils, combined with higher earthworm activity (particularly in B2+E, considering it had the highest survival rate and the lowest weight loss of *E. fetida*) may have favoured the remobilization of PTEs from biochar, resulting in increased PTEs bioaccumulation by earthworms, as a possible explanation for this phenomenon.

Finally, *E. fetida* is a compost earthworm rather than a geophagous one. However, it does exhibit geophagous behaviour when added to soils without a litter layer, and this is the premise for its widespread use in ecotoxicology testing. The results of this study demonstrated that *E. fetida* was able to tolerate contaminated soils amended with a low biochar rate, promoted biochar homogenisation with the soil and facilitated phytostabilisation. Even at the highest dose of soil amendment, a biochar + earthworm synergistic effect was observed, despite a reduction in the earthworm population, and this could be ascribed to a certain influence of the earthworm necromass. Finally, it is fair to say that a self-sustaining population of *E. fetida* in contaminated soil in cold climates is unlikely, but it may provide short-term benefits by helping to establish a vegetative cover and create the necessary conditions for colonisation by other earthworm species.

4. Conclusions

The results obtained indicated that the combined use of sorghum, softwood biochar, and *E. fetid*a earthworms can represent an effective strategy for the assisted phytostabilisation of PTE-contaminated soils. The synergistic action of biochar and earthworms reduced mobile PTEs in soil, promoted plant growth, and increased the amount of PTEs absorbed mainly by sorghum roots. At the same time, the increased PTE mineralomasses in plants grown in biochar- and earthworm-treated soils indicated the suitability of the approach in reducing the mobility of PTEs in soil. Despite this, clear evidence of toxicity was observed for *E. fetida* in the presence of 5% biochar, raising questions on the impact that high rates of biochar can have on soil biota. So, the best combination would seem to be 2% biochar + earthworms. However, further studies are needed to establish the long-term stability of the observed effects as well as to evaluate the suitability of the earthworm- and biochar-assisted phytoremediation approach in field conditions.

