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Co-cropping vetiver grass and legume for the phytoremediation of an acid mine drainage (AMD) impacted soil

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ABSTRACT

Acid mine drainage (AMD) is a form of environmental pollution from mining activity that can negatively affect soil environments by acidification, salinisation, and metal(loid) contamination. The use of plants to remediate (phytoremediation) these impacted environments while generating plant-based value is a promising approach to more accessible and cost-benefiting restoration of post-mining, marginal lands. In this study, a 3-month growth-chamber pot experiment was conducted to investigate the influence of co-cropping two plant species, *Chrysopogon zizanioides* (vetiver grass) and the legume *Medicago truncatula* (barrel clover) with a wheat straw biochar amendment on the phytostabilisation of metal(loid)s Cr, Zn, and As and the phytoextraction of rare earth element (REE) in an AMD impacted soil from a gold mining region in South Africa. The results showed that co-cropping with vetiver significantly lowered the legume's Cr, Zn, and As root contents by 80%, 32% and 54%, respectively, and improved the plant's overall metal(loid) tolerance by increasing its translocation from root to shoot tissue. The biochar further inhibited root uptake of Cr and Zn, by 71% and 36%, and increased the legume biomass by 40%. Both plant species and cropping treatments exhibited low REE extraction capabilities by shoot tissue, which accounted for less than 0.2% of total soil REE contents. The study shows that co-cropping with vetiver and biochar amendment are effective tools for the phytoremediation of AMD impacted soil mainly by lowering plant uptake and improving plant metal(loid) tolerance. Likely mechanisms at play include the alteration of rhizosphere chemistry and species-specific physiological and molecular responses. These effects offer support for the phytostabilisation of AMD impacted soil with the generation of plant-based value through dual (and safe) cultivation (phytoprotection) rather than through REE recovery from plant biomass (phytoextraction). These techniques could allow for the simultaneous restoration of post-mining, mining-impacted and marginal lands with agricultural production.

1. Introduction

Acid mine drainage (AMD) is a form of wastewater generated by mining activity. It forms from the oxidation of sulfide-bearing minerals, predominantly pyrite (FeS_2), when exposed to water and air (Sheridan et al., 2018). The reactions produce a highly acidic solution comprising dissolved sulfuric acid (H_2SO_4) and ferric iron (Fe^{3+}) that leads to the release of potentially toxic metals and metalloids as well as valuable elements such as rare earth elements (REE) from the surrounding bedrock (León et al., 2023; Nordstrom et al., 2015). While the effects of AMD on the environment are multifarious, it is predominantly the acidity and contamination by metal(loid)s that presents a major problem for human health and natural systems. Through the ingestion of AMD-impacted soil-grown crops and AMD-impacted water, humans' risk of exposure to toxic and carcinogenic compounds increases (Vicente-Beckett et al., 2016; Fernández-Caliani et al., 2019). Further, AMD causes the deterioration of soil and water quality through acidification, salinisation, and sedimentation processes (Gray, 1997; Liu et al., 2018; Thomas et al., 2023). The impacts of AMD on the environment, on human health, and on natural systems that are so tied to food and water security also present major challenges to sustainable socio-economic development (Naidoo, 2017; Worlanyo and Jiangfeng, 2021). The

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harsh reality of AMD is that the impact extends far beyond the financial capabilities of mining companies (Younger et al., 2005), the legislative frameworks of governments (Crawford, 2015), and the geographic boundaries of mining properties and even regions (Alvarenga et al., 2021). Therefore, the remediation of AMD impacted environments is of paramount importance.

The current suite of abiotic and biological mitigation strategies focus on the treatment of AMD, which aim to lower acidity and remove high concentrations of sulfate and metal(loid)s, and more recently, extract metals or elements of high value (Naidu et al., 2019; Rambabu et al., 2020). These methods, however, are only relevant and effective for AMD in its aqueous form and can require high material and labour costs as well as advanced technical skills (Sheridan et al., 2018). They do not address the remediation needs of greater AMD impacted environments, which include the more heterogeneous media of soils and sediments as well as their diverse ecosystems and land uses (Thomas et al., 2021). Therefore research is needed to investigate additional remediation strategies that are suitable for soil environments, diverse land users, and that offer profitable land-use benefits (Worlanyo and Jiangfeng, 2021).

Phytoremediation is the use of plant-based systems to manage contaminated soil, sediment and water and has been widely considered to be lower cost and resource-intensive than traditional soil remediation techniques (Tonelli et al., 2022). Research has shown that in AMD conditions, acid tolerant and metal(loid) accumulating plant species are best suited for stabilising (*phytostabilisation*) select contaminants, that various soil amendments aid in plant growth or metal(loid) immobilisation through temporary pH-mediation and addition of nutrients, and that the conditions are suitable for growing bioenergy crops (Honeker et al., 2019; Gałuszka et al., 2020; Punia, 2019; Thomas et al., 2021). Concurrently, co-cropping or intercropping, the planting of two or more different plant species, has been shown to increase metal(loid) bioavailability, increase plant biomass, improve plant metal(loid) tolerance, and increase soil bacterial diversity, soil organic matter, and nutrient availability in phytoremediation plant systems (Chamkhi et al., 2022; Wiche and Heilmeier, 2016; Tang et al., 2020; Cid et al., 2020; Bian et al., 2021; Arco-Lázaro et al., 2017; Saad et al., 2018; De Conti et al., 2019). Therefore, co-cropping shows promise as phytoremediation tool for profitable land use, either through the production of high biomass generating plants for biofuel, the extraction of valuable elements through phytoextraction, or the safe production of other valuable crops. The effect of co-cropping on phytoremediation strategies for AMD impacted soil environments has yet to be studied.

Phytoremediation for the extraction of REE offers a new opportunity for developing plant-based value from AMD impacted soil media (Thomas et al., 2021). The REE comprise the lanthanide series of elements on the periodic table (La–Lu) in addition to yttrium (Y) and sometimes scandium (Sc), and are further divided into “light” rare earth elements (LREE) (La–Eu) and “heavy" rare earth elements (HREE) (Gd–Lu, Y) based on their atomic mass and ionic radii (Thomas et al., 2023). Concentrations of REE in AMD impacted soil have been found exceeding 500 mg kg^{-1} in some mining regions (Liu et al., 2021). These elements are considered critical elements to the future of sustainable energy development and there has been extensive research dedicated to their recovery from various waste streams (European Commission, 2023). Recent research investigating plant extraction of REE under phytoremediation has largely been limited to the use of plant species deemed REE hyperaccumulators (those that can extract >1000 mg REE per plant dry weight) (Zhang et al., 2020; Chen et al., 2022) and the use of non-mining impacted soil substrate (Gawroński et al., 2022; Okoroafor et al., 2022). However, it is known that plant mechanisms of element uptake and homeostasis is both highly species and environmentally dependent. Given the environmental conditions associated with AMD impacted soil, there is potential for REE phytoextraction from AMD impacted soil by non-hyperaccumulator plant species as a form of profitable plant-based value. At present, there have been no investigations into the potential for REE extraction from AMD impacted soil media acting in parallel to standard phytoremediation techniques. Therefore, this study aimed to assess the effects of co-cropping and a biochar soil amendment as phytoremediation tools on the phytostabilisation of AMD impacted soil with additional generation of plant-based value.

2. Materials and methods

2.1. Soil sampling

Soil samples were collected from a major gold mining region in Gauteng Province of South Africa with many well-documented cases of AMD (McCarthy, 2010; Tutu et al., 2008; Thomas et al., 2023). Sampling occurred near the outflow of a previously AMD polluted stream, the Tweelopiespruit, which had flooded on numerous occasions since 2002 with untreated and treated mine wastewater from the West Rand Basin Treatment Plant (Tutu et al., 2008) (Fig. 1).

