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Research article

Combining biochar and grass-legume mixture to improve the phytoremediation of soils contaminated with potentially toxic elements (PTEs)

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ABSTRACT

The combination of soil amendments with plants can be a viable option for restoring the functionality of PTEs-contaminated soils. Soil recovery could be further optimized through the mixed cropping of plant species (e.g. legumes and grasses) with different physiological characteristics. The aim of this study was to assess the phytoremediation ability of *Vicia villosa* Roth. And *Lolium rigidum* Gaud. Grown alone or in mixture in a soil contaminated with PTEs (C), i.e. Cd (23 mg kg^{-1}), Pb (4473 mg kg^{-1}) and Zn (3147 mg kg^{-1}), and amended with 3% biochar (C + B). Biochar improved soil fertility and changed PTEs distribution, reducing soluble fractions and increasing the more stable ones. The addition of biochar increased the plant biomass of hairy vetch and annual ryegrass, both in monoculture and when in mixture. For example, shoot and root biomass of the C + B intercropped hairy vetch and annual ryegrass increased 9- and 7-fold, and ~3-fold respectively, compared to the respective C plants. The biochar addition decreased PTE-uptake by both plants, while mixed cropping increased the uptake of PTEs by shoots of hairy vetch grown in C and C + B. The bioaccumulation, translocation factors, and mineralomass showed that hairy vetch and annual ryegrass behaved as phytostabilising plants. PTE mineralomasses proved that mixed cropping in C + B increased the overall capacity of PTE accumulation by plant tissues, particularly the root system. Therefore, the combination of biochar and legumes/grasses mixed cropping could be an effective solution for the recovery of PTEs-contaminated soils and the mitigation of their environmental hazard.

1. Introduction

Widespread environmental degradation caused by soil pollution is becoming unsustainable and restoring the functionality of contaminated sites is a major global challenge [1]. Among the main sources of soil pollution (e.g., industrial activities, urban and industrial waste mismanagement, unsustainable agricultural practices and transport), mining activities are considered the main

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anthropogenic source of potentially toxic elements (PTEs, e.g. Pb, Cd, Cu, Zn, As, Sb). Due to their ubiquity, toxicity, persistence and accumulation, PTEs are responsible for severe soil pollution, which is why the United States Environmental Protection Agency and the European Union defined PTEs as priority pollutants.

In this context, the restoration of PTEs-contaminated soils is one of the most important environmental priorities also in relation to the SDG goal 15. Among soil clean-up strategies, phytoremediation is the simplest, cheapest, nature-based and most environmentally friendly option; it also improves soil quality and can be used in combination with other physico-chemical treatments [2,3]. Various types of phytoremediation can be employed, depending on the clean-up interventions and the characteristics of the plants selected [4]. Importantly, selected plants should have high tolerance to PTEs, rapid growth rate, great biomass yield, and an extensive root system [5]. In this context, the employment of legume-species in phytoremediation seems to be promising [6,7]. Among these species, hairy vetch (*Vicia villosa* Roth.), used as a cover crop especially in semi-arid temperate environments, behaved as a phytostabilising plant for Pb [8] and As [9]. Hairy vetch improves soil carbon balance and nitrogen fixation, soil structure and water-holding capacity, and prevents erosion [10,11]. Forage grasses have also received considerable attention as promising candidates for the recovery of PTEs-polluted soils. Among them annual ryegrass (*Lolium rigidum* Gaud.), due to its fast growth, adaptability to Mediterranean climate, self-reseeding capacity [12] and tolerance to contaminants, appears to be suitable for the revegetation and remediation of PTEs-contaminated soils [13].

Phytoremediation can be strengthened by mixed cropping mixtures of plant species (e.g. Fabaceae and Poaceae) with complementary characteristics [14]. For instance, Zu et al. [15] suggested the intercropping of *Sonchus asper* L. and *Vicia faba* as a phytoremediation strategy of Cd-contaminated soils. Such intercropping increased the biomass of both plants, as well as the Cd concentration and translocation from the roots to the shoots of *S. asper*. In contrast, Cd concentration in *V. faba* (both in roots and shoots), as well as its transfer coefficient decreased. Cui et al. [16] proposed the mixed cropping of perennial ryegrass (*L. perenne* L.) and alfalfa (*Medicago sativa* L.) as a strategy to increase biomass and reduce Pb uptake in forage plants. Based on the literature (albeit not many papers are available), the mixed cropping of legumes and grasses, e.g. hairy vetch and annual ryegrass, due to their characteristics (i.e., extensive root system and nitrogen fixation by hairy vetch, and the fast growth and PTEs tolerance by annual ryegrass) could allow an effective environmental recovery of PTEs-contaminated soils. Although their mixture has proven to be a suitable strategy for sustainable agricultural systems [17], to the best of our knowledge, mixed cropping of hairy vetch with annual ryegrass has not yet been investigated as a phytoremediation solution of PTEs-contaminated soils.

The effectiveness of plant-based approaches for the recovery of PTEs-contaminated soils can be hindered by the hostile habitat due to high concentrations of PTEs in bioavailable form, poor soil structure, reduced organic matter content, low nutrients and extreme pH values [18]. In such degraded soils, the use of organic amendments can be helpful to improve soil physico-chemical and biological properties and reduce the content of labile PTEs. In turn, this can promote plant growth which can more easily contribute to soil restoration through the so-called “assisted phytoremediation” [19,20]. Recently, among organic soil amendments, there has been growing interest in biochar, a carbon-rich by-product obtained by the pyrolysis of different biomasses at high temperatures under oxygen-deficient conditions. Biochar amendment showed beneficial effects on soil structure, pH, organic carbon content and stability, and therefore plant growth [21]. Biochar also revealed a good adsorbent for PTEs, reducing their mobility and stimulating revegetation of contaminated soils [22,23]. Furthermore, the use of biochar derived from the pyrolysis of contaminated biomass (e.g., plants grown on PTEs-contaminated sites) represents an added value in the perspective of the circular economy and can be a viable way to produce soil amendments [24,25]. The biochar effect on PTEs mobility and uptake by the plants depends on the intrinsic biochar characteristics such as porosity, specific surface area, surface functional groups, cation exchange capacity, and pH among the others [26,27], as well as on PTE type, and plant species [28,29].

