

Article

Effectiveness of Phytoextraction of Cu, Zn, Ni, Sr and P from Mining Soils Using Three Successive Engineering Cycles in Bioreactors

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Featured Application

This study demonstrates that element recovery yields in agromining systems is not constant over time and is strongly influenced by substrate conditions after the first harvesting cycle. The substantial reduction in metal bioaccumulation observed following the initial cultivation highlights the need for active substrate management to sustain long-term recovery efficiency. In this context, the application of soil amendments and/or crop rotation strategies may therefore constitute effective management practices for maintaining metal phytoavailability and ensuring stable recovery yields over successive cultivation cycles.

Abstract

Phytoextraction is a sustainable strategy for removing potentially valuable elements from contaminated substrates while contributing to site remediation. However, the effectiveness of repeated phytoextractive cycles remains poorly investigated. This study evaluated the phytoextraction performance of *Helianthus annuus* (HA) cultivated on mining soil from Complejo Minero Fabril Sierra Pintada (Argentina) containing elevated concentrations of Ni, Zn, Sr, P, and Cu over three successive three-month cultivation cycles in TRL-4 bioreactors (BRs). The scalability of the process was subsequently assessed through projection to TRL 6 using a Vegetable Depuration Module (VDM). Elemental concentrations in soil and biomass were determined by X-ray fluorescence, while bioaccumulation coefficients, translocation factors, arbuscular mycorrhizal colonization and glomalin-related soil proteins (GRSP) were assessed to identify biological factors associated with phytoextraction performance. Projected bioextractive potential at TRL 6 (VDM) during the first



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cycle reached 16.15 ± 2.40 g Cu, 20.81 ± 2.19 g Zn, 7.43 ± 1.15 g Ni, 11.39 ± 1.19 g Sr, and 126.87 ± 15.42 g P. Phytoextractive efficiency declined substantially after the first cultivation cycle, indicating that a single crop harvested at the flowering stage maximized element removal under the tested conditions. The accumulation of economically relevant elements in sunflower biomass could be integrated with downstream metal recovery processes, highlighting the potential of HA for phytomining applications.

Keywords: agromining; metal(loid)s; phytoextraction; TRL 4–6; bioavailability; *Helianthus annuus*; engineering cycles

1. Introduction

Phytomining represents a promising and sustainable approach for recovering valuable and critical elements from contaminated soils, low-grade ores, and secondary resources. The phytomining process comprises three main stages: (i) phytoextraction, in which plants are used to extract target elements from soils; (ii) biomass processing and enrichment, involving the concentration of these elements within plant biomass; and (iii) metal recovery, which consists of extracting the target elements from the enriched biomass, thereby completing the phytomining cycle. Recent advances have expanded the range of elements considered suitable for phytomining to include Au, rare earth elements (REEs), and base and trace metals such as Mn, Cu, Cd, and Zn. Although laboratory studies and field trials have demonstrated the technical feasibility of phytomining for these elements, scaling up remains a major challenge [1].

In recent years, substantial progress has been made in enhancing plant-based metal(loid) accumulation, as well as in developing efficient methods for biomass processing and recovery. Advances in plant biotechnology include genetic engineering, the application of soil amendments, and the identification of hyperaccumulating plant species, aimed at increasing the concentrations of chemical elements of interest in plant tissues [2]. Concurrently, increasing attention has been devoted to the development of cost-effective and environmentally sustainable extraction technologies, including electrochemical and bioleaching methods, that reduce environmental impact [3]. Despite these advancements, phytomining remains in the early stages of industrial implementation and requires further multidisciplinary research.

Phytoextraction is a promising technology that enables the remediation of contaminated soils while simultaneously recovering part or all of the remediation costs. When economically valuable elements are recovered, phytoremediation may even generate economic returns that exceed remediation costs [4]. More than 700 hyperaccumulator plant species have been reported. Van der Ent (2015) [5] identified two hyperaccumulator plants: *Haumaniastrum robertii* and *Aeolanthus biformifolius*. The most extreme Co hyperaccumulator, *H. robertii*, was able to accumulate up to 1 wt% Co, whereas the most extreme Cu hyperaccumulator, *A. biformifolius*, can accumulate up to 1 wt% Cu. Based on this accumulation capacity, large-scale cultivation of *H. robertii* could yield up to 200 kg Co ha^{−1} yr^{−1} from approximately 20 t of dry biomass. Li et al. (2003) [6] investigated planting methods, population density, weed control practices, harvesting schedules and methods, pollination control, and seed processing. Such crop management studies have improved phytoextraction efficiency and provide a tool for farmers to conduct commercial production.

Agromining involves the cultivation of selected hyperaccumulator plant species ('metal crops') on low-grade ore bodies or mineralized (ultramafic) soils, or anthropogenic metal-rich materials (e.g., contaminated soils, mine spoils, industrial sludge), followed by

harvesting and incineration of the biomass to recover target metals or salts. Full commercial agromining of Ni is underway in Europe (e.g., the Balkans, France) using Brassicaceae (notably *Odontarrhena chalcidica*—formerly *Alyssum murale*), and major trials in Malaysia are underway using *Phyllanthus rufuschaneyi*. Variable prices of commodity metals add constraints on the development of commercial agromining [7]. Bani et al. (2025) [8] assessed the use of biomass from Ni hyperaccumulator plants and explored the potential of biomass generated from fruit trees, olive trees, citrus and grapevines for bioenergy production in Albania, where also *Odontarrhena chalcidica* can be cultivated across 11% of the country, with potential yields exceeding 150 kg of Ni per hectare. Li et al. (2003) [6] demonstrated that, in fact, hyperaccumulators and ‘normal’ plants absorb metals from the same available (labile) pool of metals; however, hyperaccumulator plants exhibit an exceptional capacity to deplete this pool. For example, a single crop of *N. caerulescens* was shown to take up more than 20% of the available soil Cd [9] and up to 40% when soils were acidified to maximize Cd phytoavailability. Hyperaccumulators therefore act as effective “metal sinks”, preferentially extracting bioavailable metals and potentially reducing the metal burden available for uptake by co-cultivated edible crops in contaminated agricultural systems. Accordingly, intercropping systems combining hyperaccumulator and food crops have been proposed as a risk-management strategy to limit metal transfer into the human food chain, while simultaneously generating metal-rich biomass. Morais et al. (2015) [10] demonstrated that metals are much more available when present in leaf litter than in the soil. Consequently, the plant contributes to increasing the size of the available metal pool of the surface of the soil, a pool where it preferentially picks up metal ions during its life. Recent isotopic fractionation studies have revealed the contribution of the hyperaccumulator leaf litter plants to phytoavailable Ni in local ultramafic substrates [11]. However, Ni is rapidly adsorbed onto the Fe and Mn oxides within the substrate matrix. The authors emphasize that, although the Ni-rich aboveground biomass is removed through harvesting during agromining operations, Ni remains within less labile but slowly replenishing pools to sustain uptake by subsequent cropping cycles. This dynamic supports the long-term feasibility of agromining systems, where continuous replenishment of the bioavailable Ni fraction enables repeated phytoextraction cycles. The main characteristics of Ni hyperaccumulator plants that are considered for agromining include high biomass yield and high shoot Ni concentrations [12]. These species need to be fast-growing. In addition, “metal crops” with >1% Ni in shoots are excellent candidates for economic agromining operations.