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Figure captions

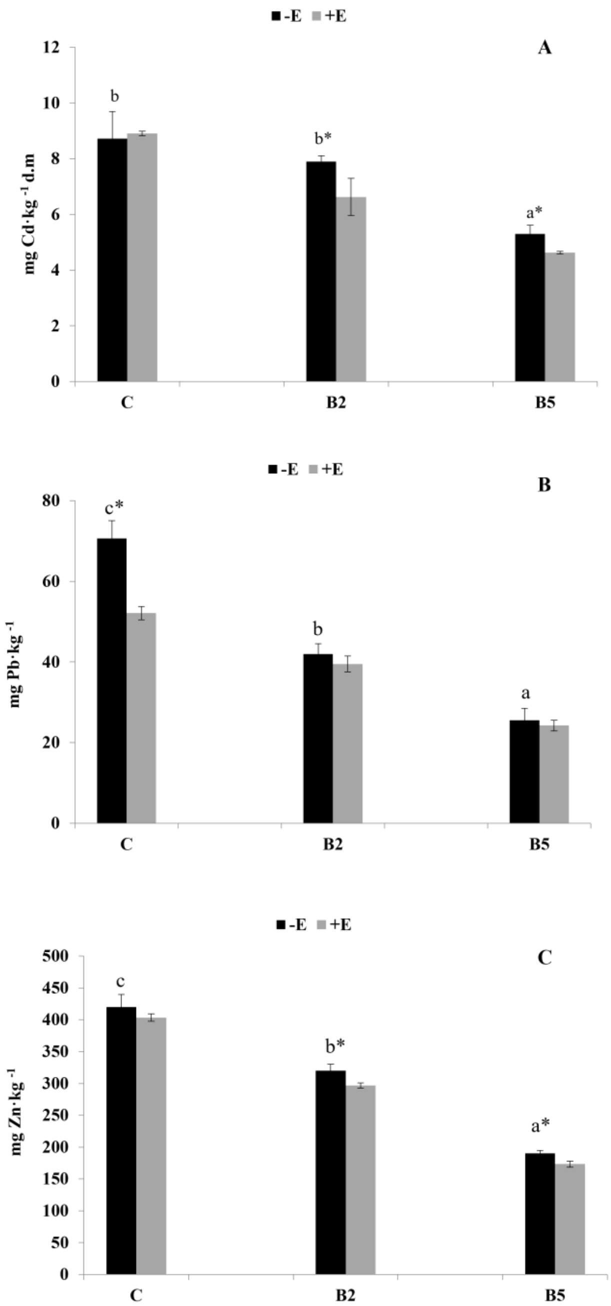


Fig. 1. Mobile (labile) fraction of Cd (A), Pb (B) and Zn (C) determined by extraction with 0.5 M $Ca(NO_3)_2$ (mean values ± SE) after *S. vulgare* growth in biochar treated (B2 and B5) and untreated (C) soils, and in the presence (+E) and absence (-E) of *E. fetida*. For each PTE, different letters on top