Based on the above considerations, we assumed that mixing two different crops with complementary characteristics (e.g. Fabaceae and Poaceae), combined with biochar addition to the soil, can ensure improved recovery of PTEs-contaminated soils. Furthermore, the use of a biochar derived from plant biomass grown on contaminated soils represents an added value that can guarantee the environmental sustainability of the remediation. However, as far as we know, there are no manuscripts in the literature investigating the formulated hypothesis. Accordingly, further research is needed to set up and evaluate a technology that combines the cultivation of different plant species with the biochar amendment and provide suitable protocols for the recovery of PTEs-contaminated soils.

Consequently, the aim of this study was to assess the suitability of the combined use of a biochar, produced by the pyrolysis of biomass grown in a Pb contaminated industrial site, with annual ryegrass and hairy vetch as monocrops or in mixtures, for the assisted phytoremediation of a mining soil contaminated with PTEs.

2. Materials and methods

2.1. Origin and properties of soil and biochar, sampling and experimental set-up

The contaminated soil was collected within a range of 3.5 km from the Montevecchio dismissed mining site located in the Southwestern Sardinia (Italy, N 39°33′34.3″; E 8°35′16.8″, Guspini). According to the World Reference Base for Soil Resources [30], the soil is classified as Lithic Leptosol (Fluvic, Technic, Toxic). Montevecchio was one of the main mining sites in Italy and was exploited for more than a century (1848–1991) to extract Pb and Zn from galena (PbS) and sphalerite [(Zn,Fe)S]. Soil samples were randomly collected from the top soil layer (0–30 cm), pooled in the laboratory to obtain a composite soil that was air-dried, sieved to <2 mm, physically and chemically characterized. Particle size was determined using the pipette method [31] which allowed to classify the soils as a sandy loam (USDA textural classification, 67% sand, 15% silt, 18% clay). The composite soil was divided in 2 subsamples: contaminated untreated soil (control, C-soil), and contaminated soil treated with 3% (w/w) biochar (C + B-soil).

Populus nigra grown on a Pb contaminated industrial site in Marcianise (Campania, Italy), was used as biomass for biochar production. After harvesting, the biomass was ground and pelletized in size L and Ø: 1 × 0.5 cm x cm. The biomass was heated to the final temperature of 465 °C and at constant heating rate (10 °C/min). Such a choice, which represents an added value in a circular economy perspective, shows that PTE-polluted areas can also be recovered in term of productivity, e.g. through the production of plant biomass which can be useful in the environmental recovery. The biomass and the corresponding biochar were characterized, and physico-chemical properties are listed in Table S1.

After biochar addition, treated and untreated soils were separately mixed, and then incubated for 2 months at 20 °C. During this time, they were mixed once a week and kept at 40% of their water-holding capacity (WHC). The WHC value chosen was derived from preliminary laboratory tests, and was attributable to the soil characteristics, as at values above 40% WHC the soil became muddy. After the contact, soil sub-samples were air-dried and chemical analyses were carried out to evaluate the influence of biochar on soil chemical properties (Table 1). Soil pH and electric conductivity (EC) were measured in 1:2.5 (w/v) solid to water suspension; cation exchange capacity (CEC) and the concentration of exchangeable Ca, Mg, K and Na were determined using the $BaCl_2$ and triethanolamine method; extractable P was determined using the Olsen method, while active carbonates were determined by reaction of soil with 0.1 M ammonium oxalate [31]. The dissolved organic carbon (DOC) content was estimated as previously described by Manzano et al. [32]. Total C and total N were determined using a CHN analyzer (Leco CHN 628) and Soil LCRM Leco part n° 502–697 as calibration sample. Pseudo-total PTEs concentration (i.e. Cd, Pb and Zn) was quantified in soil, after digestion with aqua regia reverse solution (HNO_3/HCl, 3:1 v/v) and microwave mineralization (Milestone MLS1200), using a PerkinElmer AAnalyst 200 flame atomic absorption spectrometer (FAAS) for Zn quantification and a PerkinElmer AAnalyst 400-HGA 900 graphite furnace atomic absorption spectrometer (GFAAS), for Pb and Cd quantification. A standard reference material (NIST-SRM 2711A) for quality assurance and quality control was included, with PTE recoveries around ±10% of the certified values.

2.2. *Influence of biochar on PTEs mobility*

The influence of biochar on Cd, Pb and Zn mobility was determined in triplicate independent soil samples collected from each mesocosm following the sequential extraction procedure proposed by Basta and Gradwohl [33]. The concentration of soluble and exchangeable Cd, Pb and Zn was determined after extraction with 0.5 M $Ca(NO_3)_2$ (Fraction 1, F1). The PTEs forming weak surface complexes were quantified after extraction with 1 M NaOAc (F2), whereas 0.1 M Na_2EDTA was used to extract strongly surface-complexed and precipitated PTEs (F3). Following each step of the sequential extraction, samples were centrifuged and filtered to separate the liquid and the solid phases. The concentration of PTEs in filtered solutions was determined as previously described. After the third step, the residual solid phase was dried at 105 °C overnight and digested using a HNO_3 and HCl mixture (3:1 v/v ratio) in a Microwave Milestone MLS 1200. PTEs were then quantified as previously described (F4).

2.3. *Plant-growth experiment and plant analysis*

After the contact period, the soil from each treatment was used to fill plastic pots (18 cm diameter, 22 cm height) each containing approximately 6 kg of soil, which were planted separately with hairy vetch seeds (vetch; 7 g m^{-2}), annual ryegrass seeds (ryegrass; 3 g m^{-2}) and hairy vetch + annual ryegrass seeds (4 + 2 g m^{-2}). A total of 24 pots were prepared, 4 replicated pots x 2 soil treatments (C: untreated soil and C + B: biochar-treated soil) x 3 plant treatments (vetch, ryegrass, and vetch + ryegrass):

Pot 1–4: hairy vetch grown in C-soil;

Pot 5–8: hairy vetch grown in C + B-soil;

Table 1
Selected chemical characteristics of control (C) and biochar amended (C + B) soils (mean ± SE, n = 3). Different letters in a line indicate statistically significant differences ($P < 0.05$).