Two main categories of plants can be suitable for phytomining: fast-growing species and hyperaccumulators. While fast-growing species such as poplar and willow generate substantial biomass, their metal accumulation capacity is generally lower than those of hyperaccumulators [13]. In contrast, hyperaccumulator plants [14] can accumulate exceptionally high metal concentrations from the soil without exhibiting signs of toxicity. The threshold concentrations used to classify a plant species as a hyperaccumulator vary depending on the element [15]. Typical threshold concentrations are 10,000 mg/kg for Mn; 3000 mg/kg for Zn; 1000 mg/kg for REEs, Pb, As, and Ni; 300 mg/kg for Cr, Cu, and Co; 100 mg/kg for Tl, Ca, and Cd; 10 mg/kg for Hg; and 1 mg/kg for Pd, Pt, and Au [16,17]. These species span approximately 130 genera across 52 plant families, with the highest numbers found in Brassicaceae (83 species) and Phyllanthaceae (59 species) [16].

Dinh et al. (2025) [1] highlighted the advantages and limitations of hyperaccumulating species in phytoextraction. Their main advantage is their exceptional ability to accumulate target metals to extremely high levels within plant tissues. However, their application is constrained by two major limitations: low biomass production, which restricts the total amount of metal that can be recovered, and the limited availability of well-characterized hyperaccumulator species. In contrast, fast-growing plant species offer the advantage of

producing substantially higher biomass, thereby enhancing the overall potential for metal recovery. In addition to classical hyperaccumulators, increasing attention has been directed toward high-biomass and stress-tolerant species capable of substantial metal removal despite lower tissue concentrations. *Atriplex halimus* has shown promising potential in saline and metal-contaminated soils due to its high adaptability, biomass production, and tolerance to harsh environmental conditions, making it a promising candidate for low-input phytoremediation and phytomining applications [18].

However, the mechanisms governing metal accumulation in plants remain poorly understood. Further research in plant biotechnology, including the identification of new hyperaccumulator species, the development and application of soil amendments, together with genetic engineering approaches, are required to enhance metal accumulation in plants and improve phytoextraction efficiency. It is also essential to evaluate phytoextraction efficiency over successive cultivation cycles involved in phytomining, since annual yield is not constant due to changes in the bioavailability of chemical elements after the first growing cycle.

As previously stated, the bioavailability of metal(loid)s varies depending on multiple factors, including crop species, intercropping systems, soil properties, the source of the chemical elements, organic matter content, pH, plant developmental stage, root exudates, and broader environmental conditions, and the composition of the soil microbiota. Arbuscular mycorrhizal (AM) fungi are recognized as key components of plant–soil interactions in contaminated environments, playing important roles in plant nutrition, stress tolerance, and phytoremediation processes [19]. Through the development of extensive hyphal networks, AM fungi can modify metal availability and influence phytoextraction processes [20]. In addition, they produce glomalin, a recalcitrant glycoprotein that is involved in C stabilization and the sequestration of heavy metals [21]. Depending on the plant species, metal type, and soil conditions, mycorrhizal associations may either promote or restrict metal translocation to aboveground tissues, thereby affecting phytoextraction efficiency [22]. However, it remains unclear how successive phytoextraction cycles affect AM fungal colonization, GRSP production, and element uptake. Crop performance in agromining/phytomining systems is commonly evaluated in terms of chemical element uptake per hectare per year, as well as element concentration per unit of plant mass. *Helianthus annuus* is a fast-growing, high-biomass hyperaccumulator species, that has been reported in previous studies to accumulate Zn, Cu, Ni, Sr, P, and other metals and metal(loid)s [23,24]; however, its performance over successive cultivation cycles for phytomining applications remains poorly understood. Another important factor in the success of biotechnology for field implementation is the progressive scaling up of technological maturity, Technology Readiness Level (TRL). Two technology development systems have been developed at the Bioenvironmental Laboratory (LAB) at a 1:100 scale for scaling up biotechnologies focused on bioaccumulation and bioleaching, among other environmental applications.

The technology readiness levels (TRLs) considered in this study range from TRL 1 (proof-of-concept development) to TRL 9, where the technology has been tested, optimized, validated, and demonstrated under real operating conditions. TRL 1–2 represents proof-of-concept testing at a small scale and in the laboratory; TRL 3–4 involves integrated laboratory-scale validation prior to scale-up to a larger scale than TRL 1–2; TRL 5–6 represents trials with all inputs, at a larger scale, under simulated environmental conditions; TRL 7 corresponds to field-scale demonstration; and TRL 8–9 involves field trials including adjustments and modifications in order to achieve reproducibility and validation under real operating conditions.

The experimental design employed two development systems developed at the Bioenvironmental Laboratory: a small-scale system designated Bioreactors (BR) at TRL 4, and

a larger-scale, modular unit designated as Vegetable Depuration Module (VDM). Unlike BR, VDM does not include hypothesis-control units, as its primary purpose is to simulate real environmental conditions and optimize the system for field deployment of the biotechnological development already validated at TRL 4.

We hypothesize that changes in soil or substrate properties induced by repeated cultivation in agromining modify chemical element bioavailability.

The objective of this study is to investigate the bioavailability of the studied elements across successive cultivation cycles of the same hyperaccumulator crop. Understanding this variability is essential for the design of agromining systems, as soil amendments or intercropping strategies are required to sustain phytoextraction efficiency.

2. Materials and Methods

2.1. Experimental Plan

The experimental workflow of this study consisted of four sequential phases:

- (i) preliminary environmental characterization and selection of representative mining materials from the Sierra Pintada Uranium Mine (Argentina);
- (ii) repeated phytoextraction cycles using *Helianthus annuus* cultivated in laboratory-scale bioreactors operated at TRL 4;
- (iii) chemical, physicochemical, and microbiological characterization of soils and plant tissues during successive cultivation cycles; and
- (iv) projection of the laboratory-scale bioextraction performance to a Vegetable Depuration Module (VDM) TRL 6 scenario.

