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Helicrysum italicum (roth) G. Don, a promising species for the phytostabilization of polluted mine sites: a case study in the Montevecchio mine (Sardinia, Italy).

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Abstract

Mine exploitations worldwide have generated a great amount of tailings, which still contain large quantities of Potentially Harmful Elements (PHEs) able to contaminate soil, water, air, wildlife, and the food chain. Phytoremediation is an option to immobilize and/or extract PHEs from polluted mining areas. This study aims at assessing the phytoremediation properties of *Helicrysum italicum* (roth) G. Don, and in particular the capacity to absorb, transfer and accumulate some PHEs, such as Cd, Cu, Ni, Pb and Zn, in the plant tissues. A restricted literature review (7 papers) is also proposed in order to outline the *H. italicum*'s behaviour and verify its possible use in phytoremediation strategy of polluted mine soils. A number of 22 contaminated sites from Montevecchio mine area (Sardinia, Italy) were sampled and the results compared with 6 uncontaminated sites. In each site both rhizospheric soil materials and *H. italicum* plants were sampled. Total composition and bioavailable fractions were analysed on soil samples. *Helicrysum italicum* roots, stems and leaves were separately analysed to extract PHEs; root/soil and leaf/root ratios were calculated to elucidate plant behaviour. Results show that Cd and Zn are the most bioavailable PHEs in contaminated sites compared to uncontaminated ones (300 and 500 folds, respectively), while Cd, Cu, Pb and Zn exceed the thresholds of the Italian environmental legislation when aqua regia extraction is executed. *Helicrysum italicum* plants growing on contaminated soils accumulate significantly more Cd, Ni, Pb and Zn than plants growing on uncontaminated soils, while no significant differences are found for Cu. For all considered PHEs the root/soil ratios are > 1 in both contaminated/uncontaminated sites meaning that *H. italicum* can be defined as a tolerant species for remediation of metal-polluted soils. The leaf/root ratios weakly > 1 of Zn, Pb and Cu and < 1 of Ni and Cd indicate *H. italicum* not suitable for phytoextraction. Our results are aligned with the available literature indicating *H. italicum* a tolerant species, especially for Cd,

Pb and Zn. The low leaf/root ratios, along with its being a spontaneous and perennial species able to propagate seeds directly on contaminated soils, recommended the use of *H. italicum* as pioneering strategy for the phytostabilization.

Keywords

Helicrysum italicum (roth) G. Don, Potentially Harmful Elements, phytoremediation, mine soils, literature review

1. Introduction

Every historical civilization' advancement has required mined resources and also future human development and green technologies will depend on extensive Earth-extracted resources (Mills, 2020). Mining activities are widespread in the world, locally modifying the original environment and impacting biota even many years after their dismissal (Camizuli et al., 2018). Estimations for Europe, China and the USA reveal that about 0.1% of the land is represented by abandoned mining sites (Arbogast et al., 2000; EUROSTAT, 2012; Lin and Ho, 2003; Perez, 2012). Mine exploitation generates large amounts of tailings, which can still contain high quantities of Potentially Harmful Elements (PHEs). Tailings are poorly colonized by vegetation because of unfavourable chemical-physical conditions for plant growth (Martínez-Sánchez et al., 2012), thus PHEs could easily spread out in the environment contaminating soil, water, air, and wildlife reaching also the food chain (Dore et al., 2020). In many cases PHEs are essential elements at a low concentration (e.g., Cu, Zn, Mn, Fe) that turn to be toxic to one or more species when reaching higher concentrations (Bini and Wahsha, 2014; Vamerali et al., 2010). Other elements (e.g., As, Cd, Cr, Ni, and Pb) show toxic impacts on plants and animals even at low concentrations (Singh et al., 2011).

Remediation is the only intervention to reduce or definitively solve the environmental contamination problem. Conventional remediation technologies are often expensive, labour-intensive, destructive, and not eco-friendly (Meuser, 2013; Yao et al., 2012). On the other hand, phytoremediation, defined as the use of plants for degradation of xenobiotics or extraction/immobilization of PHEs from water or soil substrates (USEPA, 2000), is cost-effective, widely acceptable, sustainable, applicable in large areas, and economically exploitable, particularly when native plants are used (Pandey et al., 2015; 2016).

Plants for phytoremediation must be resistant to both contaminants and unfavourable climatic conditions like drought and heat, especially in Mediterranean areas (Poschenrieder et al., 2012), and should display a high growth rate to absorb considerable quantities of toxic elements in their tissues (Mendez and Maier, 2008). For these reasons, plants are often native of the environment in which they will be used (Yoon et al., 2006).

Phytoremediation includes five types of strategies adopted by plants: phytoextraction/phytoaccumulation, phytostabilization, phytodegradation, rhizofiltration and phytovolatilization; only the first two are considered for PHEs (Mahar et al., 2016; Pandey and Bajpai, 2019). As reported by Mendez and Maier (2008), plants for phytostabilization should accumulate PHEs in roots and not transfer them to shoots, to avoid further transfer into the food chain. Instead, plants eligible for phytoextraction should be tolerant to PHEs, absorb and accumulate them in the aboveground plant parts, grow fast and be easy to harvest (Mendez and Maier, 2008).

A plant's phytoremediation capacity is generally assessed by means of a large number of different quantitative indicators (Buscaroli, 2017). These are calculated as ratios between element contents in aerial parts and roots, or as ratios between element contents in plant parts and soil. In literature, the element concentration in soil is assessed by adopting different analytical procedures such as X-ray fluorescence or by several wet extraction methods (e.g., Aqua regia, EDTA, DTPA, etc.) and thus, resulting ratios between plant parts could significantly differ. Abreu et al. (2008) defined the Bioconcentration Coefficient (BC) the ratio between the element content in leaves and available fraction of the corresponding soil element, extracted with DTPA aqueous solution. Plants are considered tolerant when the BC value is greater than 1 ($BC > 1$). Similarly, Sidhu et al. (2017) named the same abovementioned ratio Bioconcentration Factor (BCF) and stated that the BCF values >1 indicate the potential of a plant species for remediation of metal polluted soils. Regardless of the name, when the root/soil ratio is > 1, the plant is considered a tolerant species (Abreu et al., 2008) useful for remediation of metal polluted soils (Sidhu et al., 2017). Moreover, when the leaf/root ratio is > 1 the element is efficiently transferred from roots to shoots proving that the plant is a phytoextractor while, if the leaf/root ratio is < 1 no element translocation occurs, and the plant is suitable for phytostabilization (Bolan et al., 2011).

Helicrysum italicum (roth) G. Don is a perennial subshrub of the genus *Helichrysum* of the family Asteraceae, characteristic of the Mediterranean area, and it grows on barren, dry, sandy and poorly developed soils in a wide altitudinal range from the sea level up to 2200 m a.s.l. (Galbany-Casals et al., 2011; Ninčević et al., 2019). The scientific and industrial interest for *H. italicum* is increasing due to its rusticity, versatile biological activities, cosmetic and pharmaceutical applications, and ornamental uses (Bianchini et al., 2009; Melito et al., 2015; Ninčević et al., 2019). Moreover, for the utilization and commercialization of derivatives from *H. italicum* the European Union requires certified absence of chemical impurities and heavy elements (Bullitta et al., 2010).

Helicrysum spp., are indicated as metallophyte, metal tolerant plants growing on soils enriched or contaminated by several elements (Nkoane et al., 2003; 2007; Koosaletse-Mswela, 2015). *Helicrysum* spp. have already been considered for bio-remediation purposes in contaminated mine tailings (Bacchetta et al., 2017; 2018; Bini et al., 2017; Barbafieri et al., 2011; Cao et al., 2004; Leita et al., 1989, studied *H. italicum*) and soils (Brunetti et al., 2018, studied *H. italicum*) in Italy and in many other regions of the world (Conesa et al., 2006; 2011; García et al., 2002, 2005, studied *H. decumbens;* Fitamo and Leta, 2010, studied *H. odoratissiumum*; Hesami et al., 2018, studied *Helichrysum* Spp.). However, only the recent research paper by Brunetti et al. (2018) considered as many PHEs as this study, even if Brunetti's work was conducted as a pot experiment, while this work is an in situ experiment. The other papers alternatively investigated Pb, Zn, Cd and sometimes Cu. For these reasons there is still a lack of knowledge upon PHEs uptake and translocation in *Helicrysum* spp. in different environments, as well as their interaction mechanisms.

A previous study, conducted with the same criteria, was performed by Buscaroli et al. (2017) on *Dittrichia viscosa*, another rustic plant growing on the Montevecchio mine tailings. The ability of phytostabilization and translocation shown by *D. viscosa* in this environment, justifies also the interest for *H. italicum*.

The aim of this study is to evaluate *H. italicum* for phytoremediation applications in metal-contaminated sites. Major and trace elements total concentrations in soil samples were measured, while Cu, Cd, Fe, Ni, Pb and Zn were also quantified as bioavailable soil fractions to be compared with total amounts extracted from plants. Detailed objectives of this study are: i) to assess elements accumulation potential and interaction mechanisms in different parts of *H. italicum*; ii) to study elements uptake capability in the roots and the translocation to aerials plant parts; iii) to evaluate differences in plant behaviour in contaminated and uncontaminated sites; iv) to compare elements concentration and phytoremediation properties with the existing literature for *H. italicum* subspp.

2. Materials and methods

2.1. Site description and sample collection

Mining activities related to Pb and Zn extraction have been representing the main economic activity for centuries in the South-West Sardinian mining districts (Italy), largely impacting the environment (particularly soil and water) and landscape (Boni et al., 1999; Dore et al., 2020). In this area the mining activities ended in 1991 leaving many abandoned heaps of waste materials now exposed to gravity movements and water and wind weathering. It is estimated that about 297 hectares are occupied by landfills and about 4.9 million m^3 is the volume of abandoned heaps. The cost for reclaiming activities is estimated at more than 485 million Euros (Italian Government, 2001). The mining area has been included in the Italian list of polluted sites since 2001 but, until today, no remediation activities have occurred.

In this work two broad areas were selected for the sample collection: one including the contaminated sites in the Montevecchio area (CS) and the other including the uncontaminated reference sites either close to the mining area or in the Emilia-Romagna area (US) (Fig. 1).