	C	C + B
pH_{H2O}	5.95 ± 0.01^b	6.68 ± 0.04^a
EC ($ms \cdot cm^{-1}$)	3.28 ± 0.03^a	2.68 ± 0.03^b
DOC ($mg \cdot g^{-1}$)	0.02 ± 0.00^b	0.03 ± 0.00^a
Ash (%)	90.7 ± 0.05^a	86.3 ± 0.02^b
Total C (%)	1.63 ± 0.03^b	3.50 ± 0.01^a
Total N (%)	0.08 ± 0.01^a	0.11 ± 0.01^a
O.M. (% d.m.)	2.82 ± 0.05^b	6.03 ± 0.01^a
Active carbonate ($g \cdot kg^{-1}$)	17.1 ± 1.30^a	7.90 ± 1.50^b
Extractable P ($mg \cdot kg^{-1}$)	0.78 ± 0.00^b	1.17 ± 0.09^a
CEC ($cmol_{(+)} \cdot kg^{-1}$)	25.3 ± 0.02^a	25.3 ± 0.77^a
Ca^{2+} ($cmol_{(+)} \cdot kg^{-1}$)	9.44 ± 0.03^b	9.94 ± 0.12^a
Mg^{2+} ($cmol_{(+)} \cdot kg^{-1}$)	2.90 ± 0.05^a	2.46 ± 0.00^b
K^+ ($cmol_{(+)} \cdot kg^{-1}$)	0.51 ± 0.01^b	1.14 ± 0.01^a
Na^+ ($cmol_{(+)} \cdot kg^{-1}$)	0.89 ± 0.01^a	0.64 ± 0.01^b
GSB (%)	54.4 ± 0.16^a	56.1 ± 2.20^a
Cd ($mg \cdot kg^{-1}$)	22.6 ± 4.00^a	22.3 ± 3.86^a
Pb ($mg \cdot kg^{-1}$)	4473 ± 48.7^a	4468 ± 48.1^a
Zn ($mg \cdot kg^{-1}$)	3147 ± 49.1^a	3148 ± 46.8^a

Pot 9–12: annual ryegrass grown in C-soil;
Pot 13–16: annual ryegrass grown in C + B-soil;
Pot 17–20: mixed cropping (hairy vetch + annual ryegrass) in C-soil;
Pot 21–24: mixed cropping (hairy vetch + annual ryegrass) in C + B-soil.

These plant species with complementary characteristics, belonging to the Fabaceae (i.e. hairy vetch) and Gramineae (i.e. annual ryegrass) families, have been selected because they are tolerant to high PTE concentrations [8,13], are well adapted to the Mediterranean climate [8,9,12] and are potentially suitable for the successful phytoremediation of PTEs-contaminated soils. Pots were arranged according to a completely randomized design and plants were grown over 3 months, from April to July 2021, in a naturally-lit greenhouse at an average temperature of 20–25 °C and 60–70% relative humidity. The application of biochar was the only source of exogenous plant nutrients. No other fertilisation was provided to highlight the impact of biochar on the biomass of vetch and ryegrass. Plants were irrigated with tap water twice a week through a drip system.

Upon harvesting, the shoots were separated from the roots and were accurately washed with deionized water and oven dried at 55 °C for 72 h to determine their respective dry weight. The land equivalent ratio (LER) was then calculated as:

$$LER = L_{hairy}\ vetch + L_{annual\ ryegrass};$$

Where L is the ratio between the yield of vetch or ryegrass in mixed cropping and that of the same species grown in monoculture [34].

The PTEs content (i.e. Cd, Pb, and Zn) in shoots and roots was determined, after mineralization of plant tissues with H_2O_2 and a mixture of 69% HNO_3 and ultrapure H_2O (ratio 1:1), in a Microwave Milestone MLS 1200 (EPA Method 3052), using FAAS for Zn and GFAAS for Pb and Cd quantification. Peach leaves were used as standard reference material (NIST-SRM 1547).

The PTEs bioaccumulation (BAF) and translocation (TF) factors as well as mineralomasses (MM), were then calculated [35] for each plant species grown in each soil.

- BAFr: ratio between the PTE concentration in roots and that present in the soil;
- BAFs: ratio between the PTE concentration in shoots and that present in the soil;
- TF: ratio between the PTE concentration in shoots and that present in roots;
- MMr: plant root biomass x PTE concentration in roots;
- MMs: plant shoot biomass x PTE concentration in shoots.

2.4. Statistical analysis

All chemical analyses were performed in triplicate and mean values± standard errors (SE) are reported in tables and figures.

Data on soil chemical parameters, PTEs mobility, root and shoot dry weight, PTEs uptake, bioaccumulation and translocation factors, and mineralomasses were subjected to a Shapiro–Wilk normality test. Next, Levene's test was performed to verify the homoscedasticity of the data. Subsequently, data obtained by soil analysis, plant weight and PTEs uptake were analyzed using a one-way ANOVA followed by Student's t-test ($P < 0.05$); Tukey test was used to compare bioaccumulation and translocation factors, and mineralomasses when significant p-values ($P < 0.05$) were obtained.

To evaluate the effect of biochar, mixed cropping and their interaction on the plant data a two-way analysis of variance (two-way ANOVA) was used, followed by multiple means comparison test (Fisher least squares difference). The significance of statistical computations was evaluated at $P < 0.05$ unless otherwise stated. The data analysis was carried out using the Sigma Plot Software (SPSS Inc., Chicago, II, USA).

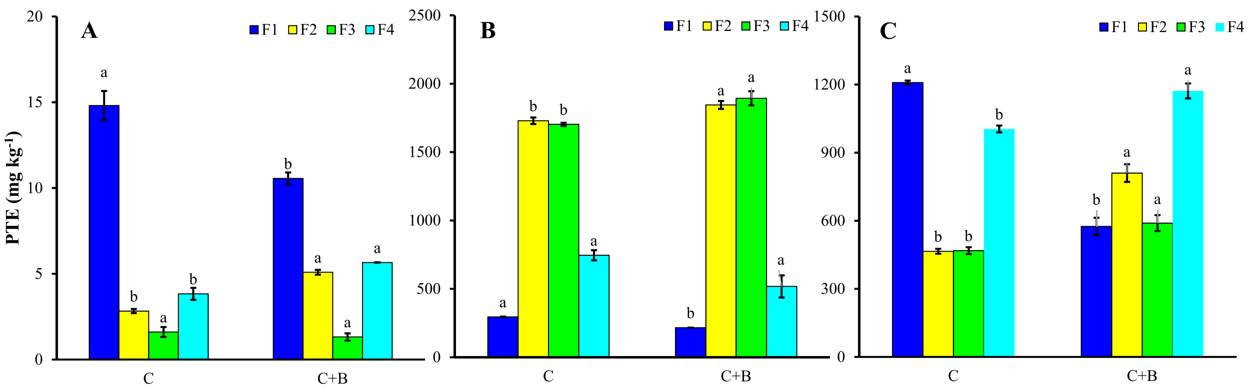

Fig. 1. Cd (A), Pb (B), and Zn (C) released after sequential extraction procedure (mg kg^{-1}, means ± SE; n = 3) in control (C) and biochar amended (C + B) soils. F1=Ca(NO_3)$_2$; F2=NaOAc; F3=Na_2-EDTA; F4=HNO_3/HCl. For each PTE and within each fraction, bars with different letters denote statistically significant differences according to the Student's t-test ($P < 0.05$).