At the time of sampling, the site was visibly impacted with wicking of sulfates, gypsum precipitation and formation of other secondary minerals on the surface of the site. Bulk samples (approx. 10 kg) were collected from seven different locations at the site from the top 15 cm of the soil. The soil samples were air-dried, crushed by mortar and pestle, and passed through a <2 mm nylon mesh sieve. To create a composite site sample, the soils were homogenised using equivalent weights of each sample. The physico-chemical properties and elemental contents of the homogenised soil was previously reported (Thomas et al., 2023) and is summarised in Table 1).

Arsenic exceeded South Africa's soil screening threshold criteria of 23 mg kg^{-1} for informal residents and background levels, and was therefore considered a moderate contaminant (Department of Environmental Affairs, 2010). Both Cr and Zn exceeded background levels and therefore were considered potential contaminants (Thomas et al., 2023).

2.2. Plant pot experiment

The pot experiment was conducted in a plant growth chamber from June–September 2022 (Fig. 2). The chambers were set to the following parameters: 60–65% humidity, 25 °C daytime and 18 °C nighttime temperatures, 16 h of daylight, and 1300 μmol/m^2/s for light intensity.

The following two plant species were selected for the co-cropping system: vetiver grass (*Chrysopogon zizanioides*) and barrel clover (*Medicago truncatula*). Vetiver grass, a high biomass producing plant, was selected for its previous use in aqueous AMD treatment systems showing positive results for acid tolerance and metal removal (Gwenzi et al., 2017; RoyChowdhury et al., 2019; Kiiskila et al., 2019, 2020, 2021; Beauclair et al., 2022) and for its seed sterility and relevance to the geographical biome (grassland) and bioregion (Highveld). Barrel clover was selected for its low genetic diversity but high ecotype variety making it a model legume for scientific purposes, and for potential plant beneficial properties in co-cropping systems (Cook, 1999; Tang et al., 2021). Six treatments were prepared to test mono-crop and co-crop planting schemes. A wheat straw biochar was added to three of the six treatments (mono-crop vetiver, mono-crop *M. truncatula* and co-crop (vetiver and *M. truncatula*). Each treatment was prepared in triplicates with 2 kg pots for a total of 18 pots. *M. truncatula* (ecotype RL08) seeds were sourced from the Department of Plant and Environmental Sciences, University of Copenhagen. Seeds were stored in their dry, intact pods at room temperature until use. Vetiver slips were purchased from Vetiver Spain (License #B93403269) and stored them in water at room temperature until use. Biochar was sourced from a wheat straw feedstock produced at a pyrolysis temperature of 550 °C and had a pH of 10.2 and carbon content of 73%. Prior to seeding, the biochar-amended soil was prepared by mixing 1.96 kg soil plus 40 g wheat straw biochar for a 2% dry weight per weight (dwt wt^{-1}) for each 2 kg pot. For the control soil, each pot was filled with 2 kg of soil.

A pilot study confirmed successful germination of seeds directly in

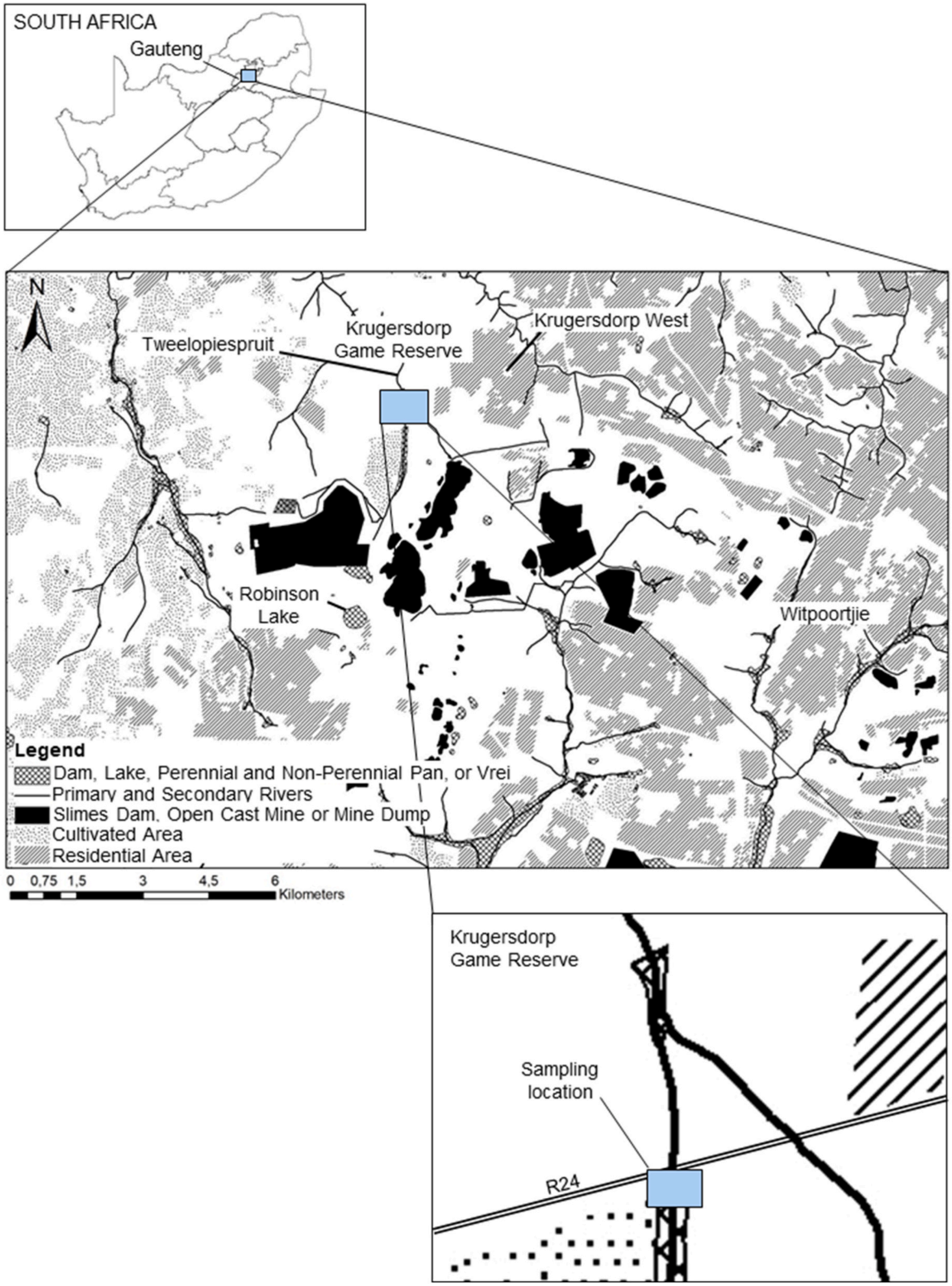

Fig. 1. Sampling location across the road (R24) from the Krugersdorp Game Reserve in Gauteng, South Africa.

the soil media. We established the vetiver and *M. truncatula* in pots by transplanting and direct seeding, respectively. For the monocrop treatments of vetiver, two slips were planted equidistant from the pot center and cut old plant slips to be equal in height. For *M. truncatula*, the seeds first had to be extracted from their pods using a corrugated rubber mat and a plasterer's hawk with handle. Fine-grained sandpaper was used for scarification of the seeds, which allows water and oxygen to penetrate the waxy coating and initiate germination. In the monocrop treatments of *M. truncatula*, three seeds were placed at two locations equidistant from the pot center. Once germinated, one seedling was selected to remain for each location to establish two plants equidistant from the pot center. The same procedures were followed in the co-crop

Table 1
Physico-chemical properties and elemental contents of the sampled AMD impacted soil compared to background contents summarised from Thomas et al. (2023).