The experimental strategy was designed to investigate the evolution of element extraction efficiency during repeated phytoextractive cycles using the same contaminated substrate. For this purpose, sunflower plants were cultivated for three consecutive cycles of three months each. At the end of every cycle, plants were harvested, chemically characterized, and new plants were sown on the same substrate to evaluate progressive changes in chemical element bioavailability and rhizosphere processes.

The selection of the experimental substrate was based on a preliminary environmental characterization of the Sierra Pintada mining area integrating remote sensing, mineralogical, and geochemical analyses described by [24]. Based on these results, sample S5P1 was identified as the most representative material for the present phytomining investigation due to its mineralogical characteristics and contamination level.

Finally, the extraction capacity observed in the laboratory-scale bioreactors was projected to TRL 6 (VDM) in order to estimate the potential bioextractive performance at a larger scale.

2.2. Study Area and Selection of Experimental Materials

2.2.1. Study Area and Sampling Sites

The Sierra Pintada Uranium mine, covering an area of 2007 ha, is located approximately 12 km to the southwest of Villa 25 de Mayo in Mendoza Province, Argentina (Figure 1). The deposit was discovered in 1968 through airborne radiometric surveys that identified a series of anomalies, which were subsequently confirmed to be associated with uranium mineralization. The study area is located within the San Rafael Block geological province, in the Sierra Pintada area. It is composed of the basement of ancient lands (Devonian and Carboniferous), affected by intense tectonism and a profuse volcanic activity that occurred during the Lower Permian that led to the accumulation of an extensive sequence of volcanic clastic rocks [25]. The large volumes of intermediate and acid ignimbrites, resulting from permo-triassic volcanism, generated favorable conditions for the concentration of uranium deposits [26]. From 1975, the Sierra Pintada Manufacturing Mining Complex

(CMFSP) operated to produce ammonium diuranate, becoming the largest uranium deposit exploited in Argentina. Mining activities were interrupted in 1997 due to the reduction in international commodity prices.

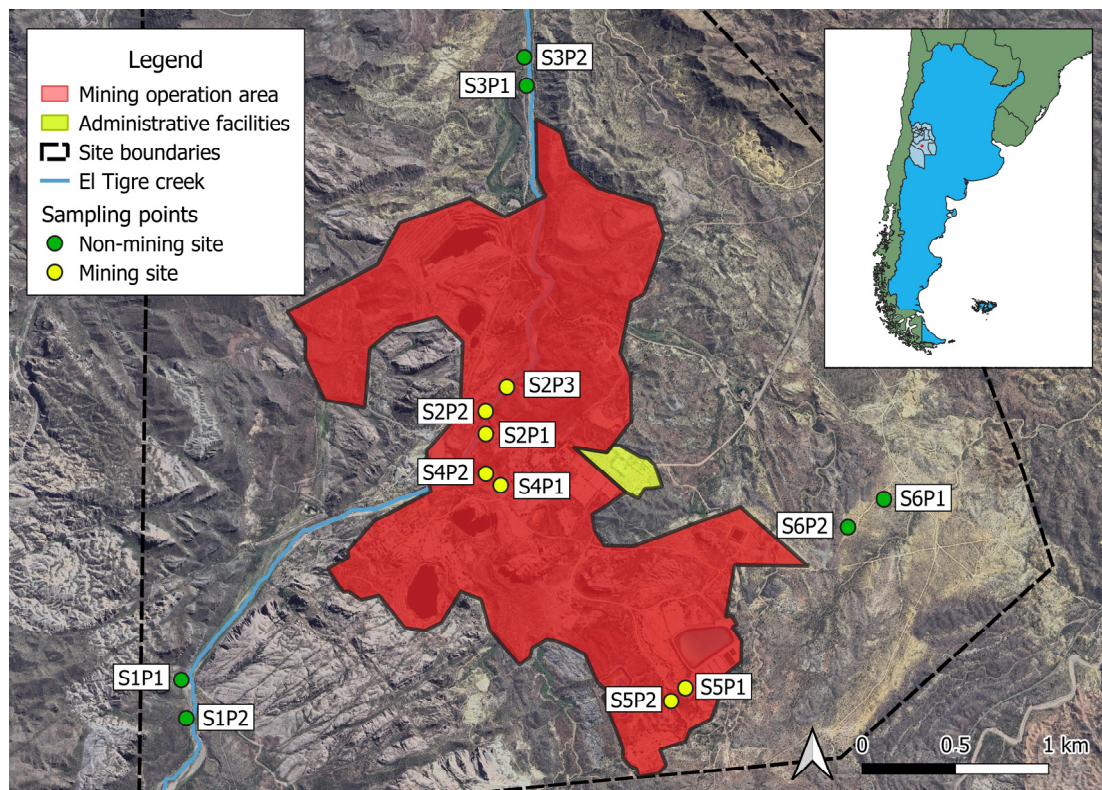


Figure 1. Study area and sampling site S5P1 in Sierra Pintada Uranium mine (Mendoza, Argentina).

2.2.2. Remote Sensing and Material Selection

The selection of experimental materials was based on the integration of remote sensing and in situ characterization data following the methodology described by [27] and [24]. A multispectral Sentinel-2A image was downloaded from the ESA Copernicus Open Access hub, acquired over the Sierra Pintada mine area on 12 December 2018. The spatial resolution of the Sentinel-2A image is 10 m in the visible and near-infrared range and 20 m in the short-wave infrared range, with a spectral resolution ranging from 490 nm to 2190 nm. A field campaign was conducted concurrently with the satellite overpass to collect soil and plant samples from 13 distinct sites. These georeferenced sampling points were overlaid on the satellite imagery to extract site-specific spectral signatures. These signatures, combined with mineralogical and chemical data, were processed using a cluster analysis—specifically the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm and the Euclidean similarity index—to identify spectrally and mineralogically consistent groups. Subsequently, a supervised pixel-based classification was performed using the Spectral Angle Mapper (SAM) algorithm. Based on the integration of these chemical-mineralogical results and the satellite-derived maps, sample S5P1 was identified as the most representative candidate for the current phytomining investigation due to its specific mineralogical profile and level of environmental impact.

2.3. Bioreactors at TRL 4 (BR) and to TRL 6 in Vegetable Depuration Module (VDM)

Four laboratory-scale bioreactors (BRs, TRL 4) were installed at the Bioenvironmental Laboratory (CNEA-FRSR, Mendoza). The reactors consisted of polycarbonate containers

(30 cm × 60 cm; height 80 cm) with a 6% slope, connected to a leachate collection chamber used to monitor percolated water volumes and flow rates.

Two BRs were filled with multiple filtration layers characterized by decreasing particle size:

- coarse gravel (10 cm thickness; average diameter 10 cm);
- medium gravel (15 cm thickness; average diameter 5 cm);
- fine gravel (20 cm thickness; average diameter 1 cm);
- upper reactive layer (15 cm thickness) consisting of mining soil, volcanic ash (1:1 *v/v*), and substrate amended with 350 ppm ZnSO₄ (CS) according to patent AR090183B1, 2022. [28,29], as previously described by [24].