3. Results and discussion

3.1. Influence of biochar on soil properties and PTEs mobility

The biochar used in this study was characterized by a very alkaline pH (i.e., 9.9), a high total carbon content (i.e., ~84%), and high concentrations of Ca, K, and Mg (Table S1). Although the biochar derived from *P. nigra* grown in a contaminated soil, its Cd, Pb, and Zn content was below the threshold limits imposed by the Italian legislation for amendments and fertilizers [36]. The addition of biochar to the soil caused an increase of pH, DOC (+50%) and organic matter (+2.14-fold), as well as exchangeable Ca and K and available P (+5, +123, and +50%, respectively) (Table 1), confirming the potential of biochar at improving the quality and fertility of PTEs-contaminated soils [22].

The results of the sequential extraction showed that, in the C-soil, the water-soluble and readily exchangeable PTEs (F1, Fig. 1) were ~66, 7, and 38% of the total Cd, Pb and Zn respectively. It is important to emphasize that the PTEs concentration in Fraction 1 in C-soil (i.e. 14.8 mg kg^{-1} for Cd, 295 mg kg^{-1} for Pb and 1209 mg kg^{-1} for Zn) already exceeded the threshold concentration (total) for potentially contaminated agricultural soils [37]. The PTEs concentration detected in F1 could pose a serious environmental hazard, as this fraction represents the most mobile and potentially bioavailable pool, accountable for environmental and human health risks [38]. The biochar application (C + B-soil) reduced the F1 of Cd, Pb and Zn by 29, 27 and 52% respectively compared to the C-soil (Fig. 1). Although the concentrations detected in C + B still represent an ecological risk, the decreases detected were however considerable. The liming effect of biochar, the PTEs precipitation promoted by its inorganic component (e.g. phosphate [39], the formation of complexes between the carboxyl and phenolic functional groups of biochar and PTEs [23], as well as the non-specific adsorption due to cation-π interactions with aromatic rings in biochar [40], were most likely the mechanisms underpinning the reduction of the PTEs exchangeable pool. The fractions extracted with NaOAc (F2) were 3, 39, and 15% of the total Cd, Pb, and Zn respectively. The concentration of Cd and Zn quantified in F2 increased after the biochar addition (Fig. 1). This was attributed to the non-specific adsorption between the biochar functional groups and Cd and Zn, which led to the formation of acid-soluble complexes, exchangeable with NaOAc. Furthermore, the pH increase in the C + B-soil and the expected release of CO_2 from the partial mineralization of biochar could have contributed to the formation of Cd and Zn bicarbonates, which were solubilized in this step. The observed effect of biochar on Cd and Zn extracted in F2 was less relevant for Pb (Fig. 1).

The relatively immobile, and not readily bioavailable or leachable pool of PTEs (F3) accounted for 7, 38 and 15% of the Cd, Pb and Zn total. The addition of biochar increased the Na_2-EDTA extractable fraction of Pb (+10%) and Zn (+26%), while the effect was not significant for Cd (Fig. 1). The fraction of Pb–F3 in C + B, which was already the most abundant in C, increased further, accounting for 42% of the total Pb, highlighting strong interactions between Pb and biochar functional groups (e.g. –COOH and –OH phenolics [41]) and, consequently, the effectiveness of biochar in immobilizing Pb.

The residual fraction of PTEs (F4), i.e. the very insoluble and/or occluded PTEs, was 17, 17 and 32% of the total Cd, Pb and Zn

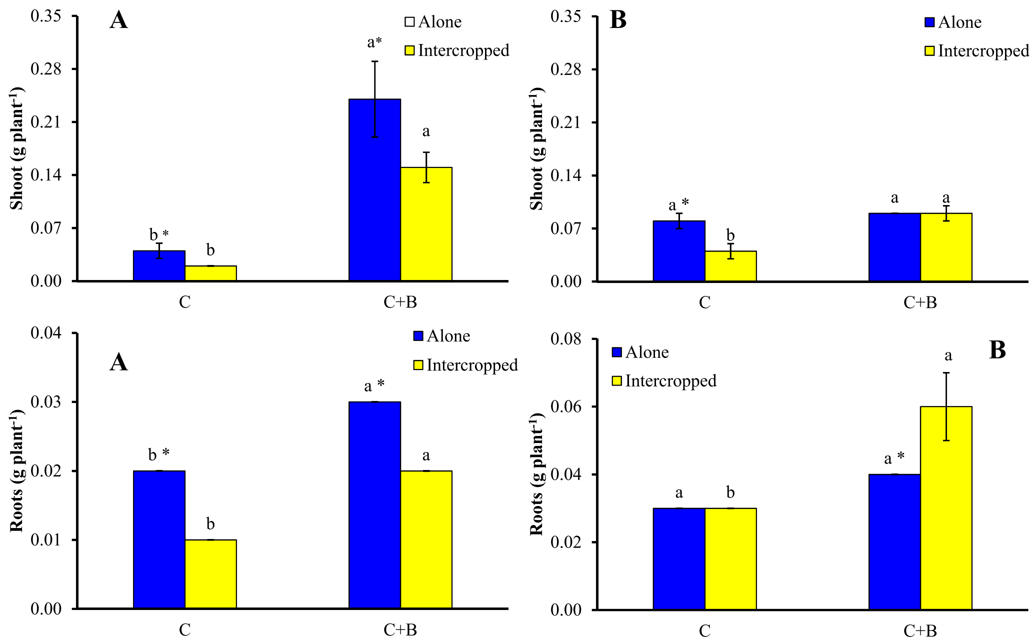

Fig. 2. Roots and shoots dry weight (g $plant^{-1}$, mean ± SE; n = 3) of A = hairy vetch and B = annual ryegrass (alone and in mixture) grown in control (C) and biochar amended (C + B) soils. For each plant part (shoots or roots) and within each cropping system (alone or in mixture), different letters denote statistically significant differences due to biochar addition (i.e., C *vs* C + B); while asterisk (*) denotes statistically significant differences due to type of cultivation (i.e., C alone *vs* C in mixture; C + B alone *vs* C + B in mixture), according to the Tukey's test ($P < 0.05$).

respectively (Fig. 1). The F4 of Cd and Zn increased by 1.5 and 1.2-fold respectively, in C + B compared to C. This result can be attributed to the formation of insoluble precipitates in the biochar's network of pores and fissures. In the C + B-soil, this fraction became the main Zn-pool. This result, also considering the effect of biochar on Zn concentration in F1 and F3, confirmed the efficiency of biochar in the immobilization of Zn [32]. The addition of biochar decreased the residual fraction of Pb (<31% in the C + B soil). This was attributed to the increased formation of weak and strong complexes between Pb and organic and inorganic biochar compounds, as evidenced by the rise of Pb in F2 and F3.