The CS were entirely located in the Montevecchio mining district, in the Southwestern Sardinia, close to Montevecchio and Ingurtosu villages (Fig. 1A). The bedrock consists of low-grade meta-sedimentary and meta-volcanic Cambrian-Ordovician rocks, with intrusions of Arburese igneous complex occurred at the end of the Hercynian orogeny (Cuccurru et al., 2016; Moroni et al., 2019 and references within). This complex is constituted by granodiorite and leucogranite with radial fractures filled with acid and basic magmatic dykes, and with quartz and metalliferous hydrothermal deposits exploited by the Montevecchio-Ingurtosu mines (Moroni et al., 2019). The ore-veins are composed of galena, sphalerite and quartz with local intrusions of carbonates (Moroni et al., 2019). During the mines' activity (1848 - 1991), approximately 3 Mt of Pb and Zn were extracted from the Montevecchio district. Nowadays several uncontrolled waste rock piles generate relevant sources of contamination (Caboi et al., 1993; Concas et al., 2006) due to the scarce vegetation cover and intense erosion.

The US include 2 sampling sites near but outside the Montevecchio mining district and 4 in the Appennine chain between eastern Emilia-Romagna and Tuscany (Fig. 1B). These sites are developed on different types of sedimentary materials. The two US near Montevecchio area were sampled on aeolian sandy deposits. In the Appennine, the bedrock is made up of alternations of sandstones and marls (Marnoso-Arenacea Formation) followed by a thin band of evaporitic gypsum (Gypsum Vein), formed during the Messinian salinity crisis. Close to the plain there are Pliocene clays and Pleistocene yellow sands (Lancianese and Dinelli, 2015). Among the four sampling sites, two were chosen on evaporitic gypsum (1 and 2 in Fig. 1B) and the other two on the Marnoso-Arenacea Formation (3 and 4 in Fig. 1B).

In these two broad areas, 28 sample sites were selected and sampled. At each site the entire *H. italicum* plants and a composite rhizospheric soil, sampled at a depth of 5-30 cm, were collected, stored in plastic bags and brought to the laboratory for analysis. Overall, 22 soil samples and 22 plants were collected in CS corresponding to the major mine tailings deposits (Fig. 1A), while 6 soil samples and 6 plants were collected in US (Fig. 1A and 1B).

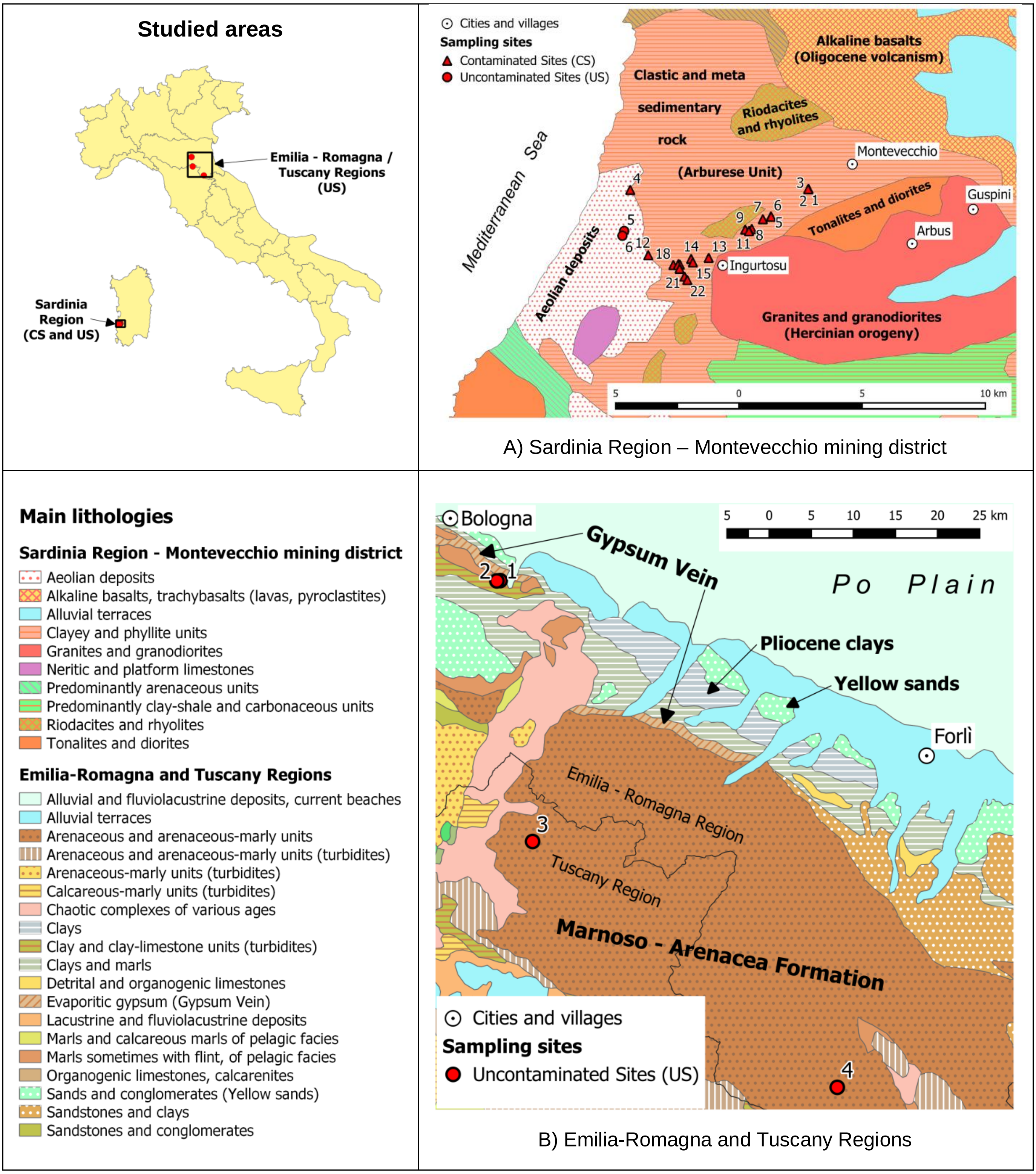

Figure 1. Location of sampling sites in the two study areas (a and b) with their main lithologies and related legend. Modified from ISPRA (http://sgi2.isprambiente.it/arcgis/rest/services/servizi/cartageologica500k/MapServer).

2.2. Chemical analysis of solid material

Soil material was air-dried at room temperature for two weeks, crushed and sieved through a 2 mm screen, and this fraction was retained for analysis. Soil sand, silt and clay determination was performed according to the hydrometer method devised by Day (1965). Soil reaction (pH) and electrical conductivity (EC) were determined in a 1:2.5 (m/V) soil-water suspension. The total limestone was determined by volumetric method according to ISO 10693 method (ISO, 1995a). Total Organic Carbon (TOC) and Total Nitrogen (TN) were determined by elemental analyser Thermo Fisher EA Flash 2000 CHNS-O according to ISO 10694 method (ISO,1995b). In order to estimate the organic and carbonate content in soil, the Loss of weight On Ignition (LOI) was determined by placing a soil sample in a muffle furnace at 950 °C for 6 h and measuring the percentage weight loss, as indicated by Heiri et al. (2001).

In this paper the soil PHEs concentrations were analysed using three different methodologies: i) extraction with a diethylenetriaminepentaacetic acid-based solution (hereafter DTPA) (element available for root uptake); ii) aqua regia extraction (hereafter AR) (pseudo-total element concentration); iii) X-Ray Fluorescence (hereafter XRF) (total element concentration) (Wang et al., 2021).

The bioavailable elements in soil (Cd, Cu, Fe, Ni, Pb and Zn) were extracted with a DTPA-based solution according to Lindsay and Norvell (1978) and ISO 14870 methods (ISO, 2001). Concentrations of Fe, Zn, Pb for all sites and Cd only in CS were measured by Flame Atomic Absorption Spectrometry (FAAS) using a Perkin-Elmer Analyst 100. Instead, concentration of Cu, Ni for all sites and Cd only in US were measured by Graphite Atomic Absorption Spectrometry (GFAAS) using a Perkin-Elmer HGS-800. The analyses were performed at Biological, Geological, and Environmental Sciences Department (BiGeA Dept.) of Bologna University using calibration standards from 0.2 to 5 mg/l and from 2 to 100 µg/l for FAAS and GFAAS, respectively. As reported by Kumpiene et al. (2017), this methodology is widely applied on non-acid soils and fitted perfectly to the US but it could be not appropriated for a few soil samples with low pH in CS. However, in order to obtain comparable results, DTPA extraction was adopted for all soil and tailing samples.

Aqua regia extraction was executed following the ISO 11047 method (ISO, 1998) and As, Cd, Co, Cr, Cu, Fe, Ni, Pb, V and Zn were quantified by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) Perkin-Elmer ELAN DRC-eat at BiGeA Dept. of the Bologna University with calibration standards from 0.01 to 5 mg/l.

The total concentrations of major and trace elements in soils were determined by X-Ray Fluorescence (XRF) using a Panalytical Axios 4000 spectrometer, following the analytical methodology by Franzini et al. (1972) and Leoni and Saitta (1976) for matrix correction methods.

To ensure accuracy and precision in soil PHEs analysis, reagent blanks and certified reference materials were used. Quality control of DTPA-extractable PHEs was performed analysing the NCSDC85102a certified reference material. The obtained recoveries (mean value ± standard deviation in %) are the following: Cd 102 ± 2, Cu 108 ± 4, Fe 90 ± 1, Ni 91 ± 2, Pb 80 ± 3, Zn 70 ± 1.

2.3. Chemical analysis of plants

After the separation into roots, stems and leaves, the plant samples were placed in an ultrasonic bath to remove soil particles, washed with deionized water, dried in a stove at 40 °C and minced.

For the determination of the total element concentrations, 250 mg of each plant part was digested with a mixture of 2 ml H_2O_2 30% and 6 ml HNO_3 65% using a microwave Milestone mls 1200 Mega. After the filtration the digested solutions were stored in 50 ml volumetric flasks. In the plant's digested solutions Fe, Zn, Pb for all sites and Cd only in CS were analysed by FAAS (Perkin-Elmer Analyst 100) while Cu, Ni for all sites and Cd only in US were analysed by GFAAS (Perkin-Elmer HGS-800). The analyses were performed at BiGeA Dept. of the Bologna University using calibration standards from 0.2 to 5 mg/l and from 2 to 100 µg/l for FAAS and GFAAS, respectively.