The reactor configuration maintained the same geometric, hydraulic, and physico-chemical characteristics implemented in the Vegetable Depuration Module (VDM, TRL 6), including slope (6%), hydraulic retention time, vertical flow regime, and physicochemical conditions (pH and Eh). The VDM dimensions were 3 m × 5 m with a dedicated collection chamber. According to [28–30] the VDM consisted of successive layers of coarse gravel, fine gravel, pellets, and remediation substrate.

The BR system maintained a scale ratio of 1:100 with respect to the VDM, allowing the laboratory-scale extraction performance to be projected to TRL 6.

100 BRs are equivalent to 1 VDM, where each BR represents a cross-sectional section of the VDM, maintaining the slope and depth. A complete description of the BR and VDM systems and their scaling variables can be found in [28,30]. The hydraulic variables considered for scaling included the irrigation inflow regime, inflow rate, outflow rate, average flow rate (*Q*), hydraulic retention time (*t_c*), permeability constant (*K_s*) of the stone filter and substrate, substrate volume, stone filter volume, water-holding capacity, porosity, Darcy velocity, and average linear velocity. In the present study, scaling was evaluated through projection only. However, all hydraulic variables were measured in the BR system to enable future implementation of the VDM under equivalent or proportionally adjusted hydraulic conditions, depending on the variable considered [30].

2.4. Phytoextraction Experiment

Six sunflower plants (*Helianthus annuus*) were cultivated across two bioreactors, with three plants per unit containing contaminated substrate from the CMFSP area, specifically from the S5P1 zone. Plants were grown for three months until post-flowering stage. At the end of each cultivation cycle, soils, roots, leaves, and flowers were collected for chemical, physical, and biological analyses. Soil pH and Eh were also monitored throughout the experiment.

At the end of the first growing cycle, the plants were harvested, and a new batch of sunflower seedlings was transplanted into the same substrate to initiate the second cycle. This procedure was repeated for the third and final cycle, allowing the evaluation of successive phytoextraction cycles in the same contaminated matrix. Upon harvest, the plants were thoroughly washed with distilled water and dried to a constant weight at 70 °C for 72 h. No soil treatment was carried out between plantings of the different cycles.

2.5. Analytical Methods

2.5.1. Chemical Analyses

The total concentration of heavy metals (Pb, Zn, Ag, Cu, Au, Cd, Ni, Cr, Sb, and V) and additional elements (P, Ca, Mg, Na, K, S, Mn, Fe, Sr, and Ba) in soils and substrates was determined by Wavelength Dispersive X-ray Fluorescence (WDXRF). Pressed pellets (28 mm in diameter) were prepared using 3 g of dried material and analyzed using a Bruker

S8 Tiger spectrometer. Quality control was performed using certified reference materials (NIST 2709a and 2710a).

2.5.2. Bioconcentration and Translocation Parameters

The phytoremediation performance was evaluated through the bioconcentration factor (BCF) and translocation factor (TF). The BCF was calculated as the ratio between chemical elements concentration in plant tissues and soil concentration, while TF was calculated as the ratio between aerial and belowground tissues. Values greater than 1 indicated efficient accumulation or translocation processes.

2.5.3. Mycorrhizal Colonization and GRSP Analyses

Percentage frequency (%F) and intensity (%I) of arbuscular mycorrhizal colonization were determined after root staining using the modified method described in [31]. Root segments were microscopically analyzed using an Olympus BX51 microscope.

Easily extractable glomalin-related soil proteins (EE-GRSP) and total GRSP (T-GRSP) were quantified as described in [32] using sodium citrate extraction, autoclave-assisted extraction procedures, and Bradford colorimetric assay at 595 nm.

2.6. Estimation of Bioextractive Potential in VDM

The bioextractive potential (BP_{BR}) was estimated by projecting the extraction capacity observed in BRs to the VDM system considering a scale ratio of 100 BR = 1 VDM. Calculations assumed a maximum density of 290 well-developed sunflower plants in the VDM.

The bioextractive potential was calculated according to the following equations:

$$BP_{BR}(mg) = \frac{C_{plant} \times Biomass_{BR}}{1000}$$

where C_{plant} is the concentration of the chemical elements in plant tissues and $Biomass_{BR}$ is the biomass produced in the bioreactor system. In this work we have considered only the extractive power of the aerial part.

2.7. Statistical Analysis

Data were subjected to analysis of variance (ANOVA), and significant differences among treatments were evaluated using Tukey's HSD post hoc test ($p \leq 0.05$). Differences in mycorrhizal colonization (%F and %I) among phytoremediation cycles were assessed using the non-parametric Kruskal–Wallis test.

3. Results

3.1. Selection of a Representative Mining Waste Area for Upscaling Experiments

The selection of S5P1 was based on the preliminary environmental characterization and remote sensing analyses reported by [24]. The integration of Sentinel-2 imagery, UPGMA clustering and SAM classification enabled the identification of a mining waste deposition area characterized by a distinct mineralogical composition, with gypsum as the dominant mineral phase, clearly distinguishing S5P1 from nearby sites such as S5P2, where quartz and albite prevailed. Therefore, the experimental substrate used in the present study is a geochemically and mineralogically representative material of the Sierra Pintada mining area. This preliminary remote sensing screening was essential for selecting the substrate used in the phytoextraction experiments and for supporting the upscaling assessment from TRL 4 bioreactors to the TRL 6 VDM scenario.

3.2. Results at TRL4 in BR

The results of the experiments carried out in BR over three successive engineering cycles are presented in Tables 1 and 2. Element concentrations measured in the different plant tissues (root, shoot and flower), relative to the initial soil concentrations of each cycle in the CMFSP mine substrate, were used to calculate the BCF and the TF.

Table 1. Chemical element concentrations (ppm), and statistical significance (Tukey's HSD) across the engineering cycles. Values represent Mean (SD) ($n = 2$). Different superscript letters within the same row indicate statistically significant differences among engineering cycles according to Tukey's HSD test ($p < 0.05$).