Overall, these results highlight the ability of biochar to improve soil fertility and immobilize PTEs through their re-distribution from soluble (potentially bioavailable, F1) to more stable (hardly bioavailable, F3 and F4) ones.

Furthermore, it is interesting to note that the transformation of biomass from contaminated soils into a valuable product such as biochar, which can be used for environmental purposes, fits the principles of the circular economy related to the creation of closed-loop agricultural systems where the value of plants waste increases and its impact decreases.

3.2. *Influence of biochar and mixed cropping on plant yield*

The addition of biochar to the PTEs contaminated soil increased vetch biomass in monoculture and in mixture (Fig. 2A). The root biomass of vetch alone increased by 1.5-fold in C + B, while shoot biomass increased by 6-fold compared to the control plants. This can be ascribed to the neutralization of pH, increased nutrient availability, as well as the immobilization of PTEs in the C + B-soil [42,43]. Our results agree with those of Helaoui et al. [44], who showed that biochar improved the adaptability of vetch to Cd-contaminated soils, and Rees et al. [45], which demonstrated that biochar was able to reduce the genotoxicity associated with the presence of Cd, Pb and Zn, thus promoting the growth of vetch in contaminated soils. The general improvement of soil quality favored by biochar appeared to be less effective on the growth of ryegrass alone (Fig. 2B).

Compared to monoculture, and probably due to the competition for resources that may limit the plant growth in a pot [46,47], in the controls the mixed cropping of vetch and ryegrass reduced both the aerial (by ~2-fold) and the root (by 2-fold in vetch) biomass (Fig. 2A and B). The mixed cropping of C + B-plants reduced by about 1.5 times the aerial and root biomass of vetch, while it did not lead to a change in the ryegrass shoot alone or in a mixture. However, comparing mixed plants grown on biochar-treated or untreated soil, the aerial biomass of C + B vetch plants increased by 7.5-fold and the root biomass by 2-fold, while the root and shoot biomass of ryegrass increased ~2-fold, compared to C plants. The total biomass (vetch + ryegrass), both root and aerial, increased in C + B compared to the control (Fig. S1), as did the LER, which was 0.73 and 1.16 in C and C + B-soil, respectively, indicating an advantage of the mixed cropping in the biochar-treated soil. Biochar, by limiting the bioavailability of PTEs, decreased their toxic effect responsible for reduced root development [48]. Overall, this led to positive effects on the growth of both plants, irrespective of their root morphological characteristics. Presumably, biochar addition, by increasing the C/N ratio of the soil, enhanced the biological N fixation of vetch [49], which in turn may have promoted increased N uptake and growth of ryegrass [50]. Similar results were reported by Rees et al. [45,51], who observed, in two separate experiments, improved growth of *Lolium perenne* and *Vicia faba* when grown alone in PTEs-contaminated soils treated with biochar. Beneficial effects of biochar on the yield of vetch and ryegrass grown in mixed cropping, but in uncontaminated soils, were also reported by different authors [e.g. 52–53]. In contrast, Liu et al. [54] observed that the addition

Table 2
Influence of biochar, mixed cropping, and mixed cropping × biochar interaction on plant biomass and PTEs concentration in plants determined by two-way ANOVA (i.e. F values are reported).

Plants	Parameter	Biochar	Mixed cropping	Biochar x Mixed cropping
	Plant yields			
Vicia villosa	Shoot biomass	33.2***	3.15	1.51
	Roots biomass	23.1***	3.50	0.76
	PTE concentration in roots			
	Cd	54.4***	18.1**	5.07*
	Pb	126***	3.38*	6.52*
	Zn	415***	1.30	2.06
	PTEs concentration in shoots			
	Cd	494***	19.8***	0.45
	Pb	5.88*	21.4***	0.00
	Zn	105***	10.2**	27.2***
Lolium rigidum	*Plant yields*			
	Shoot biomass	13.6**	5.43*	4.53*
	Roots biomass	9.16**	2.38*	17.2**
	PTE concentration in roots			
	Cd	2.05	0.86	0.01
	Pb	25.5***	3.68	0.03
	Zn	13.3***	4.97*	0.44
	PTEs concentration in shoots			
	Cd	0.91	0.02	1.50
	Pb	12.2**	0.67	1.15
	Zn	17.9***	0.08	0.12

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

of a biochar from residues pyrolysed at 800 °C resulted in a decrease and increase in the above-ground biomass of intercropped vetch and ryegrass, respectively, while a biochar from wheat straw pyrolysed at 550 °C had an insignificant effect on the biomass of the intercropped system. This points out that the effects of biochar must be evaluated on a case-by-case basis, as soil amendment could have no effect or even decrease plant growth and biomass yield with consequences on phytoremediation efficiency.

Biochar, mixed cropping, and their interaction influenced the biomass of vetch, biochar alone was ineffective on that of ryegrass (both root and aerial), while mixed cropping and their interaction with biochar affected shoots and roots biomass of plants, respectively (Table 2).

3.3. Influence of biochar and mixed cropping on PTEs uptake

The concentration of PTEs in plants alone or in mixture decreased with the application of biochar (Figs. 3 and 4).

In vetch alone (Fig. 3), shoots and roots concentration of Cd, Pb and Zn was reduced by 67 and 41%, 25 and 32% and 55 and 54%, respectively, in the amended soil. The same trend (i.e., a PTE reduction after biochar addition) was also observed for the intercropped vetch (Fig. 3). As expected, the addition of biochar to the soil, by changing the partitioning of Cd, Pb and Zn from easily exchangeable forms to more stably bound fractions, reduced their bioavailability and then their uptake by the plants.