Parameter		Element ($mg\ kg^{-1}$)		Background ($mg\ kg^{-1}$)
pH	6.71	Fe	42,000	26,000
EC (mS/cm)	4.26	Al	41,000	28,000
Carbon content (%)	1.56	Ca	37,300	4240
Particle size (%)		SO_4^{2-}	7364	14
Clay (< 2 μm)	13	Mn	2024	361
Silt (2–20 μm)	6	Cr	274	41
Fine sand (20–200 μm)	48	Zn	144	37
Coarse sand (200–2000 μm)	32	As	41	0.6
		REE (La–Lu, Y)	87	145

treatments with and without biochar for establishing each species. Fertilizer was added to each treatment due to poor nutrient conditions determined from prior analysis of elemental availability (cation exchange capacity) (Thomas et al., 2023). In the center of each pot, a single slow-release fertilizer plug was inserted with a NPK ratio of 14:3.5:9 with magnesium and micronutrients (Pindstrup Mosebrug A/S), which was selected to minimize the human-induced error resulting from daily/weekly liquid fertilizer application. Throughout the growth period, pots were hand watered to maintain a 60–65% water holding capacity (WHC) of the soil.

2.3. Harvesting and sample collection

After the 3-month growing period, all plant tissue was harvested and soil samples collected for analysis. Shoot tissue, comprising all above-ground biomass, and root tissue were separated by hand for each replicate pot and species by first cutting specimens at the base of the stem with a scalpel. Shoot tissue harvested from mono-crop treatments were combined to form as a single sample as they were the same species, while shoot tissue harvested from co-crop treatments were separated according to species. Root tissue was collected and separated by hand and tweezers. Some of the pots were root-bound and close attention was paid to accurately separating different species' root tissues. However, root tissue was not collected from the very base of these pots, as some of the fine hairs were indistinguishable. Shoots and roots were rinsed once with tap water and three times with deionized water to remove any soil particles (Huang et al., 1997) and oven dried at 50 °C for 72 h. They were weighed prior to and after oven-drying. The plant tissue was then collected in polyethylene containers and milled using zirconium silicate milling beads and a commercial electric paint shaker. After removing the plants from the soil, the soil was air-dried, crushed, and passed through a <2 mm sieve and homogenised for each replicate pot. Ten subsamples were taken from each soil allotment to create a composite soil sample of approximately 10 g.

2.4. Soil and plant analysis

Soil pH was measured by electrode using a 1:2.5 soil weight-to-volume ratio of 0.01M $CaCl_2$ with a 30-min equilibration time. Total metal(loid) content was measured on microwave oven digested (Milestone, UltraWAVE) pulverised soil samples (Pulverisette, Fritsch) using 200 mg of soil, 5 ml of 70% nitric acid (PlasmaPURE) and 1 ml 15% H_2O_2 acidified to 3.5% HNO_3 and on milled plant shoot and root samples using 100 mg of plant material, 2.5 ml 70% nitric acid (PlasmaPURE) and 1 ml of 15% H_2O_2 acidified to 3.5% HNO_3. All plant and soil digests were analysed by ICP-MS (7900 Agilent Technologies) using analytical blanks and certified references materials (NIST 2711a and NCS ZC73013) for assessment of accuracy and precision with the acceptance of 85–115% recovery and under 15% relative standard deviation (RSD). Recovery of the REE Sc, Dy, Lu, and Yb was outside of that range, at 48%, 75%, 53%, and 61%, respectively.

2.5. Data processing

Data processing and statistical analysis was done in Microsoft Excel

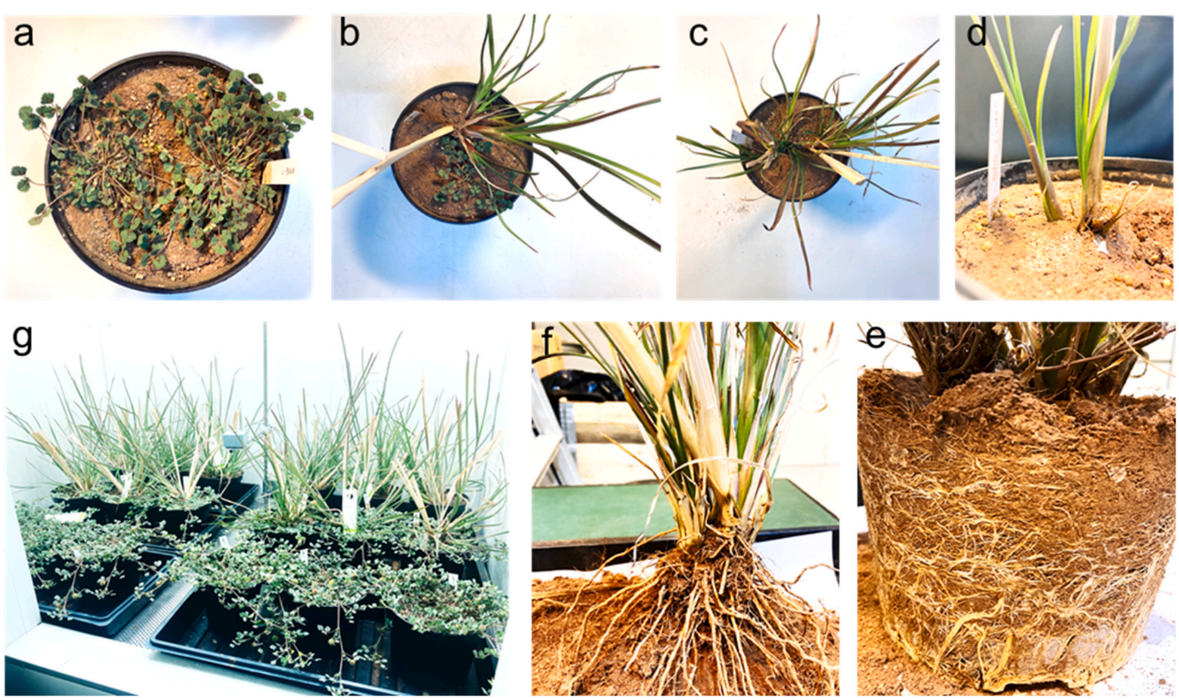

Fig. 2. Clockwise from top left a) mono-cropped *Medicago truncatula*, b) co-cropped *M. truncatula* and *Chrysopogon zizanioides*, c) mono-cropped *C. zizanioides*, d) *C. zizanioides* new shoot growth after two weeks from transplant, e) *M. truncatula* roots after 3 months, f) *C. zizanioides* shoots and roots after 3 months, and g) pot experiment set-up and plant growth after 1.5 months.

with the built-in Data Analysis Tool Pack. Values presented in the results are the averages of triplicate samples. Values for plant samples are expressed in mg per kg of plant dry weight (mg kg^{-1} DW) and for soil samples as mg per kg of soil (mg kg^{-1}). Statistically significant differences in plant data were assessed from ANOVA two factor with replication test (Supporting information, Table S1). Additional calculations were made to determine the translocation factor (TF) and bioconcentration factors (BCF), which are important parameters used to assess phytoremediation potential (Mattina et al., 2003), and were based on the following equations;

$$TF = [shoot] / [root]$$

$$BCF_{shoot} = [shoot] / [substrate]$$

$$BCF_{root} = [root] / [substrate]$$

where [shoot] is the metal(loid) concentration in shoot tissue, [root] is the metal(loid) concentration in root tissue, and [substrate] is the concentration of the metal(loid) in the soil pre-pot experiment, which is also referred to as $soil_{initial}$.