of each bar denote statistically significant differences due to biochar addition (i.e., C, B2 and B5 were compared), while asterisk (*) indicates statistically significant differences due to *E. fetida* addition (i.e., C vs C+E; B2 vs B2+E; and B5 vs B5+E) according to the Fisher's Least Significant Difference (LSD) test ($P < 0.05$).

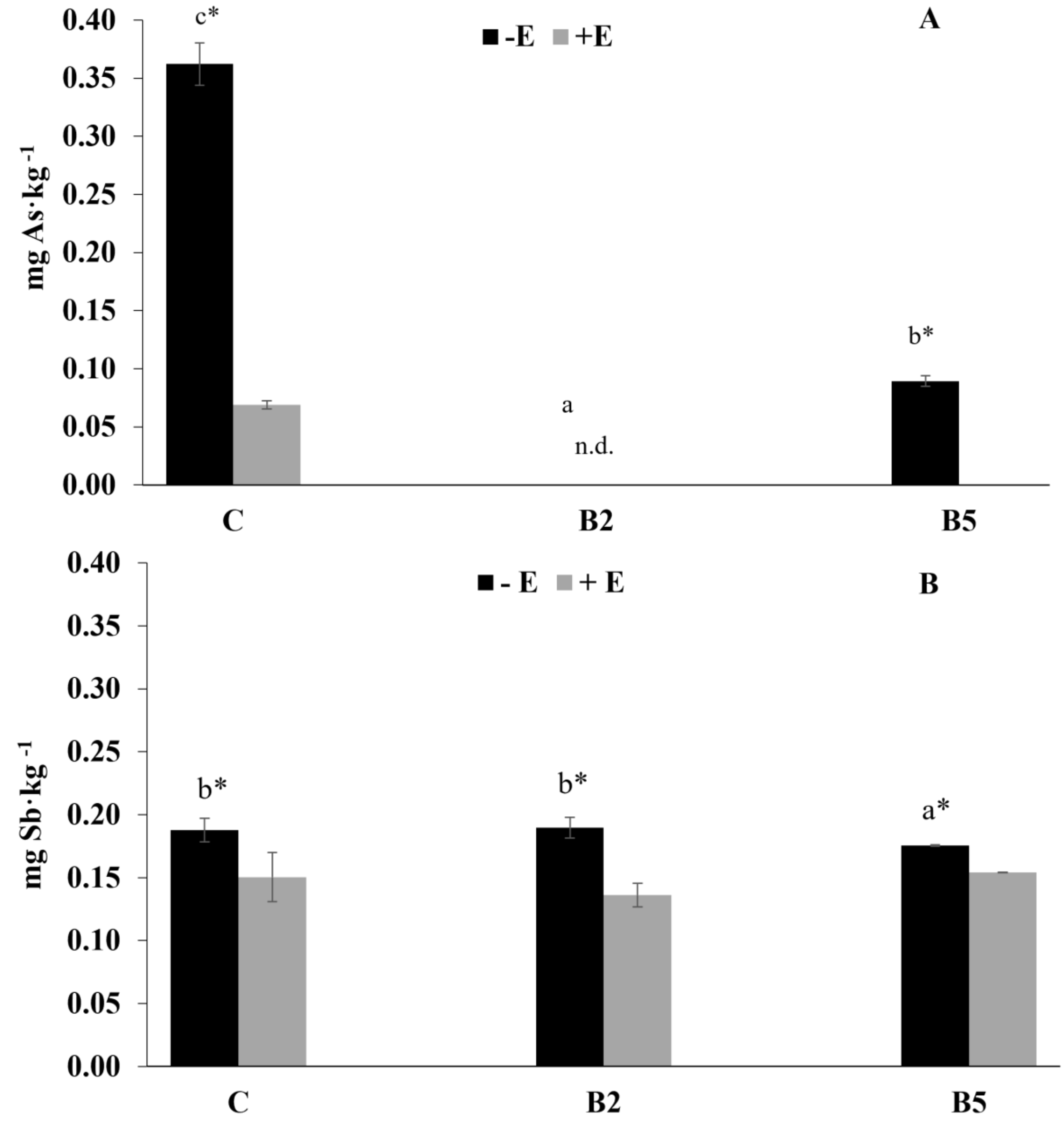
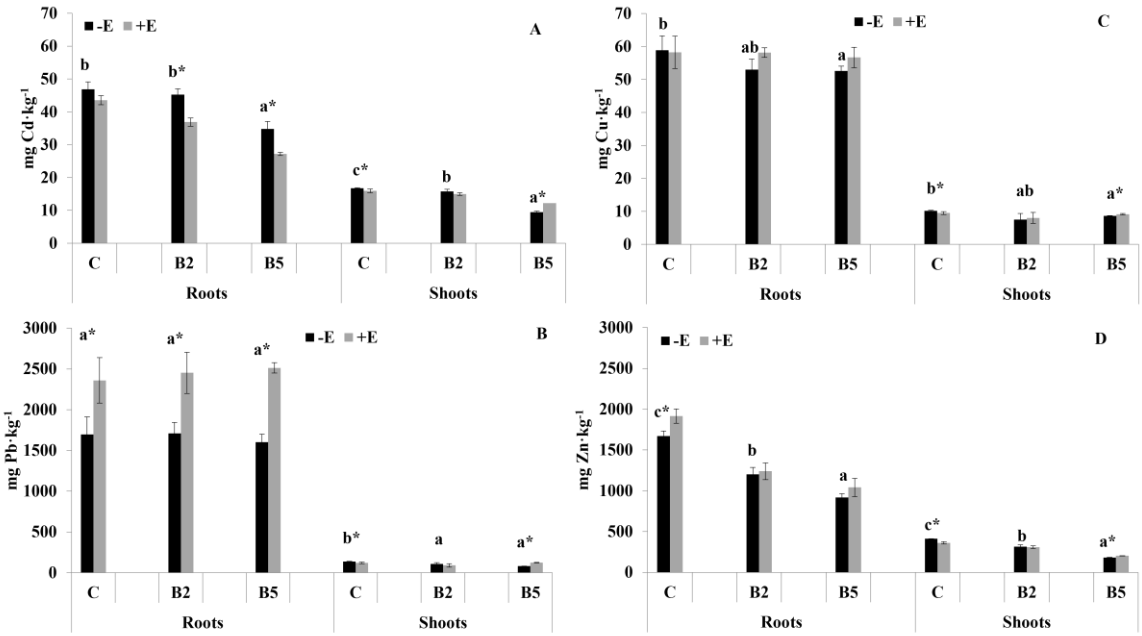