Element	Compartment	Cycle 1	Cycle 2	Cycle 3
Cu	Soil	2021.45 (3.75) ^a	1500.00 (141.42) ^a	1750.00 (212.00) ^a
	Shoot	520.00 (98.78) ^a	44.20 (4.00) ^b	93.00 (7.00) ^b
	Root	1540.00 (67.18) ^a	548.80 (44.00) ^b	640.00 (51.00) ^b
	Flower	230.00 (24.25) ^a	247.00 (2.00) ^a	36.00 (3.00) ^b
Zn	Soil	312.57 (16.69) ^a	390.50 (17.68) ^a	407.00 (35.60) ^a
	Shoot	839.50 (36.20) ^a	96.00 (8.00) ^b	79.00 (6.00) ^b
	Root	388.70 (4.53) ^a	84.00 (7.00) ^b	64.00 (5.00) ^b
	Flower	193.20 (12.37) ^a	63.00 (5.00) ^b	78.00 (6.00) ^b
Ni	Soil	71.30 (4.60) ^a	58.00 (2.83) ^a	62.00 (1.41) ^a
	Shoot	150.00 (39.32) ^a	2.00 (0.20) ^b	3.90 (0.30) ^b
	Root	370.00 (10.96) ^a	4.10 (0.30) ^b	3.10 (0.20) ^b
	Flower	160.00 (21.21) ^a	5.70 (0.50) ^b	8.10 (0.60) ^b
Sr	Soil	650.00 (34.01) ^a	491.00 (7.07) ^b	501.50 (19.09) ^b
	Shoot	431.00 (21.85) ^a	188.00 (15.00) ^b	229.00 (18.00) ^b
	Root	185.00 (15.56) ^a	119.00 (10.00) ^b	120.00 (10.00) ^b
	Flower	123.00 (9.83) ^{ab}	89.00 (7.00) ^a	129.00 (10.00) ^b
P	Soil	585.00 (256.11) ^a	1750.00 (70.71) ^b	1750.00 (535.50) ^b
	Shoot	1757.00 (216.16) ^a	1776.00 (178.00) ^a	2197.00 (220.00) ^a
	Root	636.00 (52.47) ^a	1296.00 (13.00) ^b	1218.00 (122.00) ^b
	Flower	3221.00 (302.99) ^a	2829.00 (283.00) ^a	6669.00 (667.00) ^b

Table 2. Results of parameters obtained in three engineering cycles. BCFs: Bioconcentration Factor in shoots; BCFr: Bioconcentration Factor in roots; BCFf: Bioconcentration Factor in flowers; TFs/r: Translocation Factor from root to shoot; TFf/s: Translocation Factor from shoot to flower; TFf/r: Translocation Factor from root to flower.

Cycle	1	2	3	1	2	3	1	2	3
Parameter	BCFs			BCFr			BCFf		
Cu	0.26 *	0.03	0.05	0.76	0.37	0.37	0.11	0.16	0.02
	(0.05)	(0.00)	(0.01)	(0.03)	(0.05)	(0.05)	(0.01)	(0.02)	(0.00)
Zn	2.69	0.25	0.19	1.24	0.22	0.16	0.62	0.16	0.19
	(0.18)	(0.02)	(0.02)	(0.07)	(0.02)	(0.02)	(0.05)	(0.01)	(0.02)
Ni	2.10	0.03	0.06	5.19	0.07	0.05	2.24	0.1	0.13
	(0.57)	(0.00)	(0.01)	(0.37)	(0.01)	(0.00)	(0.33)	(0.01)	(0.01)
Sr	0.66	0.38	0.46	0.28	0.24	0.24	0.19	0.18	0.26
	(0.05)	(0.03)	(0.04)	(0.03)	(0.02)	(0.02)	(0.02)	(0.01)	(0.02)
P	3.00	1.01	1.26	1.09	0.74	0.7	5.51	1.62	3.81
	(1.37)	(0.11)	(0.40)	(0.48)	(0.03)	(0.22)	(2.47)	(0.17)	(1.23)
Parameter	TFs/r			TFf/s			TFf/r		
Cu	0.34	0.08	0.15	0.44	5.59	0.39	0.15	0.45	0.06
	(0.07)	(0.01)	(0.02)	(0.10)	(0.51)	(0.04)	(0.02)	(0.04)	(0.01)

Table 2. Cont.

Cycle	1	2	3	1	2	3	1	2	3
Zn	2.16 (0.10)	1.14 (0.13)	1.23 (0.13)	0.23 (0.02)	0.66 (0.08)	0.99 (0.11)	0.50 (0.03)	0.75 (0.09)	1.22 (0.13)
Ni	0.41 (0.11)	0.49 (0.06)	1.26 (0.13)	1.07 (0.31)	2.85 (0.38)	2.08 (0.22)	0.43 (0.06)	1.39 (0.16)	2.61 (0.26)
Sr	2.33 (0.23)	1.58 (0.18)	1.91 (0.22)	0.29 (0.03)	0.47 (0.05)	0.56 (0.06)	0.66 (0.08)	0.75 (0.09)	1.08 (0.12)
P	2.76 (0.41)	1.37 (0.14)	1.80 (0.26)	1.83 (0.28)	1.59 (0.23)	3.04 (0.43)	5.06 (0.63)	2.18 (0.22)	5.48 (0.78)

* standard deviation in parentheses.

3.3. Projected Scaling from TRL4 to TRL6

The individual weights of each plant across the bioreactors and their statistical significance according to Tukey's test are presented in Table 3.

Table 3. Dry biomass production (g/plant) of *Helianthus annuus* across the three engineering cycles into two Bioreactors. Values represent the mean $\pm$ standard deviation (SD) of 6 plants per cycle. Different letters within the same column indicate statistically significant differences according to Tukey's HSD test ($p < 0.05$). The letter 'a' across all cycles confirms the absence of significant variations in biomass production.

Cycle	Bioreactor (BR)	Plant	Shoot (g)	Root (g)	Flower (g)
1	BR 1	P1	68.50	41.20	110.40
		P2	55.10	25.40	91.50
		P3	71.90	44.20	115.40
	BR 2	P4	52.00	23.50	88.50
		P5	69.20	39.80	109.20
		P6	57.50	29.00	95.40
Mean (SD)	Cycle 1		62.37 (8.12) a	33.85 (9.17) a	101.73 (11.23) a
2	BR 1	P1	54.20	24.10	90.00
		P2	70.40	43.10	114.20
		P3	52.60	22.90	89.00
	BR 2	P4	69.00	45.90	116.10
		P5	63.90	34.00	104.40
		P6	60.70	35.50	101.50
Mean (SD)	Cycle 2		61.80 (7.37) a	34.25 (9.61) a	102.53 (11.66) a
3	BR 1	P1	62.30	32.40	101.30
		P2	51.10	21.90	86.90
		P3	73.00	46.40	117.30
	BR 2	P4	53.00	24.70	89.50
		P5	71.20	43.80	113.80
		P6	60.50	33.90	101.70
Mean (SD)	Cycle 3		61.85 (8.65) a	33.85 (10.13) a	101.75 (12.15) a
Total	Biomass (g)/plant (SD)		62 (8)	34 (10)	102 (12)

The bioaccumulation and translocation results obtained during the three engineering cycles were subsequently used to estimate the potential large-scale phytoextraction performance of the system under VDM operating conditions. Considering the biomass produced

in BR, the number of sunflower plants reported by [28] in VDM (290 plants), and the ratio of 100 BR: 1 VDM, the aboveground extraction potential values were calculated.