3. Results

3.1. Plant biomass and soil pH

Of the two species, vetiver produced the greatest shoot and root biomass with approximately 20 g of shoot DW and 5 g of root DW per plant per pot (Fig. 3). This was compared to a maximum of 12 g of shoot DW and 1 g of root DW biomass per plant per pot for *M. truncatula*. Only *M. truncatula* showed any significant different in biomass based on treatment – in biochar treatments, shoot biomass increased by a factor of 1.5 and 1.6 for under mono-cropping and co-cropping (approximately 40%), respectively compared to the controls.

Soil pH increased for all pot treatments from the initial soil pH of 6.71. The treatments for the mono-cropped *M. truncatula* without and with biochar were 6.94 and 7.13, for the vetiver mono-cropped without and with biochar were 7.19 and 7.04, and for the co-cropped without and with biochar were 7.13 and 7.21, respectively. Biochar treatments showed the effect of increasing soil pH for *M. truncatula* but decreasing it for vetiver.

3.2. Metal(loid) concentration in shoot and root tissue

Compared to mono-cropped treatments, the co-cropped legume and vetiver grass system lowered As shoot content by 10% in vetiver and

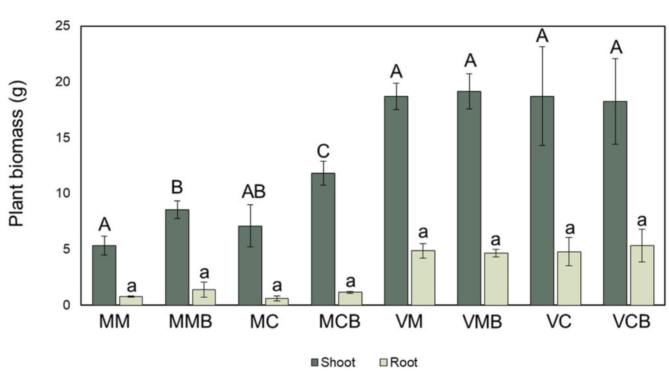

Fig. 3. Biomass per plant (g) per pot treatment based on dry weight of *M. truncatula* (M) and *C. zizanioides* (V) under mono-crop (M) and co-crop (C) treatments, without biochar () and with biochar (B). Error bars show standard deviation (n = 3). Uppercase letters indicate a significant difference ($p < 0.05$) in shoot biomass while lowercase letters indicate a significant difference in root biomass.

12% in for *M. truncatula*, and root contents by 37% in vetiver and 54% in *M. truncatula*. A decrease in Cr root uptake (80% *M. truncatula*, 46% vetiver) and Zn root uptake (32% *M. truncatula*) was also observed (Table 2).

The lower metal(loid) concentrations in co-cropped treated were only statistically significant for the *M. truncatula* and predominantly for the root tissue. Biochar treatments showed a similar effect of lowering plant metal(loid) uptake through roots but this was only significant for Cr (by 71%) and Zn (by 36%) and through shoots for As (20%) under mono-cropping. Combined, co-cropping and biochar lowered metal (loid) concentration in *M. truncatula* but had no effect on vetiver. Overall, As levels measured in *M. truncatula* shoots exceeded the WHO standards for As content in rice products (0.35 mg kg^{-1}) but fell under the equivalent dry weight limits set by the EU for beans (0.9 mg kg^{-1}) and other vegetable crops (<0.8 mg kg^{-1}) (FAO and WHO, 2019; McBride, 2013).

There was an observed decrease of REE concentration in shoot and root tissue of both plant species from mono-crop to co-crop treatments (Table 3). This was only significant for *M. truncatula* and predominantly for roots. Similarly, biochar treatments showed significantly lowered REE concentrations in *M. truncatula* roots compared to the control. The highest REE concentration in shoot tissue was found for mono-cropped vetiver, and in root tissue for the mono-cropped *M. truncatula*. Both *M. truncatula* and vetiver had shoot concentrations exceeding the normal range of REE expected from common plants (vegetables, trees, shrubs) growing in non-mining impacted substrate (Tao et al., 2022; Li et al., 2022) and there were actually relatively high concentrations found in the roots (over 60 mg kg^{-1} in *M. truncatula*). The elements Y and Ce were the most abundant in both shoot and root tissue with root concentrations ranging from 3 to 25 mg Y kg^{-1} DW and 4–18 mg Ce kg^{-1} DW and shoots concentrations ranging from 1.5 to 3.9 mg Y kg^{-1} DW and 0.4–2.9 mg Ce kg^{-1} DW. Ce is expected to be the highest of all REE found in above-ground biomass due to its similar ionic radius to Ca (Kotelnikova et al., 2021), and Y, which is isotopically lighter than all the other REE, tend to become incorporated faster due to higher reaction rates with molecules. Results from unlisted REE can be found in the supporting information file (Table S2).

3.3. Translocation factor (TF) and bioconcentration factors (BCF)

In general, the TFs for the Cr, Zn, and As increased under co-cropping treatments and decreased with the addition of biochar (Fig. 4). Vetiver treatments showed slightly, although not significantly, higher TFs than *M. truncatula* – these ranged from 0.5 to 1.1 for Zn, from 0.2 to 0.5 for Cr, and from 0.1 to 0.5 for As. The opposite trend was observed for root BCFs, which decreased under co-cropping but was only significant for *M. truncatula*. All root BCFs were higher than the shoot BCFs, which was expected given higher metal(loid) concentrations in the roots. No clear trend was observed in the shoot BCFs for Cr, Zn, and As for either plant species. Zn very clearly showed highest shoot BCFs, which was not surprising given that it is an essential nutrient. Since the BCFs shows plant tissue metal(loid) concentrations as "normalized" by the substrate concentrations, they also reflect the enrichment factor for the select metal(loid)s. Since no root or shoot BCF exceeded 1, which would indicate higher metal(loid) concentration in plant compared to soil, it is clear there is no preferential uptake or "enrichment" of Cr, Zn, and As.

Similar to Cr, Zn, and As, the TFs for the REE (La–Lu) increased under co-cropping treatments, although only significantly so for vetiver (Fig. 5). Biochar amended treatments showed no change for the TFs of *M. truncatula* and had contradictory effects for the TFs of co-cropped and mono-cropped vetiver. It was also observed that the TFs were lower for *M. truncatula* than vetiver but increased with increasing atomic mass specifically for the HREE (Gd–Lu). This same behaviour was not seen in the TFs for vetiver, although one treatment of vetiver (mono-cropped with biochar) showed a significantly higher TF (>0.5) compared to other treatments (<0.2). In general, the root BCFs for the REE (La–Lu)

Table 2
M. truncatula and *C. zizanioides* Cr, Zn, and As shoot and root concentrations of three pot replicates (n = 3) in mg element per kg plant dry weight (DW) ± the standard deviation. Significant differences at 95% (p < 0.05) and 90% confidence (p < 0.1) intervals are indicated by a (**) and (*) respectively.