As for soil, also for plant reagent blanks and certified reference materials were used.

Quality control on total PHEs concentrations in plants was performed analysing the IAEA – 359 (Cabbage - Trace elements) certified reference material in three replicas. The obtained recoveries (mean value ± standard deviation in %) are the following: Cd 127 ± 5, Cu 126 ± 8, Ni 96 ± 14, Zn 105 ± 7.

2.4 Data quality control and statistical analysis

On the results, several statistical elaborations were performed using R Software version 3.3.2: the Shapiro Wilk test (Shapiro and Wilk, 1965) was adopted to verify the normal distribution of the data; a Mann-Whitney non-parametric test was performed both between the different sampling sites and the different plant tissues for the evaluation of the statistical significance of the difference between the medians. The Spearman Rank Correlation Coefficient (SRCCs) (Spearman, 1904) was applied in the determination of correlations between elements in soils (both total and bioavailable) and in plants from CS using the R software version 3.3.2. The obtained significance correlations were compared with < 0.05 and < 0.01 p-value.

3 Results and discussion

3.1 Soils

The main compositional and textural features for the analysed soils are summarized in Table 1 (the entire dataset is available in Table S1 and S2 of the Supplementary Materials). The CS revealed a sandy loam texture with little silt and clay, whereas in the US, silt and sand were the dominant soil fractions, although a wide variation in texture was present ranging from sand to silty-loam. The CS showed a neutral-sub acid pH (5.4 - 6.7 as Min-Max range), while in the US subalkaline to alkaline pH were observed (7.6 – 9.1). EC was below 0.3 dS/m in all sites, while total lime was significant in the US and negligible in almost all the CS. In both the CS and US, TOC range was large (2 – 60 g/kg) with median values around 25 g/kg. Median values of TN content were identical (2 g/kg, for both CS and US), while the high TOC/TN ratio values (> 12) could be affected by *H. italicum* plant residues that hardly decompose in soils (Brady and Weil, 2010).

The median concentrations of major elements such as Si (282938 mg/kg), Al (89741 mg/kg), Fe (40509 mg/kg) and K (30018 mg/kg), confirmed the abundance of sheet silicates in CS (Table 1). Compared with previous results by Buscaroli et al. (2017), the dominant silicates feature was confirmed with weak increase in Fe and LOI median values and weak decrease in Ti, Al, Mg, Na and K median values. Instead, median concentrations of Ca and LOI (129691 and 211908 mg/kg, respectively) had the highest values in US reflecting the calcareous contribution of the substrate in the area as recorded in stream sediment by Lancianese and Dinelli (2015).

In terms of total concentration (XRF) the CS were characterized by high Pb and Zn contents (median 604 mg/kg and 11455 mg/kg, respectively). Compared with soils by Buscaroli et al. (2017) from the same area, soils from this study had slightly higher Pb and Cu median values, whereas Zn median concentration was double. The Ni total content (39 mg/kg) agrees with values found by Buscaroli et al. (2017). Compared with soil samples from the adjacent Ingurtosu mine area (Barbafieri et al., 2011), this study denoted around double Zn and half Pb content (7800 and 1800 mg/kg, respectively). Mean total PHEs concentrations found in this study area were comparable with concentrations of the nearby Barraxiutta mine district (De Agostini et al., 2020) and Campo Pisanu (Bacchetta et al., 2018), except for Cu and Pb that were 5 and 4 times lower, respectively. Overall, this high variability in values denoted the strong heterogeneity of mine tailings.

The US had low median total concentration of PHEs with only Cr, Ni, V and Zn ranging around 100 mg/kg (Table 1). These results were aligned to the soils from the GEMAS project (Reimann et al., 2014), to the streambed sediments of the same area analysed by Lancianese and Dinelli (2015), and also to the natural background maps by Regional Soil Service, although related to the adjacent plain area (RER, 2016).

Results of the AR extraction showed Cd and Zn median values (67 and 9771 mg/kg, respectively) much higher than the Italian environmental legislation thresholds (Italian Government, 2006) for an industrial use in CS (15 and 1500 mg/kg, respectively). If the thresholds for public and residential destinations are considered (for As, Cd, Co, Cu, Pb and Zn are 10, 2, 20, 120, 100, and 150 mg/kg, respectively), all median values exceeded the limit and for Cd, Pb and Zn all samples were above the thresholds (bold values in Table 1). The US samples did not reveal concentrations exceeding the national environmental prescriptions, except for mean and maximum As concentration (16 mg/kg and 36 mg/kg, respectively). Being part of a dominant calcareous unit, the high As concentration in US is determined by a direct control of calcite abundance on As bio-accessibility (Raimondi et al., 2021).

Regarding bioavailable element concentrations (DTPA-extracted) (Table 1), the CS were characterized by high median concentrations of Zn followed by Cd, Pb, Fe and Cu (630, 9, 6, 4, 4 mg/kg, respectively). In particular, the median concentrations of Zn, Cd, Pb and Cu were respectively enriched by 3, 2, 1 and 1 orders of magnitude in CS compared to US; the Ni was double in CS compared to US, while Fe was comparable. Except for Pb, all the considered PHEs showed higher maximum values compared to Buscaroli et al. (2017). In comparison with this study Bacchetta et al. (2018) reported lower mean bioavailable concentrations of Cd, Pb and Zn (3.9, 13 and 117 mg/kg, respectively) in Campo Pisanu mine district. The lower bioavailable concentrations measured in US were aligned with unpolluted data by Buscaroli et al. (2017).

The SRCCs for elements in soils from CS revealed significant positive correlations only between bioavailable Cd and Ni (0.56), bioavailable Cd and Zn (0.66) and between total and bioavailable Zn (0.72). Negative significant correlation existed between bioavailable Fe and total Zn and bioavailable Zn (-0.69 and -0.68, respectively). The negative correlation between bioavailable Zn and Pb was weak but significant (-0.52) (Table S3 of the Supplementary Materials).

Table 1. Minimum, median, maximum and mean values for soils main characteristics in sampling areas. Contaminated sites (CS) (n = 22) and Uncontaminated sites (US) (n = 6). Bold values in the aqua regia results exceed the Italian environmental legislation (Italian Government, 2006) for soils in public and residential areas.

Areas		CS				US			
Statistics		**Min**	**Median**	**Max**	**Mean**	**Min**	**Median**	**Max**	**Mean**
Sand 2000-50 µm	g/kg	241	695	874	649	335	413	954	516
Silt 50-2 µm	g/kg	87	228	677	265	13	447	538	390
Clay < 2 µm	g/kg	39	83	138	86	33	52	176	93
Reaction in H_2O	pH	5.4	6.2	6.7	6.1	7.6	8.0	9.1	8.2
EC	$dS \cdot m^{-1}$	0.2	0.3	0.3	0.3	0.1	0. 2	0.3	0.2
Total lime	g/kg	0	0	67	8	3	157	291	146
TOC	g/kg	2	22	34	20	2	26	60	26
TN	g/kg	0	2	3	2	0	2	5	2
TOC/TN		5	13	20	13	9	12	31	15
Major elements and LOI									
Si	mg/kg	245288	282938	353616	293225	54917	164808	408674	275377
Ti	mg/kg	1352	4040	5085	3638	499	1615	3559	1844
Al	mg/kg	33902	89741	115742	80668	22022	41253	69146	43051
Fe	mg/kg	22962	40509	78330	43247	5875	16208	39645	20045
Mn	mg/kg	845	1396	3809	1641	152	552	697	462
Mg	mg/kg	3973	7593	11630	7692	2749	7112	26934	11770
Ca	mg/kg	1102	4483	48660	7089	18775	129691	407810	149083
Na	mg/kg	1077	5142	12834	5481	824	4554	8531	4576
K	mg/kg	12274	30018	43922	28494	5767	13933	23878	14454
P	mg/kg	271	558	1006	554	128	572	700	469
LOI	mg/kg	30032	55600	122774	67108	19900	211908	234504	152533
Total elements (XRF)									
As	mg/kg	19	94	191	103	2	6	50	13
Co	mg/kg	11	16	43	19	1	3	13	5
Cu	mg/kg	1	102	706	170	2	10	26	12
Cr	mg/kg	26	57	99	55	22	65	144	80
Ni	mg/kg	11	39	73	40	5	40	105	49
Pb	mg/kg	138	604	4619	1240	10	18	22	17
V	mg/kg	39	64	114	66	10	60	161	79
Zn	mg/kg	5020	11455	41200	14873	8	29	93	39
Extractable elements (AR)									
As	mg/kg	17	**80**	**333**	**98**	10	11	**36**	**16**
Cd	mg/kg	**5**	**67**	**228**	**67**	0	0	1	0
Co	mg/kg	16	**23**	**33**	**23**	1	3	19	6
Cr	mg/kg	6	28	49	31	3	30	127	49
Cu	mg/kg	12	**130**	**485**	**147**	1	8	40	13
Ni	mg/kg	7	34	62	36	2	20	91	36
Pb	mg/kg	**170**	**819**	**3442**	**1235**	5	8	16	10
V	mg/kg	4	53	149	63	14	42	88	42
Zn	mg/kg	**1227**	**9771**	**27286**	**9377**	10	23	63	31
Bioavailable elements (DTPA)									
Cd	mg/kg	1	9	29	9	0	0	0	0
Cu	mg/kg	0	4	26	5	0	1	2	1
Fe	mg/kg	0	4	39	7	1	4	8	4
Ni	mg/kg	0	1	3	1	0	0	1	0
Pb	mg/kg	0	6	143	29	0	1	1	1
Zn	mg/kg	101	630	894	555	0	1	2	1

The Table 2 reports the DTPA/total concentration ratios expressed as a percentage and used to evaluate the elements' behaviour. The percentages were generally low in CS, especially regarding Fe, although the maximum values reached 45% and 27% for Cu and Pb, respectively. The differences between CS and US were limited and significant only for Fe in US and Ni in CS. Regardless of the soil conditions in CS and US, Cu and Pb showed highest bioavailability followed by Zn, Ni and Fe although no systematic order was observed. Previous work by Buscaroli et al. (2017) in Montevecchio district (in brackets in Table 2) reported lower median values of Cu, Pb and Zn, even if maximum values were aligned with the ones from this study. The US ratios from this study weakly differ from Buscaroli et al. (2017) confirming that North Appennine district was well characterized by the collected samples.