Each BR represents a physical cross-section of the VDM, maintaining equivalent depth and slope attributes. Consequently, the performance of the 3 plants developed per BR can be scaled up proportionally to a projected population of 300 plants growing concurrently within the VDM volume. Based on the hydraulic, physical, chemical, and biological boundary conditions described by [30] for upscaling, the net extractive potential of the sunflower aerial biomass can be inferred. Root uptake was excluded from the upscaling calculations since a major percentage of the root biomass remains embedded within the soil matrix upon harvesting.

The following hydraulic parameters were used for scale-up from TRL 4 (BR) to TRL 6 (VDM), based on Darcy's law:

- Irrigation inflow regime: vertical and laminar flux
- Inflow rate: (Q_i): $2.64 \text{ m}^3/\text{d}$
- Outflow rate: (Q_e): $0.83 \text{ m}^3/\text{d}$
- Average Q_i and Q_e (Q): $1.74 \text{ m}^3/\text{d}$
- Liquid retention time (exit to the collecting chamber) (t_c): 32 s (stone filter).
- Permeability constant (K_s) of the stone filter and soil substrate: $193 \text{ m}^3/\text{d}/\text{m}^2$
- Substrate layer volume: (last layer with the soil); 0.0225 m^3
- Stone filter volume: 0.09 m^3
- Water holding capacity (WHC): $0.14 \text{ kg H}_2\text{O}/\text{kg}$ substrate; Total water volume retained at capacity (per BR): 2 L (with zero outlet discharge).
- Porosity (ϕ): 0.4
- Darcy's velocity (VD): $11.57 \text{ m}/\text{d}$
- Average linear velocity (V_a): $29 \text{ m}/\text{d}$
- EE-GRSP and T-GRSP (first cycle): 0.4 (0.2) mg/g dry soil and 1.3 (0.3) mg/g dry soil respectively

The pH and Eh during the experiment ranged from 6.8 to 7.2 and from +300 to +320 mV, respectively. The outlet to the collection chamber was used during system calibration; however, it remained closed during the experimental runs to prevent leaching.

CMFSP soil contained AM fungal propagules capable of establishing mycorrhizal associations; however, overall fungal colonization levels remained very low. The frequency of AM root colonization (%F) in *H. annuus* plants grown in CMFSP soil remained low across all three cycles, with no significant differences detected among cycles ($p > 0.05$; $p = 0.0944$) (Figure 2). In the first cycle, colonization was minimal ($2.2 \pm 2.2\%$) (mean $\pm$ standard error), increased during the second cycle ($13.3 \pm 4.7\%$) and stabilizing in the third cycle ($13.3 \pm 5.0\%$). Similarly, AM colonization intensity (%I) ranged from 30% to 40% during the first two cycles and decreased in the third cycle ($15.3 \pm 1.3\%$), but without significant differences among cycles (Figure 2).

GRSP content showed an overall increasing trend across phytoremediation cycles in both fractions (EE-GRSP and T-GRSP) (Figure 3). However, the concentration of EE-GRSP did not vary significantly among cycles, with values of $0.4 \pm 0.2 \text{ mg g}^{-1}$ dry soil) in the first cycle and a slight increase observed in the second and third cycle (0.6 ± 0.3 and $1.2 \pm 0.2 \text{ mg g}^{-1}$ dry soil, respectively). In contrast, T-GRSP differed significantly among cycles, with concentrations in the first cycle ($1.3 \pm 0.3 \text{ mg g}^{-1}$ dry soil) being significantly lower than those recorded in the third cycle ($2.8 \pm 0.6 \text{ mg g}^{-1}$ dry soil).

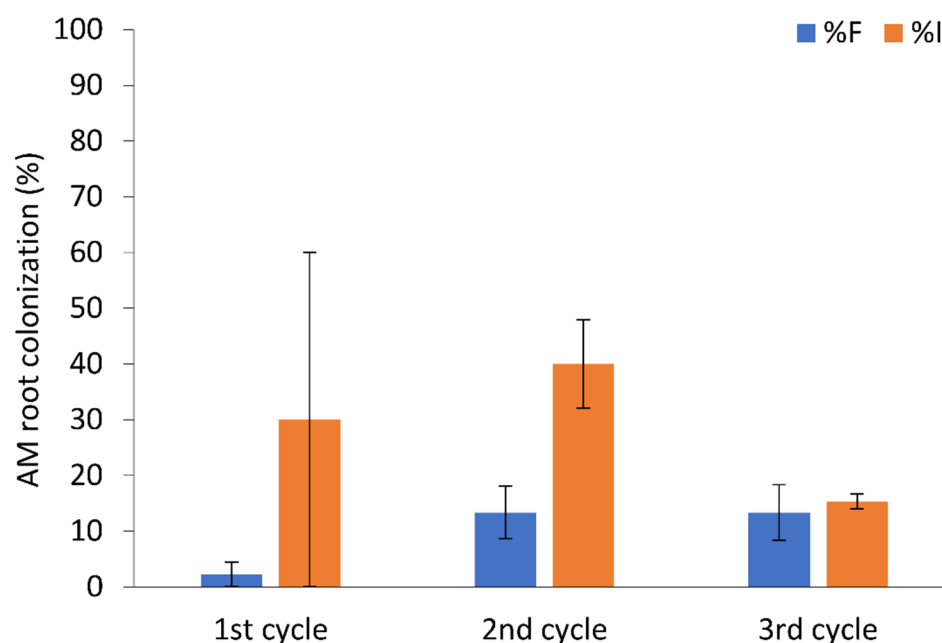


Figure 2. Percentage of frequency (%F) and intensity (%I) of AM root colonization in *Helianthus annuus* plants grown in the bioreactors over three phytoremediation cycles. Values represent mean and standard error. No significant differences among cycles were detected ($p > 0.05$).