		Mono-crop		Co-crop	
		M. truncatula	*C. zizanioides*	*M. truncatula*	*C. zizanioides*
		Chromium (mg kg^{-1} DW)			
Control	*Shoot*	2.44 ± 3.15	3.62 ± 0.40	2.49 ± 1.16	2.74 ± 0.94
	Root	106 ± 3.22	23.9 ± 8.31	21.1 ± 7.01**	13.1 ± 2.53
Biochar	*Shoot*	2.14 ± 1.21	2.98 ± 1.91	1.22 ± 0.41	1.76 ± 0.71
	Root	64.5 ± 13.4**	23.5 ± 8.31	18.7 ± 7.74**	11.6 ± 1.48
		Zinc (mg kg^{-1} DW)			
Control	*Shoot*	31.6 ± 5.48	19.4 ± 0.79	39.7 ± 4.13	36.4 ± 1.89
	Root	88.6 ± 16.1	41.9 ± 9.89	60.6 ± 5.28**	40.6 ± 4.27
Biochar	*Shoot*	45.5 ± 1.83	27.5 ± 14.1	31.7 ± 9.10**	20.6 ± 2.09**
	Root	73.8 ± 8.01*	38.4 ± 8.03	47.3 ± 9.70	27.3 ± 0.10*
		Arsenic (mg kg^{-1} DW)			
Control	*Shoot*	0.84 ± 0.49	0.80 ± 0.08	0.74 ± 0.19**	0.72 ± 0.18
	Root	12.7 ± 0.22	6.04 ± 1.52	5.91 ± 1.12**	3.83 ± 0.73
Biochar	*Shoot*	0.67 ± 0.16*	0.82 ± 0.43	0.45 ± 0.16	0.52 ± 0.14
	Root	11.7 ± 4.34	6.42 ± 2.12	5.26 ± 1.33	3.51 ± 0.23

Table 3
M. truncatula and *C. zizanioides* Y, Ce, Nd, Dy, Gd, and REE (La–Lu, Y) shoot and root concentrations of three pot replicates (n = 3) in mg element per kg plant dry weight (DW) ± the standard deviation. Significant differences at 95% (p < 0.05) and 90% confidence (p < 0.1) intervals are indicated by a (**) and (*) respectively.

		Mono-crop		Co-crop	
		M. truncatula	*C. zizanioides*	*M. truncatula*	*C. zizanioides*
		Yttrium (mg kg^{-1} DW)			
Control	*Shoot*	1.84 ± 0.90	3.94 ± 0.36	2.54 ± 0.57	3.14 ± 0.90
	Root	25.4 ± 7.63	6.39 ± 2.32	13.84 ± 9.95*	3.20 ± 1.10
Biochar	*Shoot*	1.90 ± 1.34	1.84 ± 0.92	1.46 ± 0.91	2.91 ± 0.76
	Root	13.2 ± 7.34*	5.04 ± 2.32	8.42 ± 5.66	3.86 ± 2.00*
		Cerium (mg kg^{-1} DW)			
Control	*Shoot*	1.11 ± 0.68	0.92 ± 0.18	0.67 ± 0.24	0.74 ± 0.19
	Root	18.1 ± 3.43	6.63 ± 1.85	6.30 ± 1.19**	3.89 ± 0.60
Biochar	*Shoot*	0.62 ± 0.21	2.86 ± 3.40	0.35 ± 0.13	0.54 ± 0.23
	Root	11.5 ± 5.35*	4.85 ± 3.76	5.26 ± 1.17	4.89 ± 2.30
		Neodymium (mg kg^{-1} DW)			
Control	*Shoot*	0.41 ± 0.25	0.33 ± 0.05	0.24 ± 0.08	0.27 ± 0.08
	Root	6.50 ± 2.26	2.45 ± 0.67	2.48 ± 0.51**	1.46 ± 0.24
Biochar	*Shoot*	0.24 ± 0.09	1.09 ± 1.34	0.13 ± 0.04	0.20 ± 0.09
	Root	4.18 ± 1.79	1.79 ± 1.40	2.09 ± 0.43	1.83 ± 0.83
		Gadolinium (mg kg^{-1} DW)			
Control	*Shoot*	0.09 ± 0.05	0.07 ± 0.01	0.05 ± 0.02*	0.06 ± 0.02
	Root	1.42 ± 0.43	0.54 ± 0.15	0.54 ± 0.11**	0.32 ± 0.06
Biochar	*Shoot*	0.05 ± 0.02	0.23 ± 0.28	0.03 ± 0.01	0.04 ± 0.02
	Root	0.92 ± 0.40*	0.39 ± 0.30	0.46 ± 0.09	0.40 ± 0.19
		Dysprosium (mg kg^{-1} DW)			
Control	*Shoot*	0.05 ± 0.03	0.04 ± 0.01	0.03 ± 0.01*	0.03 ± 0.01
	Root	0.80 ± 0.25	0.32 ± 0.08	0.33 ± 0.07**	0.19 ± 0.03
Biochar	*Shoot*	0.03 ± 0.01	0.13 ± 0.16	0.02 ± 0.00	0.02 ± 0.01
	Root	0.53 ± 0.21*	0.23 ± 0.18	0.28 ± 0.05	0.24 ± 0.10
		REE (mg kg^{-1} DW)			
Control	*Shoot*	4.31 ± 1.91	5.91 ± 0.37	3.97 ± 0.76	4.74 ± 0.88
	Root	63.6 ± 13.2	20.7 ± 5.11	27.9 ± 8.22**	11.7 ± 1.55
Biochar	*Shoot*	3.27 ± 1.47	9.85 ± 7.35	2.18 ± 0.38	4.07 ± 0.39
	Root	37.1 ± 13.1*	15.5 ± 8.47	20.2 ± 6.66	14.5 ± 5.64

decreased in co-cropping and biochar amended treatments, and overall, were higher for *M. truncatula* than for vetiver. The shoot BCFs for the REE (La–Lu) were relatively low (<1), which was expected given lower shoot concentrations. A slight negative Ce anomaly was observed in the root and shoot tissue of both species, which was reflected in both root and shoot BCFs showing lower Ce uptake from the substrate compared to other REEs. The same trend as the TFs was observed in *M. truncatula* where the shoot BCFs increased with increasing atomic mass for the HREE.

3.4. Metal(loid) extraction by plant shoots

The amounts of Cr, Zn, As, and REE extracted by the plant shoots in all treatments were very low (<1% for Cr, Zn, As, and REE) (Table 4). In general, the treatments with vetiver grass (i.e. vetiver mono-cropped and co-cropped) had higher metal extraction rates, which was attributed to its comparatively higher biomass than for *M. truncatula*. Data from individual REE shoot extraction can be found in the supporting information file (Table S3).