Fig. 2. Mobile (labile) fraction of soil As (A) and Sb (B) determined by extraction with 0.05 M $(NH_4)_2SO_4$ (mean values ± SE) after *S. vulgare* growth in biochar treated (B2 and B5) and untreated (C) soils, and in the presence (+E) and absence (-E) of *E. fetida*. For the meaning of the letters and asterisk (*) on top of each bar, see the caption of Fig. 1. n.d.: under detection limit (i.e. <0.2 $\mu g \cdot L^{-1}$).


A
B
C
D
-E +E
mg Cd·kg-1
mg Pb·kg-1
mg Cu·kg-1
mg Zn·kg-1
C B2 B5 C B2 B5
Roots
Shoots

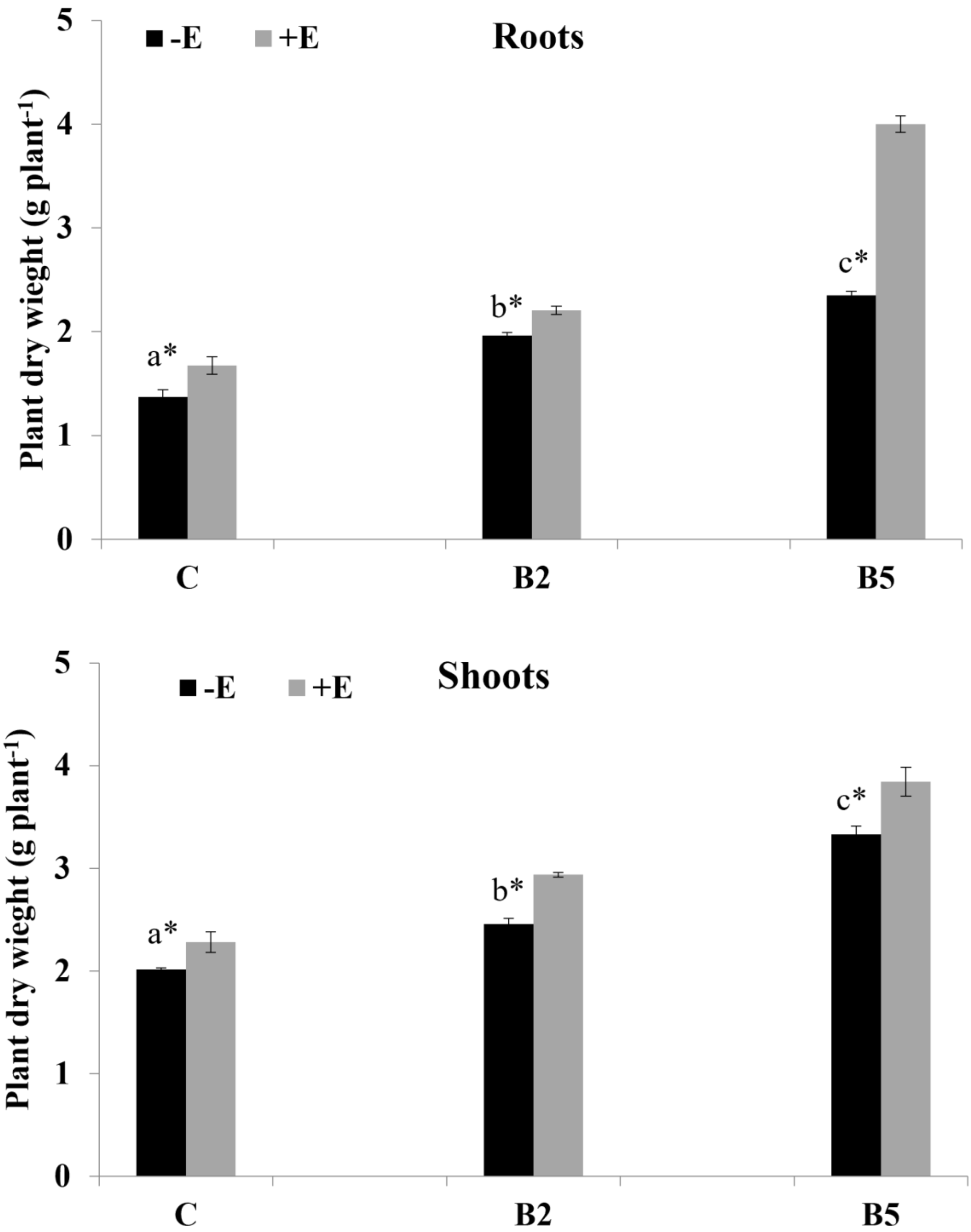


Fig. 3. Root and shoot dry weight (mean values ± SE) of *S. vulgare* grown in biochar treated (B2 and B5) and untreated (C) soils, and in the presence (+E) and absence (-E) of *E. fetida*. For each plant part, for the meaning of the letters and asterisk (*) on top of each bar, see the caption in Fig. 1.