It has been reported that mixed cropping can not only affect plant biomass production, but also change the accumulation of PTEs in plants [55]. In C or C + B vetch, mixed cropping did not affect Pb and Zn concentration in roots (compared to monoculture plants, Fig. 3), whilst it reduced Cd concentration by 27% and 18% in the roots of C and C + B respectively; a similar reduction was observed by Zu et al. [15] in Cd concentration in *V. faba* roots when intercropped with *S. asper*. By contrast, irrespective of biochar, PTEs uptake by shoots increased in intercropped vetch compared to monoculture (e.g. +16, 47 and 42% for Cd, Pb and Zn respectively in C-plants). Biochar and mixed cropping thus appear to have an opposite effect on the uptake of PTEs by vetch; in particular, mixed cropping seems to favour, independently of biochar, the phytoextraction of PTEs by vetch.

Regarding ryegrass, in both plant-alone and mixed cropping treatments, biochar reduced the Pb and Zn uptake in roots and shoots, while it did not affect Cd concentration (Fig. 4). These results are in line with the reduced uptake of PTEs due to biochar addition observed in vetch, and agree with those of Karami et al. [56], who observed reduced Pb levels in ryegrass after amendment of a contaminated soil with a biochar derived from oak, ash, sycamore and birch. Mixed cropping, when compared with monocrops of ryegrass, reduced Zn concentration by 20% in roots, and Pb concentration by 15% in shoots in C soil (Fig. 4). This is consistent to what observed by Cui et al. [16], which reported a reduction in Pb concentration in the shoots and roots of perennial ryegrass when intercropped with alfalfa, and suggests that the ryegrass-vetch mixed cropping pattern could improve the quality of forage crop cultivated in a Pb-contaminated soil. Biochar and mixed cropping show the same reducing effect on the uptake of Pb and Zn in ryegrass; while the treatments had no influence on the uptake and partitioning of Cd.

Overall, biochar, mixed cropping and their combination influenced PTEs uptake by plants differently (Table 2). Biochar proved to

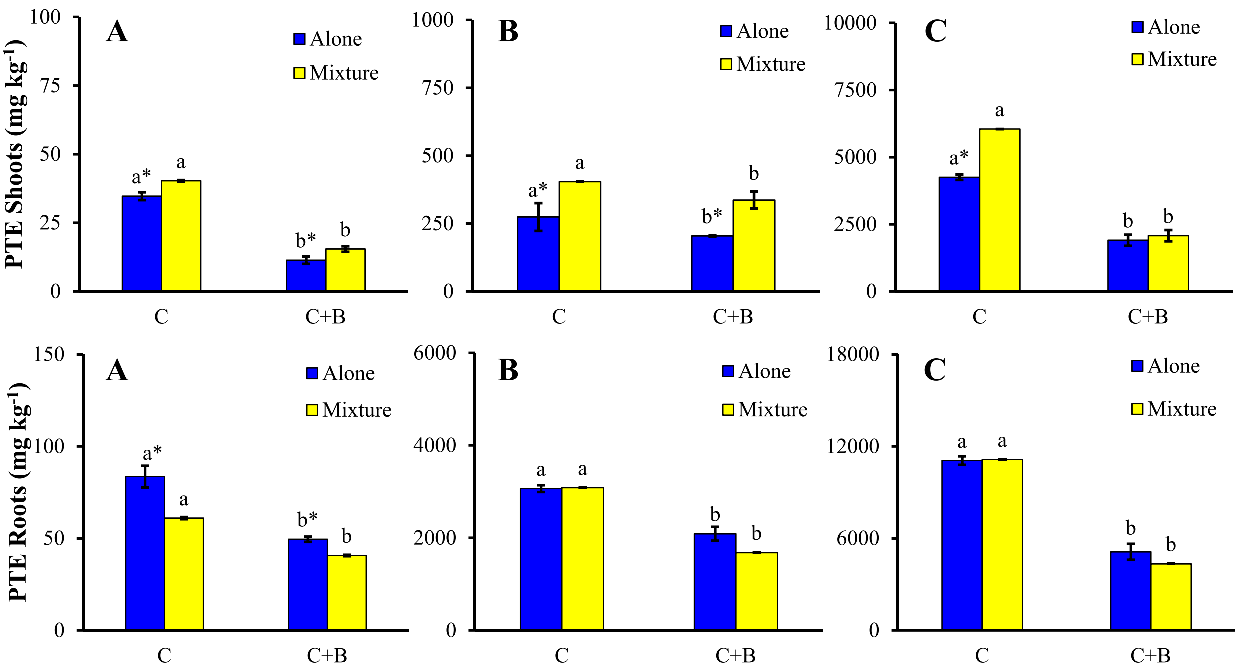

Fig. 3. Cd (A), Pb (B), and Zn (C) in shoots and roots (mg kg^{-1}, mean ± SE; n = 3) of hairy vetch grown alone or in mixture, in control (C) and biochar amended (C + B) soils. For the meaning of the letters and asterisk (*) on top of each bar, see the caption of Fig. 2.