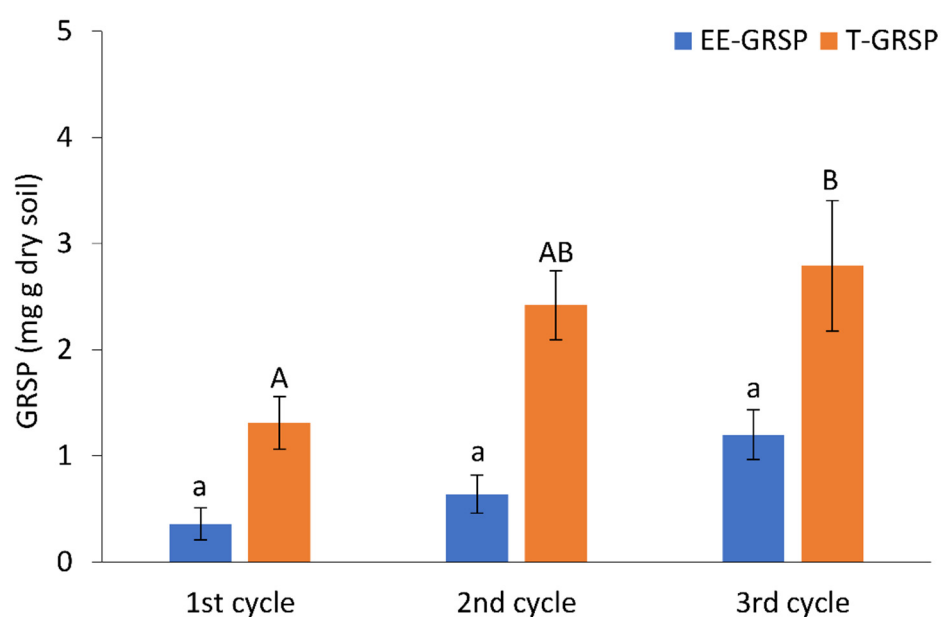


Figure 3. Concentration of easily extractable Glomalin related-soil proteins (EE-GRSP, mg g^{-1} dry soil) and total GRSP (T-GRSP) in rhizosphere soils of *Helianthus annuus* plants grown in bioreactors over three phytoremediation cycles. Values represent mean and standard error). Lowercase letters indicate differences among cycles for EE-GRSP; uppercase letters correspond to T-GRSP. Different letters denote significant differences according to ANOVA followed by Tukey test ($p \leq 0.05$).

4. Discussion

The present study demonstrated that *Helianthus annuus* cultivated in CMFSP-derived substrate exhibited high phytoextractive performance for the studied elements during the first cultivation cycle followed by a substantial decline in bioaccumulation efficiency during subsequent cycles. The observed trends suggest that the phytoextraction performance of the system was strongly influenced by progressive depletion and immobilization of

the bioavailable metal fraction. In parallel, translocation patterns and rhizosphere-related parameters indicated that plant–soil–microbial interactions evolved throughout the experimental cycles. Therefore, the discussion is structured around four main aspects: (i) the initial phytoextractive performance of *H. annuus*, (ii) the temporal decline in bioaccumulation efficiency, (iii) changes in metal translocation dynamics, and (iv) the potential role of arbuscular mycorrhizal fungi and glomalin-related soil proteins (GRSP) in regulating metal bioavailability and rhizosphere stabilization.

During the first cultivation cycle, *H. annuus* exhibited hyperaccumulator-like behaviour for Cu and Ni reaching values of 544.73 (95.16) mg Cu, 192.67 (30.94) mg Ni, 230.01 (29.83) mg Sr, 429.00 (55.62) mg Zn and 2318.77 (331.60) mg P per kg of total dry biomass. These concentrations were calculated by converting the values expressed in mg/plant to mg/kg of dry mass, (198 g/plant) (Table 4).

Table 4. Aboveground bioextraction potential (BP) during the first cycle and projection to VDM scale. BP aerial is the sum of shoot biomass and flower biomass. BP Projection refers to 290 plants grown in 1 m³ of soil.

Element	Root (mg/Plant)	Shoot (mg/Plant)	Flower (mg/Plant)	Accumulation (mg/Plant)	BPAerial (mg/Plant)	BP Projection to VDM (g/m ³)
Cu	52.13 (14.30)	32.24 (7.50)	23.46 (3.49)	107.83 (16.52)	55.70 (8.28)	16.15 (2.40)
Zn	13.16 (3.57)	52.05 (7.12)	19.71 (2.64)	84.92 (8.39)	71.76 (7.56)	20.81 (2.19)
Ni	12.52 (3.41)	9.30 (2.72)	16.32 (2.87)	38.14 (5.22)	25.62 (3.97)	7.43 (1.15)
Sr	6.26 (1.78)	26.72 (3.74)	12.55 (1.77)	45.53 (4.50)	39.27 (4.11)	11.39 (1.19)
P	21.53 (6.10)	108.93 (19.45)	328.54 (49.08)	459.00 (53.14)	437.48 (53.16)	126.87 (15.42)

These concentrations did not exceed the threshold values proposed by [15–17,33] for Sr and Zn hyperaccumulation, but exceeded the hyperaccumulator threshold for Ni and Cu. The results obtained during the first cycle are also consistent with previous studies reporting significant accumulation capacity of *H. annuus* for several metal(loid)s [34–38], although this behaviour was not maintained during the subsequent cycles in this work.

The projected aboveground BP yield of Ni reached 7.43 g/m³ of VDM substrate (Table 4) Bani et al. (2025) [8] reported that *Odontarrhena chalcidica*, one of the most studied agromining species, may achieve yields exceeding 150 kg of Ni per hectare under optimized field conditions. Assuming a soil depth of 0.30 m, the Ni extraction value reached is 50 g/m³ of soil, while the BP aerial values obtained in the present study differ by only one order of magnitude from those reported by Bani et al., suggesting that *H. annuus* may represent a promising high-biomass candidate for short-term agromining-oriented applications under specific geochemical conditions.

A marked decrease in bioaccumulation coefficients was observed after the first cultivation cycle for Cu, Zn, and Ni in shoots, roots, and flowers (Table 2). For example, Zn BCFs decreased from 2.69 in the first cycle to 0.25 and 0.19 in the second and third cycles, respectively, while Ni BCFs decreased from 2.10 to 0.03 and 0.06. These results indicate that phytoextraction performance cannot be considered constant over time, since extraction efficiency strongly depends on the persistence of the labile and phytoavailable metal fraction within the substrate. Furthermore, by analyzing the increase in Zn and P in the soil, 312.57 ppm in Cycle 1 to 407.00 ppm in Cycle 3 and 585.00 ppm to 1750.00 ppm by Zn and P respectively, we can infer that one of the main mechanisms limiting bioavailability is the accumulation of heavy metals (and metalloids) in the spores and mycelium of arbuscular mycorrhizal (AM) fungi. These effects could interfere with the decrease in the concentration of elements in the soil. This result could be related to microbial activity, which might

increase in the mycorrhizosphere due to changes in the abundance of microorganisms associated with AM hyphae, such as phosphorus-solubilizing bacteria [30].

This behaviour is consistent with the findings of [7], who demonstrated that both hyperaccumulator and non-hyperaccumulator plants extract metals from the same bioavailable (labile) pool, although hyperaccumulators exhibit a markedly greater depletion capacity. Similarly, ref. [9] reported that a single crop of *N. caerulescens* removed more than 20% of the available Cd fraction from contaminated soil and even 40% if the soil were acidified to maximize Cd phytoextraction. Therefore, the strong decrease observed in the present work may reflect progressive depletion of the readily available metal fraction after the first engineering cycle.