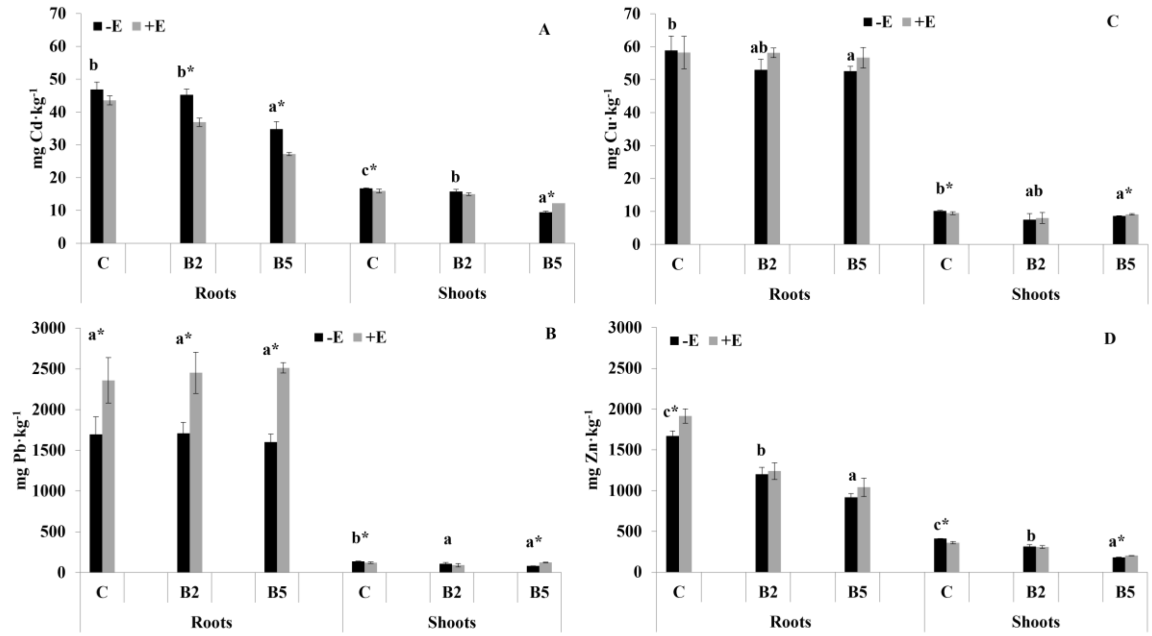


Fig. 4. Cd (A), Pb (B), Cu (C) and Zn (D) in roots and shoots of *S. vulgare* grown on biochar and *E. fetida* treated and untreated soils. For the meaning of the letters and asterisk (*) on top of each bar, see the caption of Fig. 1.