Table 2. Minimum, median and maximum bioavailable/total element ratio in soils, expressed as percentage (%). Contaminated sites (CS) (n = 22); Uncontaminated sites (US) (n = 6). The letters (C for Contaminated, U for Uncontaminated) indicate the presence of statistically significant differences between percentages of each element in each area according to the Mann–Whitney test. No letter means absence of statistically significant differences between areas. The considered significant levels are p-value < 0.1 (*) and < 0.05 (**). Values between brackets are by Buscaroli et al. (2017).

Areas	Statistics	Cu	Fe	Ni	Pb	Zn
CS	Min	0.67 (1.28)	0.0003 (0.003)	0.67 (0.48)	0.09 (1.2)	1.34 (3.87)
	Median	3.96 (6.66)	0.008 (0.01)	1.41 U** (1.03)	1.53 (5.51)	3.25 (7.07)
	Max	45 (9.2)	0.116 (0.05)	6.2 (1.79)	26.6 (23.3)	10.4 (10.82)
US	Min	0.41 (1.74)	0.02 (0.01)	0.13 (0.25)	1.44 (3.8)	1.53 (0.18)
	Median	5.38 (7.75)	0.03 C* (0.03)	0.64 (0.71)	5.47 (6.03)	3.14 (1.96)
	Max	19.5 (12.6)	0.04 (0.07)	1.32 (1.52)	9.45 (12.6)	8.19 (2.63)

3.2 Plants

The total PHEs concentrations in the different parts of *H. italicum* plant are shown in Table 3 grouped into CS and US, while the entire database is presented in Table S4 of the Supplementary Materials. The PHEs concentrations in plants were higher in CS than in US. In particular, the differences were statistically significant for Cd, Fe, Ni, Pb and Zn ("U" in Table 3), while there were no significant differences for Cu, although median concentrations in plants were slightly higher in CS than US.

The median concentrations in leaf were generally higher than the other plant parts, although statistical significance (p-value < 0.01) occurred only for Cu, Fe and Zn in CS (leaves marked with "r" and "s" in Table 3). In US no significant differences in element concentrations among plant parts were found. Only Cu in leaves was statistically different from Cu stem concentration. In both CS and US, the concentrations of PHEs in the stems were lower than in the other parts of the plant, although they were significantly different only for Cd (compared to root content), Cu, Fe, Pb and Zn in CS and for Cu in US, mainly compared to the leaves (Table 3). The ratio among median element concentrations in plant parts from CS and US revealed the following enrichment ranking: Pb > Zn > Cd > Fe > Ni > Cu (Table 3).

The median concentrations of Pb in plants were 40 mg/kg in roots, 27 mg/kg in stems and 64 mg/kg in leaves with the CS/US ratios ranging from 33 to 64 for stems and leaves, respectively (Table 3). The median values of Zn in plants were 576 mg/kg in roots, 391 mg/kg in stems and 1206 mg/kg in leaves, this last proven statistically different from the others (Table 3). *Helicrysum Italicum* plants showed from 13 to 26 times more Zn in CS than in US. The median concentrations of Cd in plant were quite homogeneous: 3.1 mg/kg in stems, 6.0 mg/kg in leaves and 5.7 mg/kg in roots, these last two significantly higher than in stems. These concentrations of Cd were from 10 to 15 times more enriched in CS than in US. Median concentration of Fe in leaves (1523 mg/kg) was significantly different from roots (701 mg/kg) and stems (62 mg/kg) and *H. Italicum* plants were from 3 to 5 times more Fe-enriched in CS than in US. The median concentrations of Ni were similar in plants: 4.4 mg/kg in roots, 3.5 mg/kg in stems, 4.6 mg/kg in leaves. Plants from CS had only 2 – 3 times more Ni than the ones from US. The median concentrations of Cu in CS plants were 13 mg/kg in roots, 10 mg/kg in stems and 20 mg/kg in leaves, this last significantly different from roots and stems. No differences of Cu content existed between CS and US (median ratio was 1 in Table 3).

Few authors studied element distribution in *H. italicum* plant parts including stems. In an adjacent mine area, Barbafieri et al. (2011) showed similar Cd concentrations and distribution in the same plant parts with stems as the lowest accumulation site. Instead, the same authors reported increasing concentrations in Pb and Zn from roots to leaves, but compared to this study mean concentrations were one order of magnitude higher for Pb and slightly lower for Zn. Also Brunetti et al. (2018) evaluated Cd, Cu, Ni, Pb and Zn abundance in roots, stems and leaves of *H. italicum* grown in a polluted soil in Apulia Region (Italy). Their abundances of Cu and Ni in plants were aligned with the concentrations of this study, while Cd, Pb and Zn were one order of magnitude lower. Brunetti et al. (2018) concluded that *H. italicum* stores PHEs in the roots with stems as the least concentrated part. Although not clearly evident in this study, the trend to accumulate Pb in roots rather than in other aerial parts is widely demonstrated in *H. italicum* (Barbafieri et al., 2011; Brunetti et al., 2018) and also other plants such as *Oryza sativa* (Ashraf et al., 2020) and *Crambe abyssinica* (Gonçalves et al., 2020), confirming the poor Pb translocation.

In a pyrite-mine site in Tuscany (Italy), Bini et al. (2017) showed preferential accumulation of PHEs in *H. italicum* roots. Compared to this study, Fe and Pb concentrations in plants were aligned, Cd and Ni were 10 times lower, Zn was three orders of magnitude lower and only Cu resulted 10 times higher. For *H. italicum* subspp. *tyrrhenicum* (*H. tyrrhenicum*), in the adjacent mine area of Campo Pisanu, Bacchetta et al. (2018) detected higher concentrations in roots and leaves for all considered elements (Cd, Pb and Zn) and, especially for Pb, the differences with this study were quite important.

Pot trials at different soil contamination of Cu and Pb executed on *Helichrysum splendidum* Less revealed a reduction of chlorophyll content (phytotoxicity sign) only when the Cu leaves concentration was about 290 mg/kg, while for the Pb a constant chlorophyll reduction was evident starting from 90 mg/kg in leaves (Banda et al., 2021). *Helichrysum italicum* never reached high concentrations of Cu in this work, while maximum concentrations of Pb exceeded 90 mg/kg in CS plants. Since it was not within the aims of the work, no surveys regarding the health status of the plants were conducted, therefore the presence of *H. italicum* plants with phytotoxic symptoms in CS could not be excluded.

Table 3. Minimum, median, maximum and mean element concentration in plant parts for Contaminated Sites (CS, n = 22) and Uncontaminated Sites (US, n = 6). Concentrations are in mg/kg. The letters R, S and L represent roots, stems and leaves, respectively. According to the Mann–Whitney test, the Statistical Significative Difference (SSD) between the analysed plant tissues within each area was indicated by the letters r and s. According to the Mann–Whitney test, the letter U indicates statistically significant difference of the plant tissue from CS with US. No letter means absence of statistically significant differences. The considered significant levels are p-value < 0.1 (*), < 0.05 (**) and < 0.01 (***).

		CS					***US***					***Median CS/US ratio***
Elements	**Plant parts**	**Min**	**Median**	**Max**	**Mean**	**SSD**	**Min**	**Median**	**Max**	**Mean**	**SSD**	
Cd	**L**	0.5	6.0	90.0	12.4	**U****	0.1	0.4	1.3	0.5		15
	S	0.6	3.1	22.3	5.8	**U****	0.1	0.3	1.1	0.4		10
	R	1.4	5.7	50.9	11.1	**s**U****	0.1	0.5	1.7	0.6		11
Cu	**L**	7.8	20.2	98.4	26.0	**r*s*****	9.4	13.9	29.8	16.8	**s***	1
	S	3.2	10.2	32.3	12.6		6.3	9.3	11.9	9.1		1
	R	6.5	12.9	103.2	22.4		6.3	9.8	19.0	10.9		1
Fe	**L**	359	1523	5991	1994	**r**s***U****	174	290	677	343		5
	S	267	662	1905	748	**U****	112	206	427	228		3
	R	142	701	7140	1245	**U****	150	252	581	319		3
Ni	**L**	1.3	4.6	11.3	4.8	**U****	0.6	1.7	4.1	1.9		3
	S	2.0	3.5	5.6	3.8	**U****	0.4	1.3	2.1	1.2		3
	R	0.6	4.4	11.0	4.7	**U***	0.8	1.8	2.4	1.7		2
Pb	**L**	5.9	63.7	288.4	74.5	**s**U****	0.6	1.0	13.0	3.6		64
	S	7.8	26.5	181.4	37.4	**U****	0.5	0.8	5.9	1.8		33
	R	5.2	39.5	384.4	76.5	**U****	0.4	0.9	4.4	1.6		44
Zn	**L**	177	1206	9837	1764	**r*s**U****	28	47	112	61		26
	S	132	391	1959	630	**U****	19	30	51	32		13
	R	220	576	3337	936	**U****	26	28	46	32		21

3.2.1 Interaction mechanisms of PHEs in *H. italicum* plants

The interaction mechanisms between PHEs could reveal synergistic or antagonistic effects able to improve or reduce element uptake and translocation in plant species. Only for plants collected in CS (n = 22), the SRCCs and their significance levels were calculated for each element (Cd, Cu, Fe, Ni, Pb and Zn) and for different plant parts and results are presented in Table 4.

Results of this study indicated that Zn in *H. italicum* was positively and significantly correlated with Cd in all considered plant parts (around 0.90 for R, S and L). Moreover, Zn and Cd were also themselves correlated with values of 0.92 and 0.9 between leaves and stems. The Zn and Cd, together with Pb were the most enriched elements in *H. italicum* compared to US (Table 3). Nevertheless, Zn and Cd were also positively and significantly correlated with all the other elements in leaves (from 0.55 to 0.7), except for Pb that showed correlations only with Cd in leaves (0.67). The relation between Zn and Cd is known in literature and depends by the element similarity (Fernández et al., 2017). Indeed, Cd plant uptake is hindered by high soil Zn concentrations (Choudhary et al., 1995; Oliver et al., 1994) because they share the same transportation protein in plants and Zn is selectively preferred (Hart et al., 2002). Kutrowska et al. (2017) documented the synergistic effect between Cd and Zn in *Brassica juncea* where Zn increased the accumulation of Cd in leaves. The same authors reported that Pb increased the Cd in stems, as identified also in the present study (Table 4).

