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An integrated geochemical and mineralogical investigation on soil-plant system of *Pinus halepensis* pioneer tree growing on heavy metal polluted mine tailing

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ABSTRACT

The plant species *Pinus halepensis* grows spontaneously on heavily polluted mine tailings dumps of Campo Pisano (Sardinia, southwestern Italy). The area is characterized mainly by Zn, Pb, and Cd. Sampling campaign was done, related to soils and plant materials (roots, barks, wood, and needles), aimed at evaluating the main mineralogical characteristics, metal content, plant accumulation, and translocation behavior. The polluted substrates were composed of pyrite, dolomite, calcite, quartz, gypsum, and barite with iron sulphate, and iron oxide. Zn ore minerals (smithsonite) and muscovite detected mostly in the deeper soil layers. Zn was the most abundant metal in the substrate as well as plant tissues. Roots accumulated high metal concentrations (664.65-2710 Zn, 58.39-735.88 Pb, and 4.86-11.02 mg kg^{-1} Cd) reflecting high metal contamination in soil. The biological accumulation and translocation values were reported below one for all plant tissues. Pb, Zn and Cd Translocation Factor (TF) in needles ranged 0.03-0.32, 0.03-0.19, 0.04-0.14. Biological Concentration Factor (BCF) estimated up to 0.17, 0.18, and 0.19, respectively.

Results indicate that *P. halepensis* is an excluder, tolerates high Zn, Pb, and Cd concentrations, restricts their accumulation and translocation to the aerial parts and may be applied for long-term phytostabilization and revegetation processes in abandoned mine tailing sites.

Keywords: Mine tailing, Heavy metals, Phytostabilization, Phytoremediation, *Pinus halepensis,* Translocation factor, Biological concentration factor

1. Introduction

The dispersal processes of mine wastes pose a significant threat to the surrounding environment and cause an adverse impact on soil, water, wildlife, human health due to the high metal concentrations (Cao et al., 2008; Bacchetta et al., 2015). The impact of mine tailings on the environment is severely worsened by the effect of drought, heat, heavy seasonal rainfalls, and limited vegetation cover, which intensify the weathering processes, water run-off, soil, and wind erosion and favor the mobilization of contaminants (Gray, 1997; Concas et al., 2006; Mendez and Maier., 2008; Barbafieri et al., 2011). Metal sulphides in mine waste that are often disposed of in open dumps oxidize and dissolve due to exposure to atmospheric agents, and can be dispersed by wind and water erosion (Mendez and Maier., 2008). This pollution can seriously contaminate and spread a high range of trace element contaminants in soils and groundwater as well as in the vast surrounding areas of abandoned mines and tailing dumps (Cao et al., 2009; Lai et al., 2015; Concas et al., 2015). This situation is common to most of the mine sites throughout the world and calls for effective sustainable remediation (Párraga-Aguado et al., 2013; Conesa and Pàrraga-Aguado., 2019).

Phyto-management through phytostabilization is considered one of the most feasible and effective tools for soil stabilization and metal immobilization in mine polluted sites (Mendez and Maier, 2008). Available scientific findings suggest that the more suitable phytoremediation plant species are the metal tolerant autochthonous plant species that are pioneer, locally adapted with complementary ecological functions of the contaminated site, and well-adjusted to drought conditions, high salinity, and low soil fertility without interfering with the local biodiversity (Cao et al., 2009; Concas et al., 2015; Lai et al., 2015; Bacchetta et al., 2018). In general, phytostabilization is considered a suitable technique for long-term projects where plant species translocate small amounts of metals to epigean organs. The goal is to reach some acceptable level of environmental risks (Concas et al., 2015).

The body of literature on the use of grasses, half- shrubs, and shrubs for phytostabilization of metal-polluted soils in semiarid areas is growing (Bacchetta et al., 2015; De Giudici et al., 2015 and 2018), but few available findings are on the use of trees despite their great potential for stabilization of mining sites (Domínguez et al., 2009; Disante et al., 2010; Conesa and Pàrraga-Aguado, 2019). One of the main constraints of using trees on contaminated lands is related to their slow growth rates compared to herbs or shrubs (Evangelou et al., 2012), which can be more than counterbalanced by their long-term life span. This favor the practices of availing of the use of evergreen tree species or deciduous species (Pàrraga-Aguado et al., 2014a).