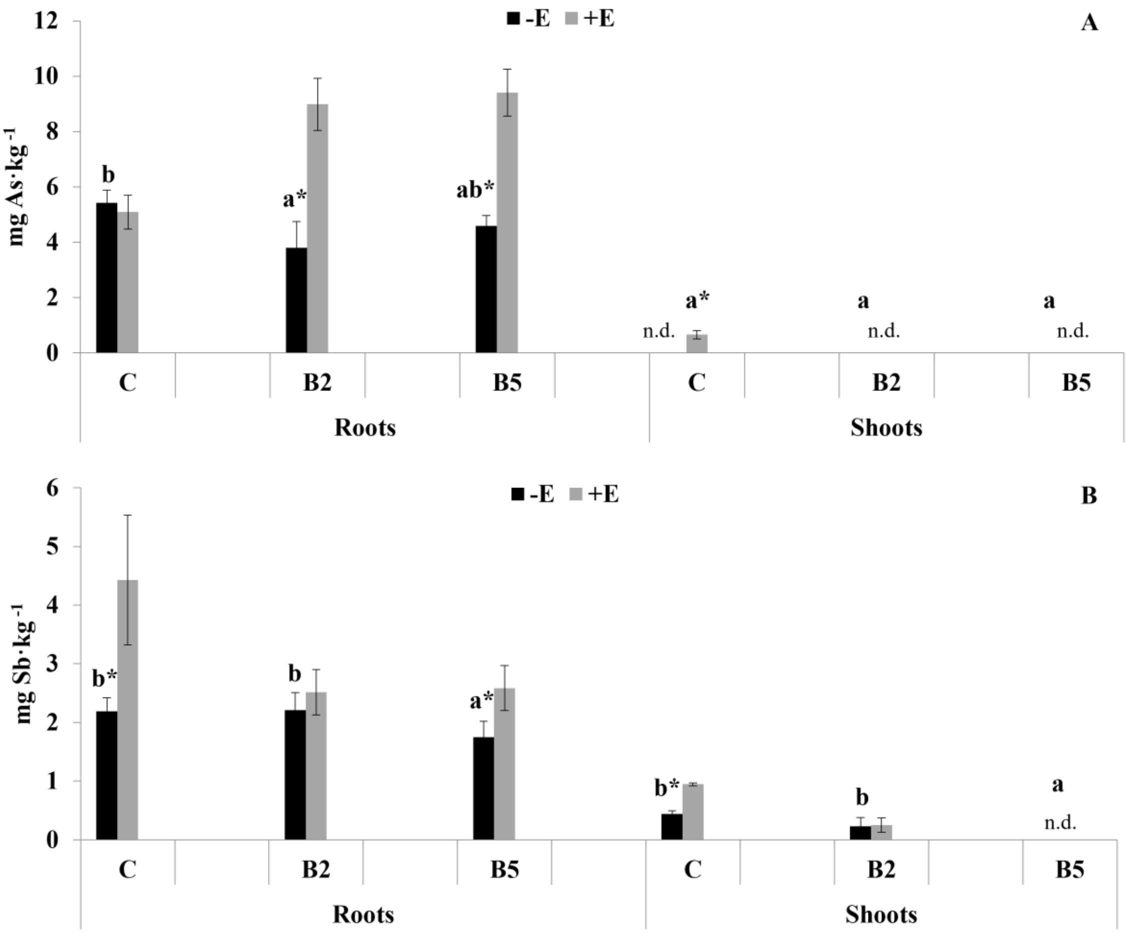


Fig. 5. As (A) and Sb (B) in roots and shoots of *S. vulgare* grown on biochar and *E. fetida* treated and untreated soils. For the meaning of the letters and asterisk (*) on top of each bar, see the caption in Fig. 1. n.d.: under detection limit (i.e. $<0.2\ \mu g \cdot L^{-1}$).

Table 1

Characteristics of the untreated (C) and biochar treated soils (B2 and B5) with (+E) and without *Eisenia fetida*, after *S. bicolor* growth.

	C	C+E	B2	B2+E	B5	B5+E
pH	6.19±0.00^{a*}	6.33±0.01	6.50±0.02^{b*}	6.56±0.01	6.75±0.00^{c*}	6.83±0.01
EC ($mS \cdot cm^{-1}$)	888±14.14^{a*}	834.5±16.26	967.5±6.36^{b*}	936.5±10.60	1005±15.55^{c}	1011.5±14.85
Total organic matter (%)	3.80±0.15^{a}	3.88±0.19	4.92±0.19^{b*}	5.69±0.22	8.93±0.59^{c}	8.28±0.25
Total N (%)	0.17±0.01^{a}	0.18±0.02	0.15±0.02^{a}	0.16±0.01	0.17±0.01^{a}	0.18±0.01
DOC ($mg \cdot g^{-1}$)	0.12±0.00^{a*}	0.19±0.03	0.14±0.01^{b*}	0.77±0.01	0.16±0.05^{b*}	0.30±0.01
Total P ($g \cdot kg^{-1}$)	2.45±0.12^{a}	2.52±0.04	2.34±0.02^{a}	2.34±0.07	2.28±0.13^{a}	2.27±0.13
P available ($mg \cdot kg^{-1}$)	26.58±0.04^{a*}	24.62±0.10	26.92±0.34^{a*}	25.82±0.42	28.75±0.32^{b}	28.38±0.16
Cation Exchange capacity (CEC, $cmol_{(+)} \cdot kg^{-1}$)	22.83±0.05^{a}	22.96±0.19	23.99±0.26^{b}	24.15±0.14	24.20±0.09^{b}	24.83±0.64
Exchangeable Na ($cmol_{(+)} \cdot kg^{-1}$)	1.54±0.05^{a*}	2.09±0.09	2.35±0.00^{b}	2.35±0.00	2.42±0.03^{c}	2.48±0.04
Exchangeable K ($cmol_{(+)} \cdot kg^{-1}$)	1.40±0.03^{c*}	1.28±0.08	1.23±0.00^{b*}	1.16±0.03	1.19±0.03^{a*}	1.04±0.03
Exchangeable Ca ($cmol_{(+)} \cdot kg^{-1}$)	18.94±0.72^{a}	19.07±0.77	19.76±0.96^{a}	20.03±0.47	22.50±0.92^{b}	21.71±0.54
Exchangeable Mg ($cmol_{(+)} \cdot kg^{-1}$)	1.40±0.05^{a*}	1.56±0.00	1.45±0.05^{a*}	1.56±0.00	1.56±0.00^{b}	1.56±0.00

Mean values ± SE followed by different letters within a row denote statistically significant differences due to biochar addition (i.e., C, B2 and B5 were compared), while the presence of asterisk (*) denotes statistically significant differences due to *E. fetida* addition (i.e., C vs C+E; B2 vs B2+E; and B5 vs B5+E were compared), according to the Fisher's Least Significant Difference (LSD) test ($P < 0.05$).

Table 2

PTE bioaccumulation (BAF_R and BAF_S) and translocation (TF) factors, and mineralomasses (MM_R and MM_S) in *S. bicolor* grown in untreated (C) and biochar treated soils (B2 and B5), with (+E) and without *Eisenia fetida*.