4. Discussion

4.1. Effects of co-cropping on Cr, Zn, and As plant uptake and tolerance

It was observed in this study that vetiver grass, a high biomass-generating grass species effectively lowered the uptake of Cr, Zn, and

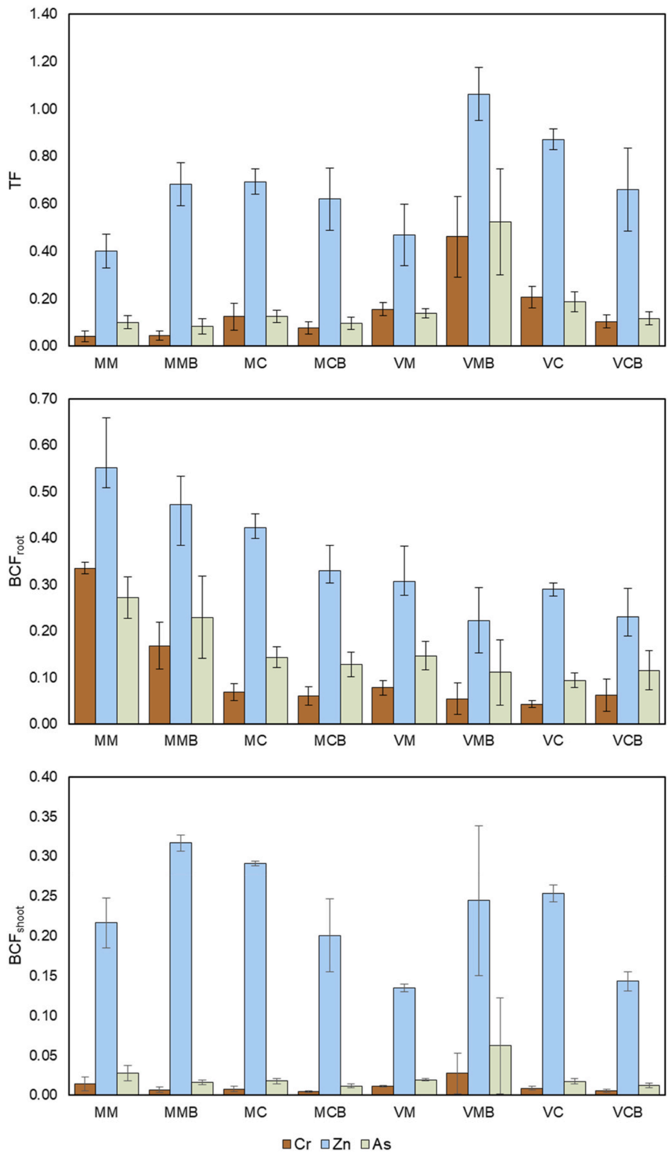

Fig. 4. TF (shoot/root), BCF_{root} (root/substrate), and BCF_{shoot} (shoot/substrate) ratios for As, Zn, and Cr of *M. truncatula* (M) and vetiver (V) under mono-crop (M) and co-crop (C) treatments, without biochar () and with biochar (B).

As in the legume species, *M. truncatula* when grown under co-cropping conditions. The decrease in root metal(loid) contents and in metal (loid) uptake from the soil, and the increase in metal(loid) translocation from root to shoot tissue of the legume indicates a clear influence of vetiver grass on the rhizosphere (soil-root interface) chemistry. These changes, either a) directly through the production of metal-chelating root exudates or b) indirectly through altered soil pH, redox, and microbial conditions, facilitate an improved tolerance of *M. truncatula* to the metal(loid)s present in the AMD impacted soil by lowering the bioavailability of those metal(loid)s as well as their actual plant uptake. The direct neutralisation of unwanted elements in the rhizosphere through complexation to organic acids and other carbon-based compounds produced by a plant's roots is considered a first-step towards improved tolerance (Bejarano et al., 2021). The stability of these complexes depends on the nature of the organic acids (higher molecular weight complexes remain insoluble in soil solution) and the soil pH conditions (lower pH induces H^+ competition for complexation), among other factors (Antoniadis et al., 2017). The slight increase in soil pH observed for *M. truncatula* under co-cropping compared to mono-cropping further supports that vetiver grass produced higher stability metal(loid) complexes and chemistry than *M. truncatula* under the oxidative stress induced by the AMD impacted soil conditions. This is supported by findings from Li et al. (2023), who demonstrated an increased production of organic acids for vetiver grass growing in an As contaminated soil under mono-crop conditions, and from Kim et al. (2008), who showed, specifically, increased dissolved organic carbon (DOC) from vetiver grass under contaminated soil conditions. Further investigation is needed to determine how the nature of these organics acids and therefore stability of chelation might be affected by high levels of other cations (i.e. Ca, Al, Fe, Mn) present in the soil solution. Other factors affecting the root uptake of Cr, Zn, and As by the legume include changes to root biomass and morphology or transpiration rates due to competition for growing space and water under co-cropping conditions. Vetiver grass obviously produced a higher root biomass than *M. truncatula*, which could have led to a smaller soil exploration zone and therefore access to metal(loid)s for the legume.

The increased translocation of Cr, Zn, and As into the legume's shoot tissue under co-cropping shows that the plant's internal regulation mechanisms to achieve homeostasis was affected in addition or in response to the changes in the rhizosphere chemistry. The redistribution of nonessential metal(loid)s (As) from roots to shoots occurs mainly as a detoxification strategy to sequester and compartmentalize them in plant cell walls and vacuoles while for essential metal(loid)s (Zn and Cr), they can be stored for future functions (Antoniadis et al., 2017). This defense mechanism exhibited by the legume is more likely of a symbiotic nature rather than from competition with vetiver grass for nutrients where storage in the above ground biomass acts as a reserve as some form of allelopathy (Thomas et al., 2021). Not only were the nutrient needs sufficiently met since fertilizer was used in every treatment, but the improved tolerance of *M. truncatula* was observed to come at no expense to and even with some benefits for the vetiver grass – metal(loid) uptake was largely unaffected or even decreased.

As reported in previous studies on co-cropping for phytoremediation, one of the plant species typically increases its metal uptake to compensate for the lowered uptake by the other (Saad et al., 2018; Cid et al., 2020; Ma et al., 2021). This behaviour may be a factor of a) co-cropping in more highly contaminated soils of which the available metal(loid) concentrations are high enough or are not strongly influenced by any rhizosphere changes that occur or b) species or family-conserved traits that cater for metal(loid) tolerance through accumulation rather than exclusion. For example, De Conti et al. (2019) found increased concentrations of DOC from the grass species *Paspalum plicatulum*, which lowered Cu availability in the soil and Cu content in co-cropped grapevines – this soil was, however, dosed with specific concentrations of Cu from solution indicating an already high availability compared to historically metal polluted soils. Likewise, legume's symbiosis with plant growth promoting rhizobia, ability to fix nitrogen, and production of nitrogen-rich residues have been shown to be beneficial for nutrient availability, uptake and plant growth, which may increase in effect with the introduction of diverse microbial communities from other plant species (Martínez-Hidalgo et al., 2022; Ramani et al., 2022). Other beneficial species or family-specific reactions to metal (loid) translocation might only be triggered by other plants (and their microbiota), such as antioxidant stress responses (Cid et al., 2020), nitrogen-use efficiency (Arco-Lázaro et al., 2017) or phosphorous-use efficiency (Tang et al., 2021; Chen et al., 2023).

The results from this study point towards a positive effect of vetiver grass and it's physiological and molecular tolerance mechanisms on a legume species' exclusion and redistribution of Cr, Zn, and As, which may extend to other legume and grass species and metals.