In *H. italicum* grown at CS Cu was positively correlated with itself, especially between stems and leaves (0.74), and clearly positively correlated also with Zn for almost all plant parts with a peak of 0.86 in leaves. As Fe, also Cu and Zn are micronutrients for plants and serve in physiological processes; so, their synergism was expected for US, but it was found also at elevated Zn concentration in CS (Table 3). In other species like *Brassica juncea* antagonism between Zn and Cu was demonstrated and indicated as happening not in the roots, but later during xylem loading/unloading (Kutrowska et al., 2017). A previous study (An et al., 2004) revealed that Cu and Cd act antagonistically resulting in decreased accumulation of both metals in *Cucumis sativus*. In the present study Cu and Cd were weakly correlated with coefficients around 0.6 between Cu in leaves and Cd in roots, stems and leaves (Table 4).

The Fe was significantly correlated between stems and leaves (0.8), in leaves with Pb (0.71) and in leaves with Cd (0.73 and 0.72 for stems and leaves, respectively). Moreover, Fe and Ni resulted significantly correlated in all the *H. italicum* plant parts, with coefficients ranging around 0.70 and peaks for roots (0.74) and leaves (0.71) (Table 4). Khalid and Tinsley (1980), in *Lolium perenne,* reported a common increase of Ni and Fe concentrations in shoots with increasing rates of Ni. Same synergistic effect of Ni on Fe was detected in maize with highest evidence in roots and leaves by Torres et al. (2016).

Absence of correlation existed among Pb and Cu as well as Pb and Zn in the plant parts of *H. italicum* grown at CS. An antagonistic effect of Pb on the Cu accumulation was documented also in *Brassica juncea* and related to a competition between metals at the plant uptake site (Kutrowska et al., 2017). Also for Israr et al. (2011), Pb showed antagonistic effect on the accumulation of Cu, Ni and Zn in *S. drummondii* species, probably due to the competition between metals at the plant uptake sites. Yet, Wong et al. (1986) reported a reduced uptake of Cu in the presence of Pb for *Brassica chinensis*. The inhibition of essential nutrient transfer (such as Cu and Zn) in plant biomass due to Pb elevated concentration has been also proposed by Yoon et al. (2006) for numerous plants grown in Florida contaminated site. In addition to all the side effects of Pb in plants, Pourrut et al. (2011) reported impaired uptake of essential elements, such as Mg and Fe. On the contrary, An et al. (2004) showed positive correlation of Zn and Pb in *Cucumis sativus* suggesting a synergistic effect.

Table 4. Main Spearman Rank Correlation Coefficients (SRCCs) and their significance levels, calculated for each element (Cd, Cu, Fe, Ni Pb and Zn) between the different plant parts (R=roots, S=stems and L=leaves) and, for each of them, between the different elements. The SRCCs were calculated considering only the Contaminated Sites (CS) (n=22). The considered significant levels are p-value < 0.05 (**) and < 0.01 (***).

		R	S	L	R	S	L	R	S	L	R	S	L	R	S	L	R	S	L
		Cd			Cu			Fe			Ni			Pb			Zn		
Cd	R	1																	
Cd	S	**0.76** ***	1																
Cd	L	**0.81** ***	**0.92** ***	1															
Cu	R	**0.55** **	0.42	**0.44** **	1														
Cu	S	0.22	0.30	0.31	**0.60** ***	1													
Cu	L	**0.66** ***	**0.56** ***	**0.66** ***	**0.69** ***	**0.74** ***	1												
Fe	R	0.37	0.19	0.20	**0.57** ***	-0.01	0.24	1											
Fe	S	0.36	**0.64** ***	**0.53** **	**0.47** **	0.30	0.31	**0.49** **	1										
Fe	L	**0.55** **	**0.73** ***	**0.72** ***	**0.47** **	0.30	**0.49** **	0.33	**0.80** ***	1									
Ni	R	0.27	0.08	0.17	**0.55** **	-0.01	0.24	**0.74** ***	0.32	0.37	1								
Ni	S	0.39	**0.46** **	**0.51** **	**0.65** ***	0.20	0.37	**0.55** **	**0.69** ***	**0.68** ***	**0.62** ***	1							
Ni	L	**0.64** ***	**0.66** ***	**0.75** ***	0.48	0.01	0.41	0.31	**0.52** **	**0.71** ***	**0.54** **	**0.73** ***	1						
Pb	R	0.12	-0.10	-0.07	0.39	-0.01	-0.03	**0.67** ***	0.17	0.08	**0.56** ***	0.36	0.09	1					
Pb	S	0.40	0.40	**0.48** **	0.15	0.09	0.23	0.42	**0.47** **	**0.46** **	0.42	0.35	0.41	0.53	1				
Pb	L	**0.46** **	**0.51** **	**0.67** ***	0.20	0.08	0.29	0.33	**0.53** **	**0.71** ***	0.41	**0.49** **	**0.60** ***	0.36	**0.86** ***	1			
Zn	R	**0.90** ***	**0.68** ***	**0.70** ***	**0.77** ***	0.33	**0.70** ***	**0.50** **	0.39	**0.56** ***	**0.46** **	**0.52** **	**0.64** ***	0.26	0.34	0.37	1		
Zn	S	**0.78** ***	**0.93** ***	**0.87** ***	**0.54** **	**0.45** **	**0.73** ***	0.24	**0.61** ***	**0.66** ***	0.12	0.43	**0.60** ***	-0.15	0.35	0.40	**0.74** ***	1	
Zn	L	**0.87** ***	**0.82** ***	**0.89** ***	**0.60** ***	**0.45** **	**0.86** ***	0.27	0.38	**0.59** ***	0.19	0.42	**0.60** ***	-0.09	0.28	0.42	**0.82** ***	**0.90** ***	1

3.3 Soil-plant relationship

The relationship between the soil bioavailable pool and the concentrations in plant parts of the investigated elements are represented by box plots in Figure 2. In terms of soil bioavailable pool, Fe and Ni showed comparable concentrations in CS and US even if Ni in US had a wider range of variation. At these low concentrations the *H. italicum* was able to absorb and concentrate the elements in the plant (2 and 1 order of magnitude for Fe and Ni, respectively). Although the soil bioavailable fractions were similar the plant concentrations between CS and US were statistically different (Table 3) with lower levels in the latter.

The Cu and Pb were enriched in CS compared to US, but due to the wider range of variations (Cu in US and Pb in CS) a clear separation was not evident. Regarding these elements the plant was able to absorb and concentrate them compared to the soil bioavailable pool (1 order of magnitude for Pb). The Cu concentrations in the plant were not statistically different between CS and US (Table 3) indicating that CS could not be considered polluted by Cu. In fact, Buscaroli et al. (2017) in Cu-contaminated Libiola mine and Brunetti et al. (2018) in Apulia soil reported bioavailable Cu concentration greater than this study by 1 and 2 orders of magnitude, respectively. The Pb in CS was absorbed and concentrated by *H. italicum*, while in US it was absorbed but not concentrated compared to soil bioavailable fractions (Fig. 2).

Soil bioavailable concentration of Cd and Zn were more abundant in CS than US of 2 and 3 times, respectively. In CS bioavailable Cd and Zn were elevated (more than Bacchetta et al. (2018) and Brunetti et al. (2018)) and *H. italicum* plant absorbed them, but weakly concentrated Cd and Zn in the plant tissues. Instead, in US *H. italicum* was able to absorb and concentrate Cd and Zn (Fig. 2), even if the plant concentrations remained significantly lower than CS (Table 3). Despite this behaviour within plants, *H. italicum* growing in CS accumulated two orders of magnitude more Pb and one order of magnitude more Zn and Cd compared to US (Fig. 2).

Bacchetta et al. (2018) for *H. tyrrhenicum* in the adjacent mine area of Campo Pisanu, detected higher concentrations in plant parts for all considered elements (Cd, Pb, and Zn). Yet, bioavailable pools for Cd and Zn were a quarter the levels of the present study, while the bioavailable pool of Pb was three times higher. Brunetti et al. (2018), starting from bioavailable pools like Bacchetta et al. (2018), presented notably lower plant concentrations. This behaviour could be related to the carbonate-soils studied by Brunetti et al. (2018) that contain high exchangeable Ca. This latter could compete with heavy metals limiting their uptake. The antagonistic effect of Ca on Cd, Cu, Fe, Ni, Pb and Zn uptake was found by Kabata-Pendias (2010) and observed also for *D. viscosa* by Buscaroli et al. (2017).

Possible interaction mechanisms between elements in soil and plant from CS were investigated through SRCCs and results are presented in Table S3 of the Supplementary Materials. In general, the correlations between soil and plant concentrations s were scarce. The only positive significant correlation existed between total Pb in soil and Pb in roots (0.75). There were weak significant negative correlations between total Ni and Cu in stems (-0.56) and leaves (-0.59), between bioavailable Pb and Cd in stems (-0.53) and leaves (-0.52), and Zn in stems (-0.54) (Table S3).