	C	C+E	B2	B2+E	B5	B5+E
BAFr						
As	0.19 ± 0.05^{a}	0.18 ± 0.02	$0.14\pm0.03^{a*}$	0.32 ± 0.09	$0.16\pm0.01^{a*}$	0.34 ± 0.07
Sb	$0.04\pm0.00^{a*}$	0.07 ± 0.01	0.04 ± 0.01^{a}	0.04 ± 0.01	0.03 ± 0.00^{a}	0.04 ± 0.01
Cd	1.64 ± 0.08^{b}	1.52 ± 0.05	$1.58\pm0.06^{b*}$	1.29 ± 0.05	$1.00\pm0.08^{a*}$	1.22 ± 0.04
Cu	0.28 ± 0.02^{a}	0.27 ± 0.02	0.25 ± 0.02^{a}	0.27 ± 0.01	0.25 ± 0.01^{a}	0.27 ± 0.02
Pb	$0.16\pm0.03^{a*}$	0.22 ± 0.03	$0.16\pm0.01^{a*}$	0.22 ± 0.03	$0.15\pm0.01^{a*}$	0.23 ± 0.01
Zn	0.59 ± 0.02^{c}	0.67 ± 0.11	0.42 ± 0.03^{b}	0.43 ± 0.06	0.32 ± 0.02^{a}	0.36 ± 0.04
BAFs						
As	–	0.02 ± 0.00	–	–	–	–
Sb	$7.03\cdot10^{-3}\pm0.01^{b*}$	$15.22\cdot10^{-3}\pm0.00$	$3.66\cdot10^{-3}\pm0.01^{ab}$	$4.08\cdot10^{-3}\pm0.00$	$0.00^{a}\pm0.00$	0.00 ± 0.00
Cd	0.59 ± 0.00^{b}	0.56 ± 0.02	0.55 ± 0.02^{b}	0.52 ± 0.02	0.38 ± 0.02^{a}	0.43 ± 0.03
Cu	0.05 ± 0.00^{b}	0.04 ± 0.01	0.04 ± 0.01^{a}	0.04 ± 0.01	0.04 ± 0.00^{a}	0.04 ± 0.00
Pb	0.01 ± 0.00^{b}	0.01 ± 0.00	0.01 ± 0.00^{ab}	0.01 ± 0.00	0.01 ± 0.00^{a}	0.01 ± 0.00
Zn	0.14 ± 0.00^{c}	0.13 ± 0.01	0.11 ± 0.01^{b}	0.11 ± 0.01	0.06 ± 0.00^{a}	0.07 ± 0.01
TF						
As	$–^{a*}$	0.13 ± 0.01	$–^{a}$	–	$–^{a}$	–
Sb	0.20 ± 0.01^{c}	0.21 ± 0.06	0.10 ± 0.01^{b}	0.10 ± 0.01	$–^{a}$	–
Cd	0.36 ± 0.02^{b}	0.37 ± 0.01	$0.35\pm0.02^{b*}$	0.41 ± 0.01	$0.27\pm0.01^{a*}$	0.45 ± 0.02
Cu	0.17 ± 0.02^{a}	0.16 ± 0.02	0.14 ± 0.03^{a}	0.14 ± 0.03	0.16 ± 0.00^{a}	0.16 ± 0.01
Pb	$0.08\pm0.02^{b*}$	0.05 ± 0.01	0.06 ± 0.02^{a}	0.04 ± 0.02	0.05 ± 0.00^{a}	0.05 ± 0.00
Zn	$0.25\pm0.01^{b*}$	0.19 ± 0.04	0.26 ± 0.02^{b}	0.25 ± 0.02	0.20 ± 0.01^{a}	0.20 ± 0.02
MMr						
As	$7.42\cdot10^{-3}\pm0.08\cdot10^{-3a*}$	$8.51\cdot10^{-3}\pm0.02\cdot10^{-3}$	$7.44\cdot10^{-3}\pm0.89\cdot10^{-3a*}$	$19.83\cdot10^{-3}\pm0.81\cdot10^{-3}$	$10.76\cdot10^{-3}\pm0.88\cdot10^{-3b*}$	$37.63\cdot10^{-3}\pm1.39\cdot10^{-3}$
Sb	$3.00\cdot10^{-3}\pm0.31\cdot10^{-3a*}$	$7.41\cdot10^{-3}\pm0.85\cdot10^{-3}$	$4.34\cdot10^{-3}\pm0.33\cdot10^{-3b*}$	$5.55\cdot10^{-3}\pm0.70\cdot10^{-3}$	$4.10\cdot10^{-3}\pm0.64\cdot10^{-3b*}$	$10.34\cdot10^{-3}\pm0.98\cdot10^{-3}$
Cd	$0.06\pm0.00^{a*}$	0.07 ± 0.00	$0.09\pm0.00^{b*}$	0.07 ± 0.00	$0.08\pm0.01^{b*}$	0.11 ± 0.00
Cu	$0.08\pm0.00^{a*}$	0.10 ± 0.01	$0.10\pm0.01^{b*}$	0.13 ± 0.00	$0.12\pm0.00^{c*}$	0.23 ± 0.01
Pb	$2.33\pm0.04^{a*}$	3.95 ± 0.06	$3.36\pm0.03^{b*}$	5.41 ± 0.08	$3.76\pm0.02^{b*}$	10.05 ± 0.03
Zn	$2.29\pm0.01^{a*}$	3.21 ± 0.05	$2.36\pm0.02^{a*}$	2.74 ± 0.04	$2.15\pm0.02^{a*}$	4.16 ± 0.04
MMs						
As	$–^{a*}$	$1.57\cdot10^{-3}\pm0.08\cdot10^{-3}$	$–^{a}$	–	$–^{a}$	–
Sb	$0.88\cdot10^{-3}\pm0.01\cdot10^{-3b*}$	$2.16\cdot10^{-3}\pm0.05\cdot10^{-3}$	$0.56\cdot10^{-3}\pm0.18\cdot10^{-3b}$	$0.75\cdot10^{-3}\pm0.20\cdot10^{-3}$	$–^{a}$	–
Cd	0.03 ± 0.00^{a}	0.04 ± 0.01	0.04 ± 0.01^{b}	0.04 ± 0.01	0.03 ± 0.01^{a}	0.04 ± 0.02
Cu	0.02 ± 0.00^{a}	0.02 ± 0.00	0.02 ± 0.00^{a}	0.02 ± 0.00	0.03 ± 0.00^{b}	0.04 ± 0.01
Pb	0.28 ± 0.01^{a}	0.27 ± 0.00	0.26 ± 0.01^{a}	0.27 ± 0.00	$0.27\pm0.01^{a*}$	0.48 ± 0.02
Zn	0.83 ± 0.03^{b}	0.82 ± 0.03	$0.78\pm0.02^{b*}$	0.91 ± 0.01	$0.60\pm0.01^{a*}$	0.78 ± 0.01