4.2. REE uptake in vetiver grass and M. truncatula

In this study, neither plant species exhibited high REE uptake based on the accumulation in their above ground biomass, which resulted in very little of the total soil's REE content being extracted. This was

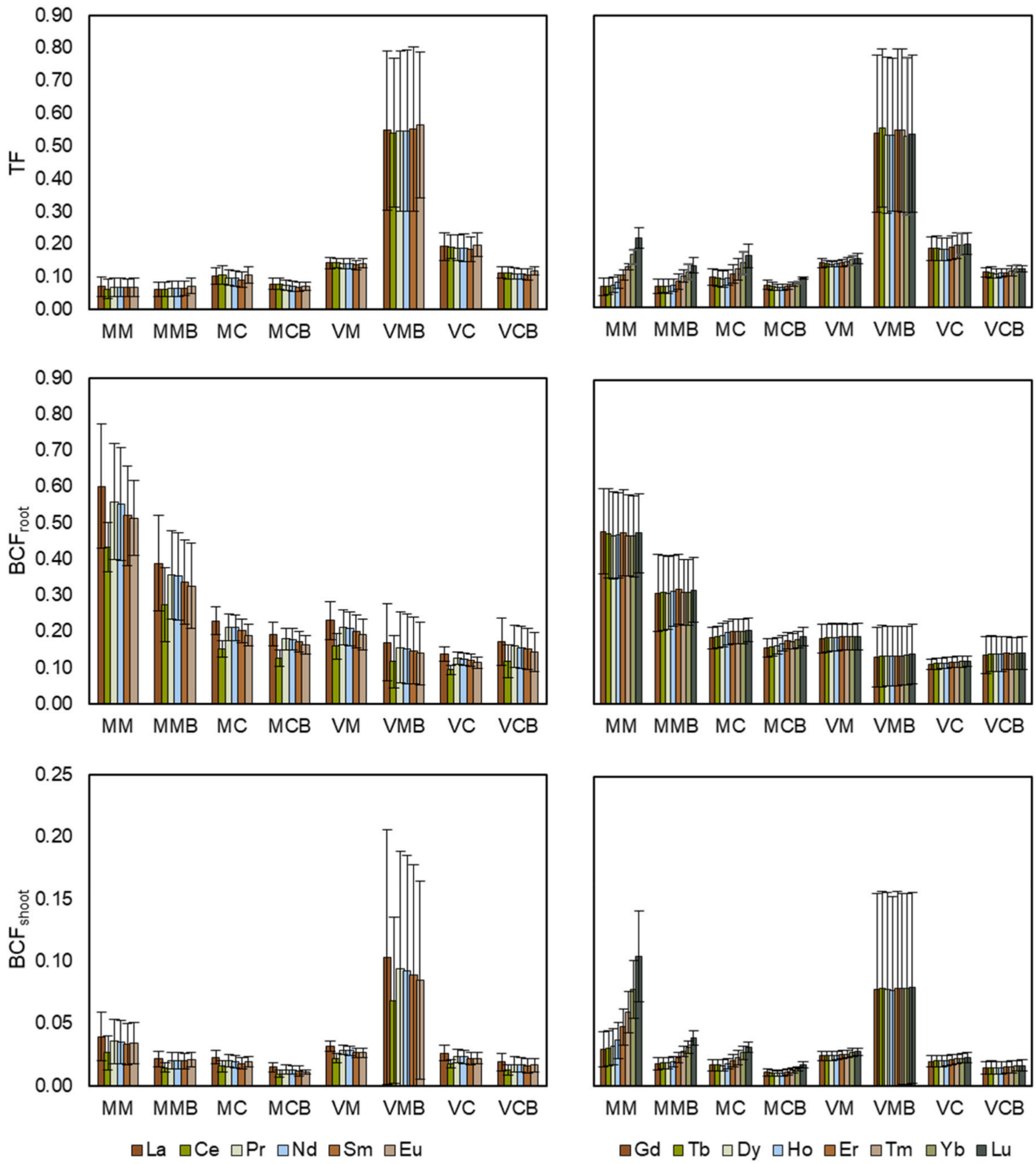

Fig. 5. TF (shoot/root), BCF_{root} (root/substrate) and BCF_{shoot} (shoot/substrate) for La–Lu of *M. truncatula* (M) and vetiver (V) under mono-crop (M) and co-crop (C) treatments, without biochar () and with biochar (B).

surprising given that it was expected for the conditions of the AMD impacted soil to be conducive to a high fraction of plant available REE – not only due to the acid dissolution processes from which they originate, but also the high content of sulfate from which REE form soluble and plant available complexes (Liang et al., 2008). Of course many other soil factors, including pH, clay content, and soil organic matter influence REE speciation and solubility, which also influence plant uptake (Kotelnikova et al., 2021). Given that root contents of REE were relatively high (over 60 mg kg^{-1} for *M truncatula*) and that shoot concentrations were higher than for average crops grown on uncontaminated soil, it seems unlikely that the low total REE uptake was due to physiological or molecular mechanisms of exclusion rather that the soil offered only a small pool of REE to begin with. This topsoil was likely already leached of its most soluble fraction, suggesting greater reserves of bioavailable REE existed below the 20 cm depth limit from which the soil was collected leaving the more recalcitrant forms at the surface. This is consistent with leaching studies conducted on mining-impacted media that show strong leachability of REE in acidic and calcareous soil conditions (Liu et al., 2018; Pan et al., 2023). This also explains a major difference from previous studies that report high REE uptake by plants grown in AMD and mining impacted environments. These studies are conducted in-situ, which allow plant roots to extend into far deeper soil horizons (Dinh et al., 2022; Khan et al., 2017; Liu et al., 2019). This would be particularly important with a plant like vetiver whose roots can reach over 2 m depth.

In this study, it was observed that *M. truncatula* exhibited a certain preference for exclusion of LREE and accumulation of HREE, each becoming more pronounced with increasing atomic number. This was

Table 4
Amount of Cr, Zn, As and REE (La–Lu) extracted by shoot tissue per pot in mg and as a percent (%) based on initial soil metal(loid) concentrations and the dry weight of shoot tissue per pot: *M. truncatula* and vetiver mono-crop treatments without biochar (MM and VM) and with biochar (MMB and VMB) and co-crop treatments without biochar (C) and with biochar (CB).

		MM	MMB	VM	VMB	C	CB
				Chromium			
$Soil_{initial}$	mg kg^{-1}			308 ± 12.0			
Shoot extraction	mg	0.05 ± 0.02	0.04 ± 0.02	0.13 ± 0.01	0.31 ± 0.28	0.07 ± 0.02	0.04 ± 0.02
	%	0.01	0.01	0.02	0.05	0.01	0.01
				Zinc			
$Soil_{initial}$	mg kg^{-1}			144 ± 4.93			
Shoot extraction	mg	0.33 ± 0.07	0.78 ± 0.09	0.72 ± 0.04	1.31 ± 0.44	0.97 ± 0.08	0.63 ± 0.18
	%	0.12	0.27	0.25	0.46	0.34	0.22
				Arsenic			
$Soil_{initial}$	mg kg^{-1}			41.1 ± 1.10			
Shoot extraction	mg	0.01 ± 0.00	0.01 ± 0.00	0.03 ± 0.00	0.09 ± 0.08	0.02 ± 0.00	0.01 ± 0.00
	%	0.01	0.01	0.04	0.11	0.02	0.02
				REE			
$Soil_{initial}$	mg kg^{-1}			87.5 ± 1.99			
Shoot extraction	mg	0.05 ± 0.02	0.06 ± 0.02	0.22 ± 0.01	0.36 ± 0.26	0.11 ± 0.03	0.09 ± 0.02
	%	0.03	0.03	0.13	0.21	0.07	0.05

not expected as most common (i.e. non-hyperaccumulator) plants either show no preference for uptake of LREE or HREE *or* a depletion of HREE in leaf and shoot tissue (Chen et al., 2022b). The behaviour is likely related to the inability for certain LREE or a complexed species to penetrate the cell wall of *M. truncatula* compared to HREE (Kovaříková et al., 2019). The uptake of individual REE is largely dependent on their ionic radii being similar to that of calcium, an essential nutrient for plants, and enter plant cells via calcium channels (Kotelnikova et al., 2021). Thus, the molecular response of *M. truncatula* to high calcium influx could have had an effect on channel activity. This behaviour has been noted in one REE hyperaccumulator species, *Phytolacca americana* (Yuan et al., 2018; Chen et al., 2022b; Grosjean et al., 2019). Whether this or other preferential methods of adsorption or translocation are at play and are common among hyperaccumulators would need to be investigated further (Liu et al., 2022b; Zheng et al., 2023). Alternatively, the uptake behavior could exhibit symptoms of the different development stages from the two plant species, since *M. truncatula* reached its maturity (flowering) at the end of the three-month experimental period. It should also not go unnoted that different transpiration rates based on plant species and biomass could cause variations in REE fractionation in plant tissue. Regardless, further research into REE uptake mechanisms under various and more isolated stress conditions are needed to better understand the effect of the soil chemistry on select species.