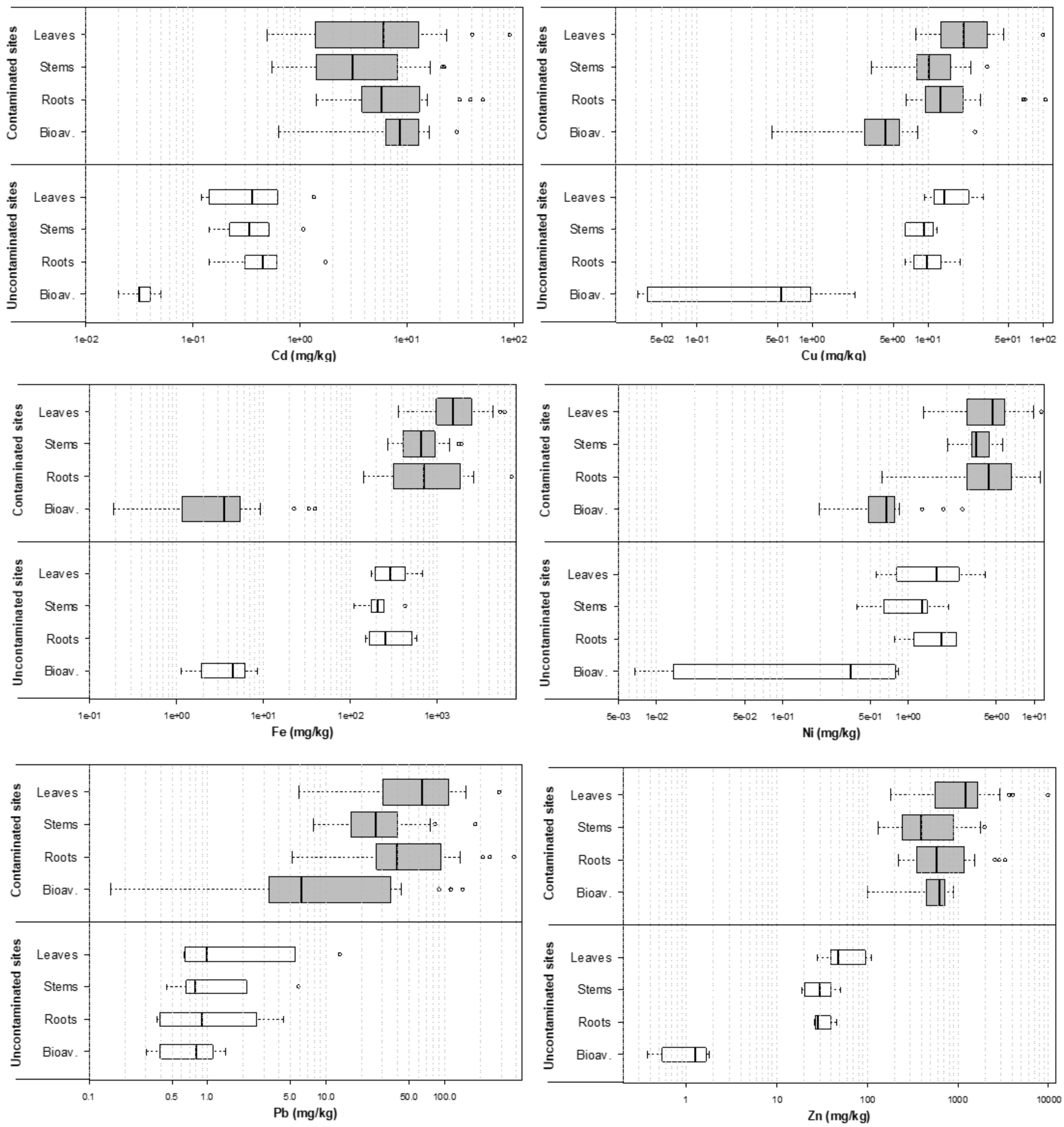

Figure 2. Bioavailable Cd, Cu, Fe, Ni, Pb and Zn in soils and their distribution in *H. italicum* plants of CS and US.

3.4 Root/bioavailable soil element concentration ratio

With the aim of studying the tolerance to PHEs of *H. italicum* and provide evidence of phytostabilization or phytoextraction capacity, the root/bioavailable soil concentration ratio (root/soil) was calculated for each element and the results are shown in Table 5. The mean values were presented only for literature comparison.

For all the elements in CS and US the medians for root/soil ratios were > 1 suggesting that *H. italicum* was able to absorb the bioavailable elements in roots. According to root/soil median values, in the CS the elements were absorbed following the ranking Fe >> Ni > Pb > Cu, whereas the ratios for Zn and Cd were close to 1. In the US the ranking resulted Fe > Zn > Cu > Cd > Ni and Pb only slightly above 1 (Table 5).

Out of the three most abundant bioavailable elements in CS (Zn, Pb and Cd, Table 1), Cd (1.21) and Zn (1.39) had the lowest ratios indicating a limited root uptake by *H. italicum* when soil available pool is abundant. Pb was more absorbed in CS (4.17) than in US (1.21). Same behaviour for Zn, Pb and Cd was detected in *D. viscosa* for the same mine area (Buscaroli et al., 2017).

The different ratio of Cu between CS and US depended from its wide variability in US, while Ni showed similar soil/root ratios in both conditions. The Fe is a fundamental micronutrient for plants strongly related to chlorophyll content and plant growth (Terry, 1980). Despite its low availability in soil (Tables 1 and 2), *H. italicum* showed an elevated root uptake capacity for Fe, especially in CS (Fig. 2).

Many other authors calculated the root/soil ratios of *H. italicum* (Barbafieri et al., 2011; Bini et al., 2017; Cao et al., 2004; Leita et al., 1989) but only Bacchetta et al. (2018) and Brunetti et al. (2018) applied the DTPA extraction, allowing a direct comparison with ratios from this study. In particular, Bacchetta et al. (2018) found ratios of 21, 29 and 9.6 for Zn, Pb and Cd, respectively, for the *H. tyrrhenicum* in a Sardinian mine site. These ratios were higher than this study, but with lower bioavailable concentrations and significantly higher root concentrations. Recalculated ratios by Brunetti et al. (2018) showed for Cu, Ni, Pb and Zn slightly lower values, under reduced bioavailable soil pool compared to CS of this study. Instead, Cd showed root/soil ratio one order of magnitude higher and bioavailable concentrations one order of magnitude lower compared to this study in CS, but comparable with values in US (Tables 1 and 5). This confirms that Cd is strongly incorporated in roots at low soil bioavailable concentrations (Brunetti et al., 2018), while its absorption is limited when soil concentrations increase (Fig. 2 and Bacchetta et al., 2018). This represents an excluding mechanism for Cd already documented in other plant species such as *Thlaspi arvense* (Martin et al., 2012) and *Arabidopsis thaliana* (Zhu et al., 2012), but not yet in *Helicrysum* spp.

Table 5. Minimum, median, maximum and mean root/soil values of selected elements in *H. italicum*. CS n=22 and US n=6.

Area	Statistics	Cd	Cu	Fe	Ni	Pb	Zn
CS	Min.	0.14	1.45	3.60	0.81	0.36	0.35
	Median	1.21	3.03	299	6.56	4.17	1.39
	Max.	7.95	28.9	6541	29.0	52.0	5.92
	Mean	1.76	5.47	988	8.84	11.57	1.92
US	Min.	4.43	3.22	23.0	1.33	0.40	15.7
	Median	12.1	19.1	91.2	4.67	1.21	26.3
	Max.	43.1	417	135	272	8.56	74.4
	Mean	18.47	107.73	89.06	70.56	2.54	38.99
	US/CS Median ratio	10	6.3	0.3	0.71	0.29	18.9

3.5 Leaf/root element concentration ratio

The capacity of *H. italicum* to transfer PHEs from roots to leaves has been quantified for each element through the leaf/root concentration ratio (leaf/root ratio) and the results are shown in Table 6. The leaf/root ratio was extensively applied for the evaluation of the phytoextraction capacity of plants growing in mine soils (Buscaroli et al., 2017; Martínez-Sánchez et al., 2012; Wang et al., 2019; Yoon et al., 2006) or in contaminated agricultural soils (Dinu et al., 2020; Nadimi-Goki et al., 2014). As stated for root/soil ratio, also leaf/root ratio is widely used by researchers, but under different names such as Translocation factor, Transfer factor or Transportation index (Buscaroli et al., 2017).

The *H. italicum* in CS always showed leaf/root median ratios > 1, except for Cd and Ni that were 0.75 and 1, respectively (Table 6). Iron reached a value of 2.36 followed by Zn (1.54) then Pb (1.37) and Cu (1.34). The leaf/root median values in US were comparable with CS except for Fe that was half. The ratios of Zn, Ni, and Cd were almost identical, while slightly higher values were measured for Pb and Cu in US compared to CS (Table 6). However, the maximum ratios were from 2 to 10 times higher in CS than US (Table 6) for all considered elements, indicating an increased inclination of the plants to transfer elements in leaves when growing on contaminated soils.

As all the *H.* spp, also *H. italicum* is a terpene-reach species, rarely appreciated as food by wild or domestic herbivorous, reducing the possibilities of PHEs entering the food chain (Rogosic et al., 2006). Moreover, metal accumulation in aerial parts is an evolutionary adaptation that confers to plants also protection against herbivores or pathogens (Galeas et al., 2008).

In presence of a tolerant plant (root/soil ratio > 1), the higher the leaf/root ratio (e.g., > 2), the greater the capacity to transfer elements to aerial plant parts and the more adapt is the species for phytoextraction strategy (Buscaroli et al., 2017; Yoon et al., 2006). The opposite indicates the suitability of the species for phytostabilization (Rizzi et al., 2004; Yoon et al., 2006). Results from this study indicated *H. italicum* as a tolerant species weakly able to concentrate Fe, Zn, Pb and Cu in the aerial parts and unable to transfer Cd and Ni to leaves. The leaf/root ratio recalculated by Barbafieri et al. (2011) in *H. italicum* plants revealed almost identical values for Zn and Cd and double values for Pb compared to the present study. Identical values for the ratio were reported also by Bacchetta et al. (2018) in Campo Pisano mine site, but for *H. tyrrhenicum* and for the entire epigeal organs. Half values of the ratios for Cd, Cu, Pb and Zn and about one tenth for Fe were obtained by Bini et al. (2017) in a Tuscany mine district, while Brunetti et al. (2018) reported lower ratios for all elements (Cu, Ni, Pb and Zn). In the recent pot trials by Banda et al. (2021) leaves/root ratios of Cu and Pb were weakly above 1 in *Helichrysum splendidum* Less.

Only for comparison purposes, Boechat et al. (2016) reported leaf/root ratios for several Brazilian species well above 2. In particular, *Baccharis trimera* (Less) DC (5.48), *Cyperus eragrostis* Lam (3.54), *Eryngium horridum* Malme (2.91) and *Dicranopteris nervosa* (Kaulf.) (2.61) for Pb and *Senecio brasiliensis* (Spreng.) Less (2.93) for Cd.

Table 6. Minimum, median, maximum, and mean leaf/root total element concentration values of selected elements in *H. italicum*. CS n = 22 and US n = 6.