Pinus halepensis Mill. is one of the Mediterranean widespread evergreen trees that can grow mainly at the thermo- and meso-Mediterranean bioclimatic belts and in the lower altitudes, mainly, in neutral or slightly alkaline low fertile soil (Pulford and Watson, 2003; Conesa and Pàrraga-Aguado, 2019; Pàrraga-Aguado et al., 2014c). It tolerate winter temperatures below 10°C and precipitation 350-700 mm (Pérez-Piqueres et al., 2018; Querejeta et al., 2008). The *P. halepensis* high metal tolerance (Pulford and Watson, 2003; Conesa and Pàrraga-Aguado, 2019) and its ability for restoration of degraded soil in arid and semi-arid areas have been frequently noted (Querejeta et al., 2008; Pàrraga-Aguado et al., 2013); as well as its high efficiency in the use of water and nutrients in low fertility soils of mining wastes (Sardans et al., 2005). These characteristics make *P. halepensis* a potentially suitable species for revegetation and rehabilitation of highly contaminated mine sites, however, as plant survival is highly dependent on its response to heavy metal stress, a deeper understanding of the soil-plant system is required to successfully implement reforestation program.

To our knowledge, there are few available studies that have investigated *Pinus halepensis* pioneer tree growing on mine sites with multiple heavy metals contamination (Disante et al., 2010; Párraga-Aguado et al., 2013; Concas et al., 2015; Conesa and Pàrraga-Aguado, 2019). This study aims to investigate the soil-plant behavior of *P. halepensis* growing spontaneously in the heavily polluted abandoned mine tailing site of Campo Pisano (SW- Sardinia) through a multidisciplinary approach based on geochemical and mineralogical analysis of soil and plant compartments. Parts of three individual *P. halepensis* having nearly similar characteristics were sampled, along with bulk soils around their roots and core soil samples. The total and bioavailable content of Zn, Pb and Cd in soil, the metal accumulation and translocation in the compartment of the plant as well as the mineralogy of the soil-plant system were investigated. The obtained results will help to understand the performance of the plant species in response to multi-heavy metal stresses and provide data to evaluate the effectiveness and the sustainability of revegetation and phytoremediation measures.

2. Materials and Methods

2.1. Study area

The study area is the mine tailing of Campo Pisano (hereafter CP), located around the catchment basin of Rio San Giorgio near to the town of Iglesias (South-Western Sardinia, Italy). The CP mine belongs to the Metalliferous Ring of Sulcis-Iglesiente mining district. The latter was one of the most important mine regions of Europe, which has been operating since pre-Roman time. It ceased to be active in 1998 after being extensively exploited during the 19th and 20th centuries (Boni et al., 1999).

The geology of the study area is a Paleozoic carbonate with the Metalliferous Ring of the middle Cambrian limestone, and dolomites (Boni et al., 2013; Bechstädt & Boni, 1994; Boi et al., 2020). Mineralization consists of pre-Variscan sulphides (Zn and Pb sulphide), and non-sulphide deposits belonging to the Cambrian carbonate rocks.

Cerussite and anglesite are also common, generally associated with nodules and lenses of residual galena (Moore, 1972; Aversa et al., 2002; De Giudici et al., 2015). The main minerals are dolomite ($CaMg(CO_3)_2$), Calcite ($CaCO_3$), quartz (SiO_2), barite ($BaSO_4$), and iron-oxyhydroxides (Boi et al., 2019; Aversa et al., 2002). The most presented Zn-bearing minerals are smithsonite ($ZnCO_3$), hemimorphite ($Zn_4Si_2O_7(OH)_2(H_2O)$), and hydrozincite ($Zn_5(CO_3)_2(OH)_6$) (De Giudici et al., 2015).

In this study, we will refer to the mine waste samples as soil samples; we notice however that pedogenetic processes are poorly developed. CP polluted soil collected from the mine waste was previously studied by Bacchetta et al. (2012) and is affected by high concentrations of Zn (12000 mg kg^{-1}) and Pb (3250 mg kg^{-1}) as the most abundant metals; it has poor agronomic properties (total carbon 6%, nitrogen 0.01%, phosphorous 0.04%, calcium 6%, magnesium 4%) and a Cation Exchange Capacity (CEC) of about 7.19 cmol kg^{-1}. The soil physical properties have been classified as sandy-loam soil with a high content of sand (70%), a limited content of silt and clay (13% and 17%, respectively) based on USDA classification (USDA, 1998).