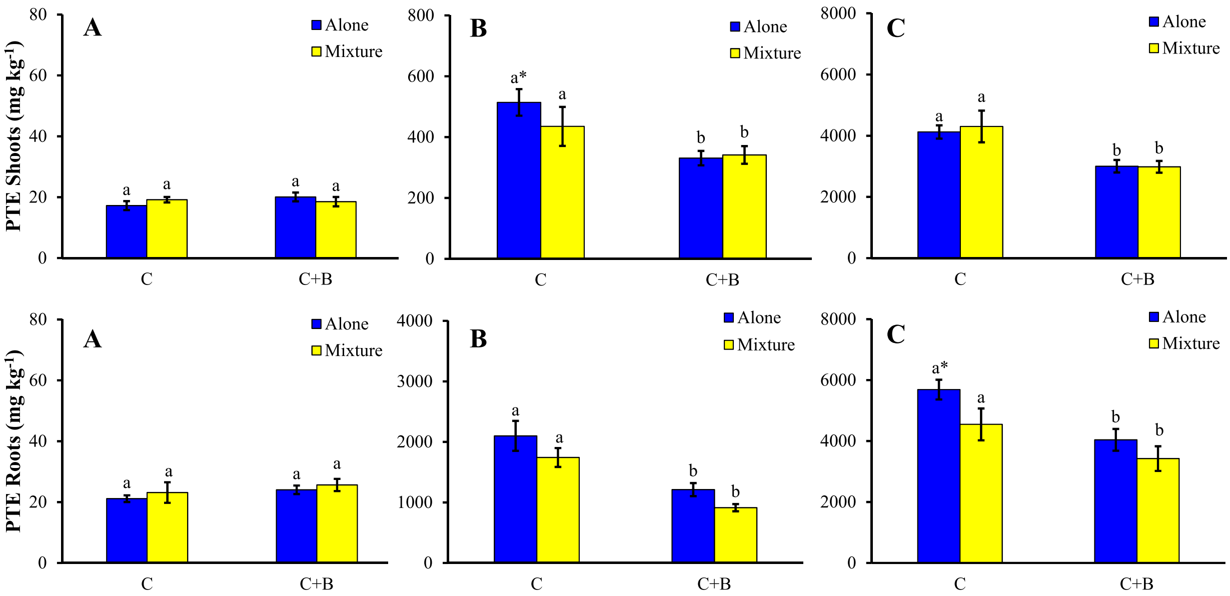

Fig. 4. Cd (A), Pb (B), and Zn (C) in shoots and roots (mg kg^{-1}, mean ± SE; n = 3) of annual ryegrass grown alone or in mixture, in control (C) and biochar amended (C + B) soils. For the meaning of the letters and asterisk (*) on top of each bar, see the caption of Fig. 2.

be the most significant factor in influencing uptake, with the exception of the Cd content in the whole ryegrass plant (which was not influenced by any of the experimental factors). Mixed cropping affected PTEs concentration in shoots of vetch and Zn content in the roots of ryegrass, whilst the biochar × cropping interaction rarely influenced the PTEs uptake by the vetch and never that of the ryegrass.

3.4. *Influence of biochar and mixed cropping on PTEs bioaccumulation, translocation and mineralomasses*

3.4.1. *Bioaccumulation factors (BAFr and BAFs)*

The bioaccumulation factors, BAFr and BAFs, which measure the plant's ability to accumulate PTEs in the roots or shoots

Table 3
PTEs bioaccumulation (BAFs and BAFr) and translocation (TF) factors, and mineralomasses (MMr and MMs) in hairy vetch grown in C and C + B, alone and in mixture with annual ryegrass. Mean values followed by different letters within a row and cropping system (alone or in mixture) denote statistically significant differences due to biochar addition (i.e., C *vs* C + B were compared), while asterisk (*) denotes statistically significant differences due to type of cultivation (i.e., C alone *vs* C in mixture, C + B alone vs in mixture C + B were compared), according to the Tukey's test ($P < 0.05$).

	C Alone	C Mixture	C + B Alone	C + B Mixture
BAFs				
Cd	1.54^{a*}	1.78^{a}	0.50^{b}	0.68^{b}
Pb	0.06^{a}	0.09^{a}	0.04^{a*}	0.07^{a}
Zn	1.35^{a*}	1.92^{a}	0.61^{b}	0.66^{b}
BAFr				
Cd	3.70^{a*}	2.70^{a}	2.19^{b*}	1.80^{b}
Pb	0.65^{a}	0.66^{a}	0.44^{b}	0.36^{b}
Zn	3.52^{a}	3.54^{a}	1.63^{b}	1.38^{b}
TF				
Cd	0.42^{a*}	0.66^{a}	0.23^{b*}	0.38^{b}
Pb	0.09^{a}	0.13^{b}	0.10^{a*}	0.20^{a}
Zn	0.38^{a*}	0.54^{a}	0.38^{a}	0.48^{a}
MMs				
Cd	1.29^{b*}	0.80^{b}	2.71^{a}	2.30^{a}
Pb	10.2^{b}	8.06^{b}	48.6^{a}	50.2^{a}
Zn	157^{b*}	121^{b}	454^{a*}	310^{a}
MMr				
Cd	1.53^{a*}	0.29^{b}	1.58^{a*}	0.99^{a}
Pb	56.0^{a*}	14.9^{b}	66.6^{a*}	41.0^{a}
Zn	202^{a*}	53.9^{b}	163^{a*}	106^{a}

respectively, were >1 for Cd and Zn in C vetch (Table 3). The BAF values of Cd and Zn were higher than Pb, indicating higher concentrations of Cd and Zn in the plant than in the soil, and a good accumulation capacity of vetch for these PTEs (Table 3). The BAFr values in all vetch plants, which were always higher than the BAFs, indicate that this plant exhibited phytostabilisation capacity for Cd, Pb and Zn. The addition of biochar, along with mixed cropping with ryegrass, reduced the BAFr of all PTEs (Table 3). This result, attributable to the reduced PTEs uptake of plants grown on biochar added soil, confirms the PTEs immobilization effect of biochar and suggests a negligible influence of the root activity on PTEs re-mobilization phenomena [57]. With the exception of Pb, BAFs also decreased in the presence of biochar, either in vetch alone or in mixture.

Also in ryegrass, PTEs bioaccumulation in roots was higher than in shoots (Table 4). The BAFr of Cd and Zn were always higher than those of Pb and >1 in all plants, with the exception of Cd-BAFr in C plants, and decreased in the biochar-treated soil. The same behavior was observed for BAFs, whose values were always <1, with the exception of Zn-BAFs in ryegrass C alone and in mixture. Biochar and mixed cropping did not seem to have any effect on Cd bioaccumulation factors.

3.4.2. Translocation factors (TF)

Translocation factors (TF, Tables 3 and 4) were <1 for both vetch and ryegrass in all treatments, indicating for both plants phytostabilising capacities and confirming BAF results. TFs for vetch were always lower than those of ryegrass, in agreement with the phytostabilising capabilities recognized to vetch [8]. With the exception of Cd in vetch, biochar did not affect the PTEs partitioning between shoots and roots in both plants when grown alone, whereas an increase of Pb-TF was observed in the C + B intercropped plants. Finally, irrespective of biochar, mixed cropping decreased Cd-TF in vetch and increased Zn-TF in ryegrass.