Area	Statistics	Cd	Cu	Fe	Ni	Pb	Zn
CS	Min.	0.22	0.41	0.17	0.39	0.12	0.80
	Median	0.75	1.34	2.36	1.02	1.37	1.54
	Max.	6.91	4.96	19.3	6.59	14.3	13.1
	Mean	1.20	1.61	3.39	1.46	2.46	2.27
US	Min.	0.40	0.73	0.38	0.30	0.71	1.04
	Median	0.74	1.78	1.08	1.04	1.87	1.51
	Max.	1.35	2.98	2.88	2.11	3.00	3.67
	Mean	0.82	1.70	1.33	1.12	1.83	1.90

3.6 Literature comparison and final remarks

The interest for the species *H. italicum* (subspp. *italicum* or *tyrrhenicum*) as a possible phytoremediation plant, especially in abandoned mine areas, goes long back in time. From Leita et al. (1989) up to Bacchetta et al. (2018) and Brunetti et al. (2018) many authors have studied the capacity of *H. italicum*, to uptake PHEs from contaminated soils. With the aim ofsummarizing the available literature on these species and outline their phytoremediation capacities, a literature research browsing the keyword “Helichrysum italicum and remediation” in the Web of Science (last time checked 08/02/2022) was performed. Only 3 publications appeared Bini et al. (2017), Brunetti et al. (2018) and Boi et al. (2020). The work of Boi et al. (2020) was dedicated to the seed germination and for this reason excluded. This literature research demonstrated that the publications already cited in this study represent the most updated articles dealing with the application of *H. italicum* for phytoremediation purpose. All the available articles considering *H. italicum* for phytoremediation (N=6) (180 total citations and maximum number 75 for Barbafieri et al., 2011) have been reviewed and their main characteristics summarized in Table 7. Particular attention has been paid to the analytical methods applied on soil samples. The DTPA extraction method for the determination of the bioavailable soil fraction has been executed only by Bacchetta et al. (2018) and Brunetti et al. (2018). This last was a greenhouse study conducted on contaminated agricultural soil in Apulia Region and not on mine tailings as all the other considered studies (Table 7). The remaining studies adopted more aggressive soil extraction techniques and, although presented, they cannot be considered in the discussion.

Table 7. Locations of the studies, quantity of analysed samples, adopted methodologies for soil and plant analysis, considered elements and *Helicrysum Spp*. from the literature review for the *H. italicum Spp.*. “n.a.” means not available information.

Literature	**N° of CS samples**	**Methodology for PHEs determination in contaminated soils**	**N° of plant**	**Methodology for PHEs determination in plants**	**Considered elements**	**Studied *Helicrysum* subspp.**	**Location in Italy**
Current study	22	DTPA extraction, Lindsay and Norvell (1978)	22	0.25 g plant +2 ml H_2O_2 + 6 ml HNO_3	*Cd, Cu, Fe, Ni, Pb, Zn*	*H. italicum*	*Montevecchio mine district (Sardinia)*
Barbafieri et al., 2011	3	SEP[(1)] with H_2O, KNO_3, EDTA	9	HNO_3/$HClO_4$ in 2.5/1 ratio	*Cd, Pb, Zn*	*H. Italicum*	*Ingurtosu mine district (Sardinia)*
Bacchetta et al., 2018	5	DTPA extraction, Lindsay and Norvell (1978)	5	0.5 g plant + 9 ml HNO_3 + 0.5 ml HF	*Cd, Pb, Zn*	*H. tyrrhenicum*	*Campo Pisano mine district (Sardinia)*
Brunetti et al., 2018	10	DTPA extraction, Lindsay and Norvell (1978)	10	HNO_3:H_2O_2:HCl mixture (5:1:1 v/v)	*Cd, Co, Cr, Cu, Ni, Pb, Zn*	*H. italicum*	*Agricultural area Alta Murgia (Apulia)*
Bini et al., 2017	n.a.	0.2 g of soil + 5 ml of aqua regia (37% HCl+65% HNO_3, 1:3) +1 ml of 48% HF + 1 ml of cold supersaturated H_3BO_3	n.a.	0.5 g of plants + 5 ml 65% HNO_3 + 3 ml 30% H_2O_2 in open vessels on the hot plat	*Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Zn*	*H. italicum*	*Noccioleta mine district (Tuscany)*
Cao et al., 2004	n.a.	SEP[(1)] with H_2O, KNO_3, EDTA	n.a.	Aqua Regia	*Pb, Zn*	*H. italicum*	*Montevecchio mine district (Sardinia)*
Leita et al., 1989	3	10 g soil + 50 ml of 0.05 M EDTA.	3	1 g plants digested in concentrated HNO_3 - HCl 3:1 at 150 °C	*Cd, Cu, Pb, Zn*	*H. italicum*	*Wide Iglesias mine district (Sardinia)*

(1): Sequential Extraction Procedure

Concerning concentrations in plants, the literature review showed that only Zn and Pb were considered in all 7 studies (including this), Cd was measured in 5, while Cu, Ni and Fe in 4, 3 and 2 studies, respectively (Fig. 3). Only this study, Barbafieri et al. (2011) and Brunetti et al. (2018) measured PHEs in stems and leaves separately. All the other studies were limited to roots and leaves and Leita et al. (1989) considered only leaves.

Mean element concentrations identified Zn, Pb and Fe as most abundant in *H. italicum* plant with concentrations around 10^3 mg/kg, $5*10^2$ mg/kg and 10^3 mg/kg, respectively (Fig. 3). Instead, Cu, Cd and Ni, were poorly absorbed by the plant and their concentrations ranged around 10 mg/kg for Cu and below 10 mg/kg for Cd and Ni. The stems were the most impoverished parts for all considered elements. Based on the thresholds provided by Van der Ent et al. (2013), *H. Italicum* cannot be considered an hyperaccumulator species.

Considering only studies from Sardinia region that shared the same soil element abundance ranking Zn > Pb > Cd, both *H. italicum* or *H. tyrrhenicum* (our study, Bacchetta et al., 2018; Barbafieri et al., 2011; Cao et al., 2004; Leita et al., 1989) showed similar abundances for Cd, Pb and Zn, with *H. tyrrhenicum* reporting higher concentrations. The experiment of Brunetti et al. (2018) was conducted on compost-contaminated clay-loam soils at basic pH with the following contaminant soil ranking: Zn > Cu > Pb > Ni > Cd, while Bini et al. (2017), in Tuscany, had different geological settings and the contaminant rank in soil was Fe > Mn > Pb > Zn > Cu > Ni > Cd. These different experimental conditions justified the evident differences in terms of element abundances in plant parts of Cd, Zn and Pb, as well as the alignment of Fe and Cu (Fig. 3).

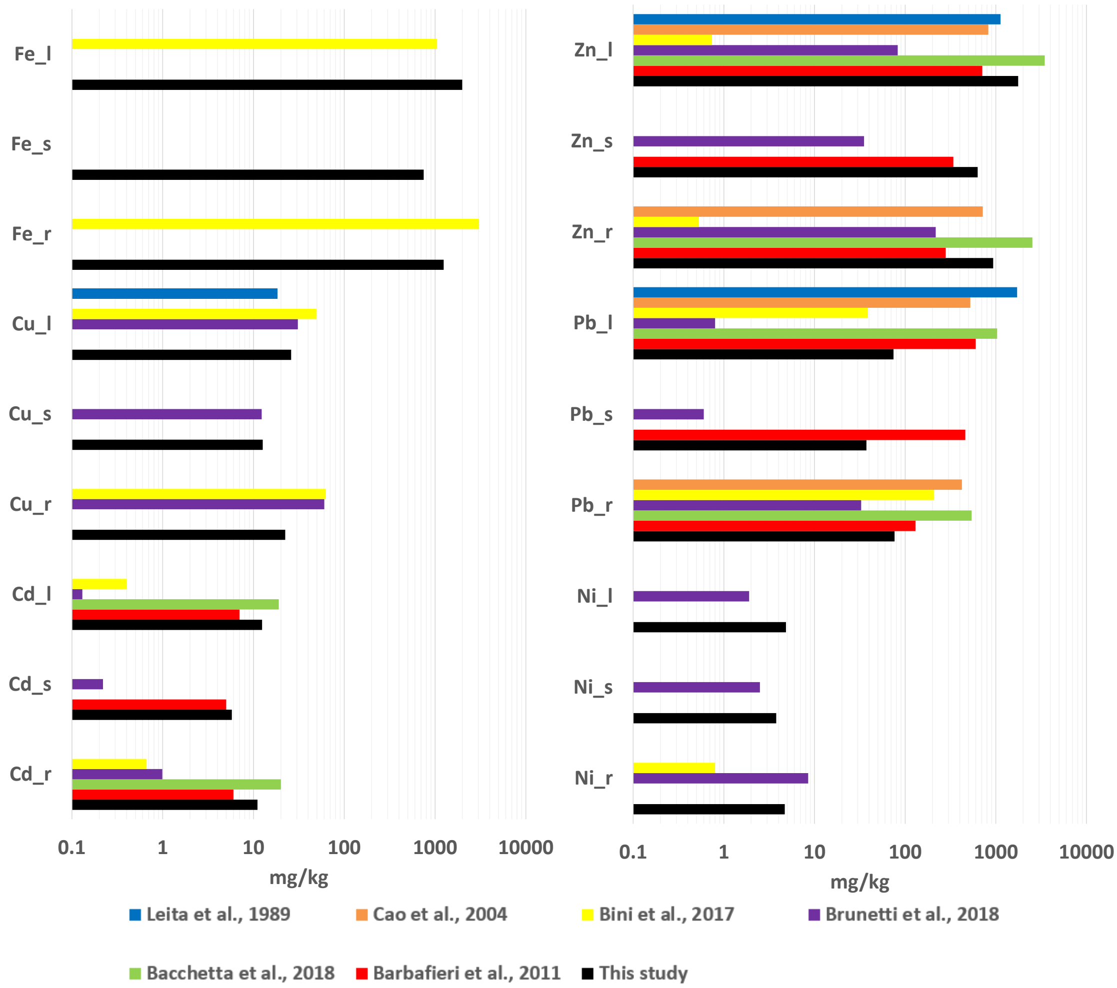

Figure 3. Mean concentration of the analysed PHEs in *H.* Spp. plant parts (r= roots; s = steams; l = leaves) from this study and from the works presented in Table 7.

As stated above, the root/soil ratio can be compared only against works that adopted the same DTPA extraction on soil samples, even if ratios from Cao et al. (2004) and Barbafieri et al. (2011) are also reported (empty bars) in Figure 4A. The uptake capacity was evident for *H. italicum* because all values were > 1 as reported here and also by Brunetti et al. (2018) and Bacchetta et al. (2018). The ratio values rarely exceed 10 (except for Fe, but it has a biological function) and when it happens, it is for *H. tyrrhenicum* subspp. for Pb and Zn (Bacchetta et al., 2018) or for Cd at low bioavailability in soils by Brunetti et al. (2018).