4.3. Influence of biochar on Cr and Zn immobilisation

Biochar is often used as a soil amendment in phytoremediation to aid in the phytostabilisation or the immobilisation of select contaminants in the soil (Natasha et al., 2022; Naveed et al., 2021). This can occur through a variety of effects of the biochar on a select meta (loid) or on soil chemical and physical conditions. Most notably, biochar stabilises metal(loid)s through their adsorption to different functional groups on the biochar's surface, physical adsorption, co-precipitation with biochar or induced metal(loid) precipitation from changes to soil pH, and reduction processes. In this study, it was observed that biochar had two main effects: 1) increasing the plant biomass of the legume and 2) lowering the Cr (and Zn) concentrations in the legume roots and in one case, lowering As contents of vetiver shoots. These effects on the legume were more substantial when complimented by co-cropping treatments. The most likely mechanism for increased plant biomass was the alteration of the AMD impacted soil's chemical and physical properties relating to plant growth – such as soil porosity, pH, organic matter, and water retention (Naveed et al., 2021). It was unlikely that nutrient availability was largely affected given the use of fertilizer in the study design. It was also unlikely that biochar lowered the toxicity of Cr, thereby improving plant growth parameters given that the Cr levels in the soil were not above safety regulations. It is more likely that the effect on biomass and the effect on lowering root uptake of Cr and Zn acted independently of one another - the former through improved chemical and physical growing conditions and the latter through increased metal sorption. Wheat straw biochar typically exhibits a negative surface charge in near neutral pH conditions (Naeem et al., 2019), which would also explain why As was not significantly affected in plant root contents of either species.

At a 2% incorporation rate of a wheat straw biochar to a coal mine contaminated soil, Zaman et al. (2022) found that there was a decrease in Cr uptake by tomato shoots, but that it was not significant. At 4% and 6% incorporation rates, the results were significant and the 6% treatment lowered the tomato shoot Cr contents from 33.67 to 18.33 mg kg^{-1} (46%). Alhar et al. (2021) showed that wheat straw possessed a particularly high sorption capacity for a selection of metal(loid)s when mixed with various mine spoils at 5% and 10%. At the 5% incorporation rate, pore water concentrations significantly decreased for Zn (6328–158175 μg L^{-1}) and As (531–32 μg L^{-1}) – Cr was not reported in the pore water analysis. This was complimented by a decrease in rye grass shoot tissue concentrations for Cr (1.20–0.22 μg L^{-1}), Zn (368–175 μg L^{-1}), and As (2.8–1.8 μg L^{-1}). These studies imply that a the addition of biochar at greater than 2% dwt wt^{-1} is more effective in significantly lowering the metal(loid) concentrations, including As, in the shoots compared to only the roots, as was observed in the present study. Further, these studies show that biochar mechanisms of at least Cr and Zn immobilisation may be similar across a range of soil textures and mediums from coarse mine spoils to loamy and clay rich soil. However, it should be carefully considered the added physical challenges that come with a high incorporation rate of biochar, which become more apparent at field-scale applications. Finally, while the observed decreases in Cr root content with biochar amendment under mono- and co-cropping can be interpreted as a positive effect on plant health and could explain the increased shoot biomass of the legume, the decrease in Zn root content can possible have negative implications for plant growth since Zn is an essential nutrient.

4.4. Implications for phytoremediation of AMD impacted soil with generation of plant-based value

The present findings indicate that AMD impacted soil has a higher potential for the generation of plant-based value through phytostabilisation with culturally and economically meaningful crop cultivation than with biofuel production from plant residues or REE extraction through phytoextraction. Previous studies recommending phytostabilisation focus on the potential benefits from the production of biomass to generate biofuel. However, in order to make this viable, biomass production would need to be done at an incredible scale. Sallustio et al. (2022) estimated that conditions in Italy for the production of up to 27 billion liters of biofuel (cellulosic ethanol) from perennial grasses (including vetiver grass) and trees, which would meet almost 70% of the current fuel consumption demands of the country – 2.8 million hectares. This does not even consider the potential detriment to soil fertility from the removal of crop residues over that amount of land. The benefit from energy crop production likely comes with great environmental costs.

Plant-based recovery of REE has been support through phytoextraction or phytomining enhanced by various organic amendments, chelating agents and microbial inoculations (Jalali and Lebeau, 2021; Liu et al., 2022a; Liu et al., 2022b). The final extraction of REE from the secondary plant sources includes applying various methods of metallurgy (pyro-, hydro-, and biometallurgy), which are costly, waste-generating and have relatively poor selectivity (Dinh et al., 2022). While a comparison is needed between the life-cycle of phytomining versus conventional mining of REE, it too must be carried out at a large-scale for financial feasibility. There still may be rational benefits for the targeted phytoextraction of REE from a plant toxicity or human health risk perspective, although the literature on this subject remains contradictory (Kotelnikova et al., 2021).

Therefore, the findings from this study support the use of phytostabilisation in tandem with agricultural production – both of culturally and economically relevant plant materials (crops for consumption, forage and fodder crops, raw material crops) – for plant-based value provided that methods are utilized to sufficiently decrease the levels of potentially harmful metals and metalloids. This study demonstrates that co-cropping and biochar amendment have great potential as effective methods to aid in metal(loid) immobilisation and lower plant uptake, and that vetiver grass exhibits phytoprotection capabilities to promote safe cultivation of secondary crops. The authors consider this phytoremediation approach to be far more accessible to different land uses and landowners, and thus fills a critical void in AMD remediation techniques.

5. Conclusion

Acid mine drainage is a geochemically complex form of pollution from mining activity that presents major problems for the remediation and restoration of impacted environments. Such environments are in need of sustainable and profit-oriented strategies that address soil and sediment contamination while producing some form of plant-based value. The purpose of this study was to assess the effects of a phytoremediation co-cropping system and wheat straw biochar amendment for the phytostabilisation of metals and the extraction of REE from an AMD impacted soil. The results showed that co-cropping treatments lowered plant As, Cr and in some cases Zn uptake, which was strongest in the legume and, more specifically, in the legume roots and increased plant tolerance to the metal(loid)s. Further, the legume displayed a selective accumulation and translocation of HREE over LREE in shoot tissue, which provides interesting insights into species-sensitive mechanisms of REE homeostasis. Overall, the study showed that co-cropping is an effective method to meet remediation and restoration goals of phytostabilisation. While the study intended to show REE extraction as that form of plant-based value, an alternative conclusion was met – that co-cropping (with vetiver) phytoprotects other plant species from metal (loid) accumulation, thereby supporting the safe cultivation of AMD impacted soils as a form of plant-based value.

Credit author statement

Glenna Thomas: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Visualization. Craig Sheridan: Methodology, Supervision, Writing – review & editing. Peter E. Holm: Methodology, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interestsGlenna Thomas reports travel was provided by Danish Agency for Higher Education and Science. Peter E. Holm, Craig Sheridan reports a relationship with Danida Fellowship Centre that includes: funding grants.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.122873.

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