Mean values ± SE followed by different letters within a row denote statistically significant differences due to biochar addition (i.e., C, B2 and B5 were compared), while the presence of asterisk (*) denotes statistically significant differences due to *E. fetida* addition (i.e., C vs C+E, B2 vs B2+E, and B5 vs B5+E were compared), according to the Fisher's Least Significant Difference (LSD) test ($P < 0.05$).

Table 3

Eisenia fetida fitness (survival and weight loss rates), PTEs concentration and bioaccumulation factors (BAF) in untreated (C+E) and biochar treated soils (B2+E and B5+E) after *S. bicolor* growth.

	C+E	B2+E	B5+E
E. fetida survival rate (%)	87.05±1.37^{b}	89.58±1.28^{b}	42.89±0.81^{a}
Weight loss rate (%)	7.75±0.97^{a}	6.13±0.69^{a}	17.84±0.45^{b}
PTEs concentration in *E. fetida* (mg·kg^{-1})			
As	9.55±0.04^{a}	22.08±0.05^{b}	n.d.
Sb	2.83±0.05^{a}	6.77±0.03^{b}	n.d.
Cd	57.85±4.92^{a}	84.14±2.23^{b}	85.90±2.45^{b}
Cu	66.45±2.78^{b}	93.93±3.84^{c}	20.46±5.69^{a}
Pb	2586±14.98^{b}	4230±85.65^{c}	460.34±12.46^{a}
Zn	857.41±7.53^{b}	1329±25.36^{c}	214.53±5.14^{a}
E. fetida BAF			
As	0.34^{a}	0.79^{b}	n.d.
Sb	0.05^{a}	0.11^{b}	n.d.
Cd	2.02^{a}	2.94^{b}	3.00^{b}
Cu	0.31^{b}	0.44^{c}	0.094^{a}
Pb	0.24^{b}	0.39^{c}	0.04^{a}
Zn	0.31^{b}	0.47^{c}	0.08^{a}

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