Helicrysum italicum can be defined as a tolerant species for Cd, Cu, Fe, Ni, Pb and Zn, but it is not suitable for phytoextraction since it shows little capacity to accumulate elements in leaves. Indeed, Cd was equally distributed in the plant and leaf/root ratio was 1 both in the present study and Barbafieri et al. (2011) and Bacchetta et al. (2018), while it was 0.6 in Tuscany mine of Bini et al. (2017) (Fig. 4B). Although the studies conducted in the Sardinia region showed an accumulation of Zn and Pb in leaves with mean leaf/root ratios around 2, and even higher in Barbafieri et al. (2011) (Fig. 4B), the high variability of the leaf/root ratios in this study does not allow a clear and univocal indication on translocation capacity of *H. italicum* (Table 6).

In this study, Fe resulted the most absorbed element (Fig. 4A) and contemporarily also the most translocated from roots to leaves (2.36 leaf/root median ratio in Table 6) because of its biological function in photosynthetic process. Despite this, the Fe leaf/root ratio by Bini et al. (2017) was widely < 1 indicating a root accumulation (Fig. 4B). Bioavailable Fe in CS and US were aligned (Table 1) and the higher absorption and translocation in CS was probably related to the lower pH and lime content in respect to US, as reported by Buscaroli et al. (2017). In the mine site investigated by Bini et al. (2017), total Fe was the most abundant element and plants preferred to store it in roots. Similar behaviour has been reported in rice species that are able to oxidise Fe at the root surface, leading to the formation of iron plaques (Green and Etherington, 1977) or accumulating Fe as ferric hydroxides (goethite and lepidocrocite) in roots (Bacha and Hossner, 1977). In both cases Fe precipitation in roots could later influence the uptake of other elements (Armstrong and Armstrong, 1988).

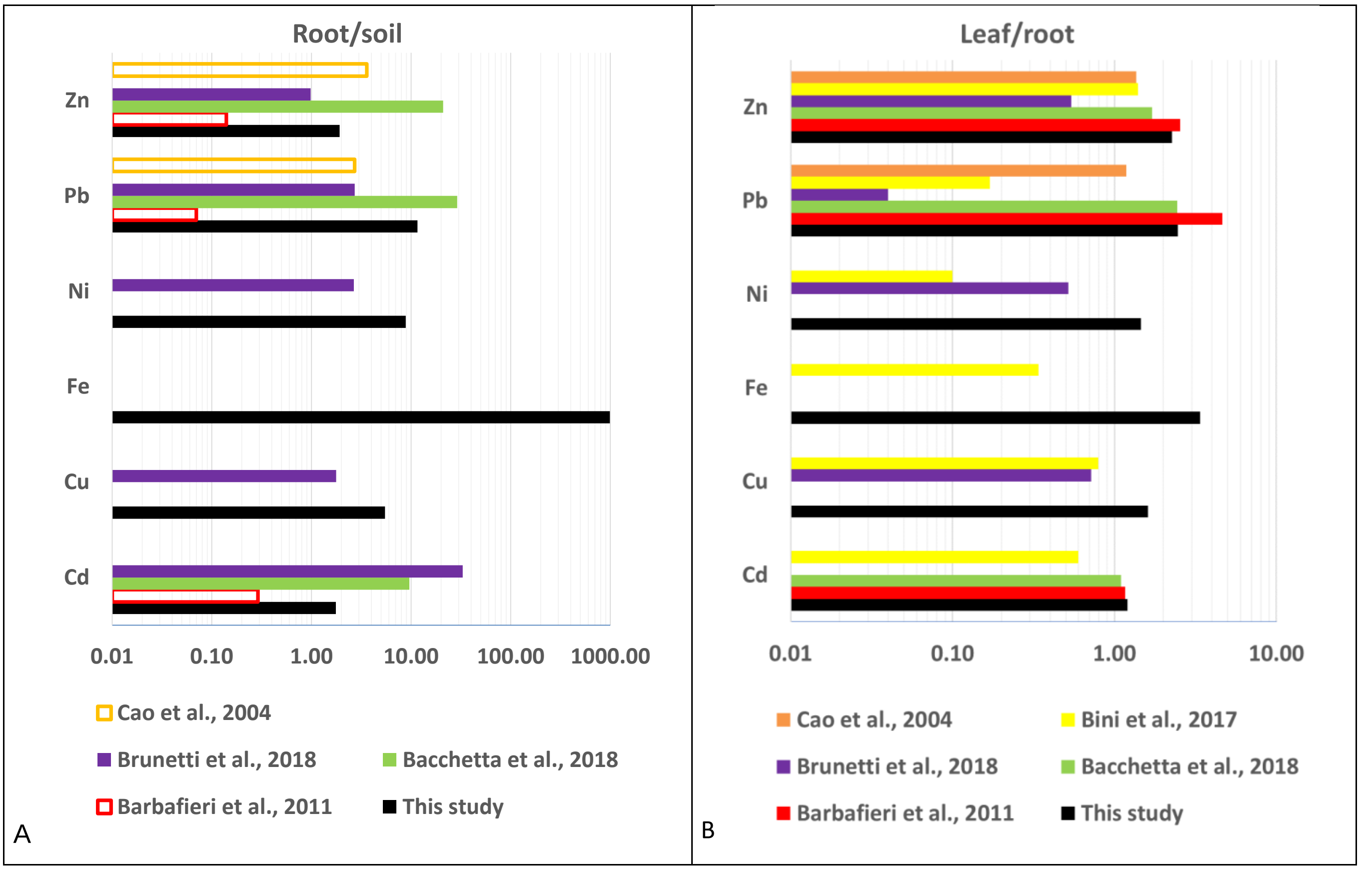

Figure 4. Mean root/bioavailable soil element concentration ratio (A) and mean leaf/root element concentration ratio (B) in *H.* Spp., calculated from this study and from the other papers presented in Table 7.

Plants suitable for phytoextraction should possess multiple abilities: first of all absorb (root/soil) and translocate (leaf/root) to aerial parts heavy metals then rusticity, fast growth, high biomass yield and easy harvesting (Jabeen et al., 2009).

Based on the literature review, the content of all investigated elements, in plant tissues makes *H. italicum* a tolerant species (especially in respect of Pb and Zn), but it does not reach the concentrations to be defined an hyperaccumulator plant (Baker et al., 2000): 100 mg/kg of Cd (of the leaf dry weight), 1000 mg/kg for Ni, Cu and Pb and 10000 mg/kg for Zn. The root/soil bioavailable ratio > 1 for all elements suggests the use of *H. italicum* for phytostabilization in mine areas as a pioneering strategy. Although the median leaf/root ratio was > 1 for Zn and Pb in plants grown in Sardinia mine districts, contrasting mean ratios were achieved for *H. italicum* in different contaminated sites not allowing a clear evidence of its phytoextraction ability.

Moreover, Boi et al. (2020) argued that few kilograms per hectare (6 - 11 kg/ha) can be recovered by *H. tyrrhenicum* (the most performant subspp. as shown in Fig. 3 and 4) and given the actual price of Zn, it does not allow economic sustainability.

Since *H. italicum* i) is a spontaneous and perennial species, tolerant to PHEs; ii) guarantees the canopy cover all throughout the year, preventing wind dispersion and water erosion; iii) influences the soil retention capacity and can itself rehabilitate the vegetation cover, reactivating pedological processes; iv) can be propagated sowing directly seeds on contaminated soils allowing cheaper propagation; v) permits the stabilization of mine tailing also from land management point of view, it can be indicated for phytostabilization in abandoned mine districts, reducing the impact of PHEs on the mine sites and surrounding environments (Barbafieri et al., 2011; Bacchetta et al., 2018; Boi et al., 2019; 2020).

4. Conclusions

This study aimed at evaluating phytoremediation properties of *H. italicum* for PHEs (Cd, Cu, Fe, Ni, Pb and Zn) by the determination of elements concentration on the roots, stems and leaves, and by the related root/soil and leaf/root ratios on plants collected from mine tailing deposits in contaminated sites (CS, Montevecchio mine, Sardinia) and in uncontaminated sites (US). Moreover, a literature review on the phytoremediation properties of *Helicrysum* Spp. has been executed in order to elucidate its phytoremediation potentiality.

The Cd, Pb and Zn resulted to be the most bioavailable PHEs in soils collected from Montevecchio mine district compared to US, also exceeding the thresholds of the Italian environmental legislation.

Element concentrations in plants from CS were higher than those from US. Leaf element concentrations were higher compared to stems and roots, although statistically significant only for Cu, Fe and Zn in CS. Interaction mechanisms (synergistic effects) between Cd and Zn, Fe and Ni, and Cu and Zn in *H. Italicum* plants grown on CS were detected.

The medians for root/soil ratio were > 1 for Cu, Fe, Ni and Pb in both CS and US, meaning that *H. italicum* was able to accumulate bioavailable elements in roots. Cadmium and Zn in CS had root/soil ratio close to one (1.39 and 1.21, respectively), suggesting their limited uptake when soil bioavailable concentrations are elevated. Based on the root/soil ratios *H. italicum* is considered a metal tolerant species.

The medians leaf/root ratio in CS were 2.36, 1.54, 1.37 and 1.34 for Fe, Zn, Pb and Cu, respectively, while ratios were < 1 for Cd (0.75) and Ni (1). Similar ratios were also calculated for US proving that *H. italicum* has a weak phytoextraction capacity.

The literature review on the phytoremediation potentiality of *H. Italicum* confirmed the analytical findings of this study. Indeed, Zn, Pb and Fe were the most abundant elements in *H. Italicum* plants grown on contaminated soils with concentrations around 10^3 mg/kg, $5*10^2$ mg/kg and 10^3 mg/kg, respectively. The Cu, Cd and Ni, were poorly absorbed by plants and their concentrations ranged around 10 mg/kg of Cu and below 10 mg/kg of Cd and Ni. In light of this, *H. Italicum* cannot be considered a hyperaccumulator species. Overall, the root/soil bioavailable ratio > 1 for all elements suggested the use of *H. italicum* for phytostabilization in mine areas as a pioneering strategy of remediation.

Given that, *H. italicum* is a spontaneous and perennial species, which guarantees the canopy cover all throughout the year, rehabilitates the vegetation cover and it can be propagated by directly sowing seeds on contaminated soil, it can be recommended for phytostabilization of abandoned mine districts and for stabilization of mine tailing from land management point of view.

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