In addition to depletion processes, the reduction in phytoavailability may also be associated with progressive immobilization mechanisms occurring within the rhizosphere. Metal(loid)s may become less mobile through sorption onto mineral surfaces, complexation with organic matter, precipitation/crystallization processes, and interactions with Fe and Mn oxides. Furthermore, rhizosphere-induced changes in pH and redox conditions, together with microbial activity and root exudation processes, may progressively stabilize metal fractions within the substrate [39,40]. On the other hand, elements immobilized within bacterial cell walls, fungal hyphae, spores, and residual root tissues remaining in the soil after harvest are no longer readily available for plant uptake. Benavidez et al. (2024) [41] reported substantial accumulation of Cu and Ni in non-bioavailable fungal structures.

Interestingly, although overall bioaccumulation decreased during subsequent cycles, some translocation factors increased over time, particularly for Ni and P. For instance, Tff/r values for Ni increased from 0.43 during the first cycle to 2.61 in the third cycle, while Zn Tff/r increased from 0.50 to 1.22 (Table 2). These trends suggest that, despite reduced overall availability, internal redistribution mechanisms remained active and may have been enhanced under progressive stress conditions. Because P is strongly associated with AM functioning and reproductive allocation, the increased P translocation toward aerial and reproductive tissues may reflect an efficient physiological response of the symbiosis [42]. Increased translocation toward reproductive tissues may also influence the long-term dynamics of phytoavailable elements within the system, since the deposition of flowers and aerial biomass onto the soil surface may contribute to the recycling of metal(loid)s into the upper rhizosphere layers, as previously discussed by [10].

The microbiological results provide additional insight into the progressive evolution of the rhizosphere during repeated phytoextraction cycles. Although AM root colonization remained relatively low throughout the experiment and no statistically significant differences were observed among cycles, T-GRSP increased significantly from the first to the third cycle. GRSP are known to contribute to soil aggregation and metal immobilization through sorption and organo-mineral interactions [19,43]. Therefore, the increase in T-GRSP may have contributed, at least partially, to the reduction in metal mobility and phytoavailability observed during the later cultivation cycles. Although metal contamination can reduce AM fungal root colonization, tolerant AM fungal communities may continue producing GRSP, which may contribute to metal sequestration and reduced element mobility in soil [26].

From an operational perspective, the results suggest that *H. annuus* may be particularly effective during the initial phytoextraction stage of agromining-oriented remediation systems, whereas prolonged multi-cycle cultivation may require substrate management strategies aimed at restoring or maintaining metal bioavailability. Such approaches may include the application of amendments, rhizosphere engineering, microbial management, or intercropping strategies in order to sustain phytoextraction efficiency over time.

These findings are consistent with the results of the present study, which revealed a marked decrease in bioaccumulation between the first and subsequent cycles (Table 2)

for Cu, Zn, and Ni in leaves, roots, and flowers. This indicates that phytoextraction performance of a crop for agromining is not constant over time, and yield can vary between cultivation cycles, either due to increased leaf litter, as noted by [10], or due to a lack of bioavailability, as found in this study, which may result from the depletion of the soluble fraction, fixation to soil mineral fractions, production of chelating compounds (e.g., organic acids and metallothioneins), interactions with microbial and plant cell walls, and regulation of plasma membrane transport processes under mycorrhizal conditions [44,45]. This information is useful because, when using *H. annuus* in agromining for the extraction of Ni, Cu, or Zn in mining soil with the geological and mineralogical characteristics described in the CMFSP, only one cycle of *H. annuus* should be used. In subsequent cycles, an amendment should be applied, or intercropping schemes should be designed. Regarding root-to-leaf translocation, we observed a decrease towards the second cycle but an increasing trend towards the third cycle, whereas translocation to flowers showed an increasing trend across cycles. This process is important because, as reported by [10], the senescence and deposition of aboveground biomass (shoots and flowers) may increase the bioavailability of elements for subsequent crops. A decrease in bioaccumulation coefficients during prolonged cultivation was also reported by [46].

5. Conclusions

Overall, the results demonstrate that the phytoextraction efficiency of *Helianthus annuus* strongly depends on the temporal evolution of element bioavailability within the substrate. The first cultivation cycle exhibited the highest accumulation and extraction performance, highlighting the potential of sunflower-based systems for short-term phytoextraction and potential agromining applications in mining substrates. However, the progressive decline in bioaccumulation coefficients observed during subsequent cycles indicates that repeated cultivation may substantially reduce extraction efficiency. Notably, empirical measurements show that the total substrate concentration did not drop drastically enough between cycles to account for the massive decline in plant tissue accumulation. This observation strongly supports our interpretation: the decline in extraction efficiency is primarily driven by a drop in the phytoavailable and labile fraction—associated with rhizosphere immobilization mechanisms such as organo-mineral stabilization by glomalin (T-GRSP) and microbial sequestration—rather than a total exhaustion of the absolute element pool in the soil. At the same time, the evolution of translocation factors and rhizosphere-related parameters suggests that plant–soil–microbial interactions progressively modify metal mobility and internal redistribution dynamics. These findings indicate that sustainable multi-cycle agromining systems will likely require active substrate management strategies aimed at maintaining phytoavailability over time. Nevertheless, the projected bioextractive potential obtained during the first cycle confirms that *H. annuus* represents a promising high-biomass candidate for phytomining-oriented remediation systems, where accumulated elements could be subsequently recovered through downstream hydrometallurgical processing.

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Abbreviations

The following abbreviations are used in this manuscript:

REEs	Rare Earth Elements
TRL	Technology Readiness Level
VDM	Vegetable Depuration Module
CMFSP	Complejo Minero Fabril Sierra Pintada
S5P1	Site with impacted mining soil used in this work
ESA	European Space Agency
SAM	Spectral Angle Mapper
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
BR	Bioreactor
WDXRF	Wavelength Dispersive X-ray Fluorescence
EE-GRSP	Easily extractable glomalin-related soil proteins
T-GRSP	Total extractable glomalin-related soil proteins
BP	Bioextractive Potential
BCFs	Bioconcentration Factor in shoots
BCFr	Bioconcentration Factor in roots
BCFf	Bioconcentration Factor in flowers
TFs/r	Translocation Factor from root to shoot
TFf/s	Translocation Factor from shoot to flower
TFf/r	Translocation Factor from root to flower
CNEA	Comisión Nacional de Energía Atómica
FRSR	Facultad Regional San Rafael
%F	Percentage of frequency
%I	Percentage of intensity
AM	Arbuscular mycorrhizal

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