3.4.3. Mineralomasses (MMr and MMs)

Mineralomasses (MMr and MMs) are indicative of the actual uptake of PTEs by plants [36]. The phytostabilising ability of vetch was confirmed by mineralomasses values, being the MMr of the plants grown in C-soil higher than the MMs for all PTEs, except for the Cd in some cases (Table 3). MMr values of vetch alone were unaffected by biochar addition, which instead increased MMs values for all the PTEs (i.e. Cd by 2.1-, Pb by 4.8- and Zn by 2.9-fold). This is due to the great aboveground biomass increase (6-fold compared to C-plants) measured for vetch plants in C + B soil. The mixed cropping reduced both MMr (between 5.3- and 3.7-times) and MMs (between 1.6- and 1.3-times) of all PTEs considered, compared to the monoculture with the same soil treatment. This indicates a lower PTEs removal efficiency for intercropped vetch, caused by the reduction of both root and aerial biomass. However, due to the beneficial impact of biochar on the growth of mixed cropped vetch, the MMr and MMs values of all PTEs of the intercropped plants in C + B were higher (between 3.4- and 2-fold, and between 6.2- and 2.6-fold, respectively) than the respective values determined for the C-plants, suggesting a positive effect of biochar on the PTEs removal efficiency of mixed cropped vetch plants.

MMr and MMs values highlighted the capability of ryegrass to store Pb preferably in the roots (MMr > MMs for all the treatments), as also reported by Radziemska et al. [58], whereas for Cd and Zn higher MMs values were found than MMr (Table 4). This in agreement with Zhang J. et al. [59] and Zhang Y. et al. [60], who reported a certain Cd and Zn phytoextraction capacity of ryegrass in a multi-contaminated soil. The effect of biochar on the MMs and MMr values of ryegrass was dependent on PTE. The raising in the acid-soluble and weakly complexed Cd pool in the C + B-soil resulted in a 1.4- and 1.3-fold increase in Cd-MMs and Cd-MMr of ryegrass alone, respectively, compared to the MM values in C plants. On the other hand, both MMs and MMr of the ryegrass alone grown in biochar-treated soil decreased by 1.3- and 1.5-fold for Pb and 1.2-fold in MMr Zn, respectively, compared to the control plants (Table 4), in agreement with the BAF factors. Conversely, in C + B mixed ryegrass plants, an increase in both MMs and MMr (although more evident in the MMr) compared to intercropped C plants was observed; this was due to the increased aerial and root biomass stimulated by biochar (Table 4). Even when comparing C + B ryegrass plants grown alone and associated, the combination of biochar and mixed cropping resulted in an increase in MMr for all PTE (i.e. +1.7-, 1.4- and 1.4-fold for Cd, Pb and Zn, respectively). This result indicated that the storage of PTEs in the root system of ryegrass was amplified in the consociation + biochar treatment. Therefore, the combination of mixed cropping and biochar could, in addition to increasing the amount of biomass, stabilise PTEs (Cd and Zn in particular) and make the contaminated soil safer. This is relevant from an environmental point of view, because the PTEs immobilization by roots reduces the risk of spreading of the contamination and the access of pollutants to the food chain.

4. Conclusions

The results highlighted that the addition of biochar to a multi-contaminated soil (i.e. Cd, Pb, and Zn) had a stimulating effect on the growth of vetch and ryegrass both in monoculture and mixture, by improving soil fertility and reducing PTEs mobility. At the same time, soil amendment reduced PTEs uptake in both plants, when grown alone or in a mixture. With exception of Cd in ryegrass, bioaccumulation factors were reduced by biochar addition, however the increased plant growth led to higher total amount of PTEs retained in plant tissues. Mineralomasses (especially MMr) were generally higher when mixed cropping was combined with the amendment, suggesting that the combined use of biochar, vetch and ryegrass is a valuable ecological protocol for the assisted phytostabilization of PTEs-contaminated soils. Furthermore, the use of biochar derived from plants grown on PTEs-contaminated sites, for environmental recovery represents an added value with a view to a sustainable circular economy.

The mixed cropping of vetch and ryegrass in contaminated soils amended with biochar can therefore be a winning strategy for the reclamation of contaminated soils. The results of this research are encouraging but are nevertheless limited by the short-term duration of the experiments and somehow by the mesocosm approach. In a real contaminated site scenario (i.e., at field conditions), some other biotic and abiotic variables can influence plant growth (e.g., root growth is severely restricted in pot where they are usually concentrated at the bottom). The approach proposed in this study should be therefore further explored and validated in real PTEs-

Table 4
PTEs bioaccumulation (BAFr and BAFs) and translocation (TF) factors, and mineralomasses (MMr and MMs) in annual ryegrass grown in C and C + B, alone and in mixture with hairy vetch. For the meaning of the letters and asterisk (*) in each row, see the caption of Table 3.

	C Alone	C Mixture	C + B Alone	C + B Mixture
BAFs				
Cd	0.76^{a}	0.85^{a}	0.89^{a}	0.82^{a}
Pb	0.11^{a}	0.09^{a}	0.07^{b*}	0.07^{a}
Zn	1.31^{a}	1.37^{a}	0.95^{b}	0.95^{b}
BAFr				
Cd	0.93^{a}	1.02^{a}	1.06^{a}	1.13^{a}
Pb	0.39^{a}	0.37^{a}	0.23^{b}	0.19^{b}
Zn	1.81^{a}	1.44^{a}	1.28^{b}	1.09^{a}
TF				
Cd	0.82^{a}	0.92^{a}	0.84^{a}	0.73^{a}
Pb	0.26^{a}	0.25^{b}	0.30^{a}	0.38^{a}
Zn	0.74^{a*}	0.97^{a}	0.79^{a*}	0.94^{a}
MMs				
Cd	1.31^{b*}	0.85^{b}	1.83^{a}	1.63^{a}
Pb	39.0^{a*}	19.3^{b}	30.2^{b}	30.0^{a}
Zn	313^{a*}	191^{b}	274^{a}	262^{a}
MMr				
Cd	0.70^{b}	0.62^{b}	0.89^{a*}	1.55^{a}
Pb	60.5^{a*}	46.6^{a}	39.3^{b*}	55.4^{a}
Zn	187^{a*}	122^{b}	150^{b*}	208^{a}

contaminated settings and its effectiveness evaluated in the medium to long-term.

Data availability

The data associated with our study has not been deposited into a publicly available repository.
Data will be made available on request.

CRediT authorship contribution statement

Maria Vittoria Pinna: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Stefania Diquattro:** Writing – original draft, Investigation, Formal analysis. **Matteo Garau:** Methodology, Formal analysis, Data curation. **Corinna Maria Grottola:** Investigation, Formal analysis, Data curation. **Paola Giudicianni:** Resources, Investigation, Formal analysis. **Pier Paolo Roggero:** Resources, Project administration, Conceptualization. **Paola Castaldi:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Giovanni Garau:** Writing – review & editing, Investigation.

Declaration of competing interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e26478.

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