The area is a Mediterranean pluviseasonal bio-climate with the lower meso-Mediterranean, and the upper thermo-Mediterranean, characterized by ombrotypes between the lower sub-humid and the upper dry (Bacchetta et al., 2009), annual mean temperature of 17°C, annual mean precipitation of 600 mm, and runoff and evapotranspiration around 24 % and 57 %, respectively (Cidu et al., 2001; Lai et al., 2015). Campo Pisano mine waste soil is neutral or slightly alkaline (pH equal to 7.3) based on the USDA classification (USDA, 1998).

Between 2008 and 2010, our research group performed an experimental phytoremediation study in the CP tailing site, aiming at assessing the behavior of two Mediterranean shrub plant species [*Pistacia lentiscus* L., and *Scrophularia canina* L. subsp. *bicolor* (Sibth. & Sm.) Greuter] and their responses to different soil amendments (Lai et al., 2015; Boi et al., 2019). An investigated site area of about 300 m^2 was subdivided into ten different experimental plots to apply various soil amendments. The best success was reported in the plot amended with compost produced from the organic fraction of Municipal Solid Waste (MSW) characterized by 31.5% Carbon and 1.44% Nitrogen content, CEC~22.6 cmol kg^{-1}, pH~ 7.6 with the total content of 45 mg kg^{-1} Pb and 235 mg kg^{-1} Zn (Bacchetta et al., 2015). The study area of this work is in the same experimental site and its surrounding area.

2.2. Pinus halepensis and soil selection and sampling

In the last ten years, several young trees of *P. halepensis* were spontaneously growing in CP mine tailings. *P. halepensis* parts were collected in November 2020 from three individual trees having nearly similar characteristics, similar height (2-3 m) and age (10-12 years old): **(i)** inside the experimental compost-amended plot (CP1: N 39° 17' 48.2", E 8° 31' 54.2") that has nearly 10 years old; **(ii)** outside of the compost- amended plot at the border of mine tailing dumps distancing 3-4 m from the compost-amended plot (CP2: N 39°17'48.7", E 8°31'54.0") with nearly 10 years old; and **(iii)** outside of the compost-amended plot at the border of mine tailing dumps with more distancing (6-7 m) from the compost-amended plot (CP3: N 39°17'49.0", E 8°31'54.6") which has 12 years old (Figure 1c).

The samples of each *P. halepensis* consist of hypogean and epigean organs: **(i)** plant roots (R) **(ii)** six young and old needles (N) of the first level, second level and third level of each plant height; **(iii)** three bark samples (B) from the main stem, the first branch and the second branch of each plant; **(iv)** a radial core drilled wood sample (W).

The core drilled wood samples were obtained through a core sampler. The wood core consisted of different growth rings from which it can be visually recognized that CP1 and CP2 have the same age (10 years), and CP3 aged 12 years old. Subsequently, each core was divided into three different growth-age rings: **(i)** plant initial age (1^{st} year), **(ii)** middle age (5 years), and **(iii)** current age (10^{th} or 12^{th} years), which were considered suitable for detecting the wood metal concentration over time.

Six bulk soil samples were collected: **(i)** three soil samples at around the roots (approximately 5 cm) of each selected *P. halepensis* (CP1, CP2, and CP3) (SR) (Figure 1c); **(ii)** three in-depth (50-110 cm) core drilled samples taken: the first one at nearly 50 cm distances from the pine growing in the compost-amended plot (CP1) (S1: N 39°17'48.2", E 8°31'54.1"), the second sample from pine growing in the experimental amended plot where there is no vegetation canopy distancing about 4-5 meters from CP1 (S2: N 39°17'47.7", E 8°31'54.4"), the third one at 1-2 m distances from CP1 in the amended plot (S3: N 39°17'48.1", E 8°31'54.1") (Figure 1d).

Core soil sampling was taken using a core sampler that allowed the recovery of samples in sealed plastic bags (Atlas Copco's COBRA). Core stratigraphy comprised layers visually recognizable by different grain sizes and colors and ranged between sand and clay. The collected core soil sample was separated into subsamples of different soil depth layers based on the visual recognizable color of each soil layer. The upper 20 cm of each collected core drilled soil sample was considered the uppermost soil where *P. halepensis* roots are also growing (in S1 and S3). Figure 1d provides the CP site location where the core drilled soils samples were collected (see more about the collected bulk soils and *P. halepensis* samples in table Supplementary Materials, S1)

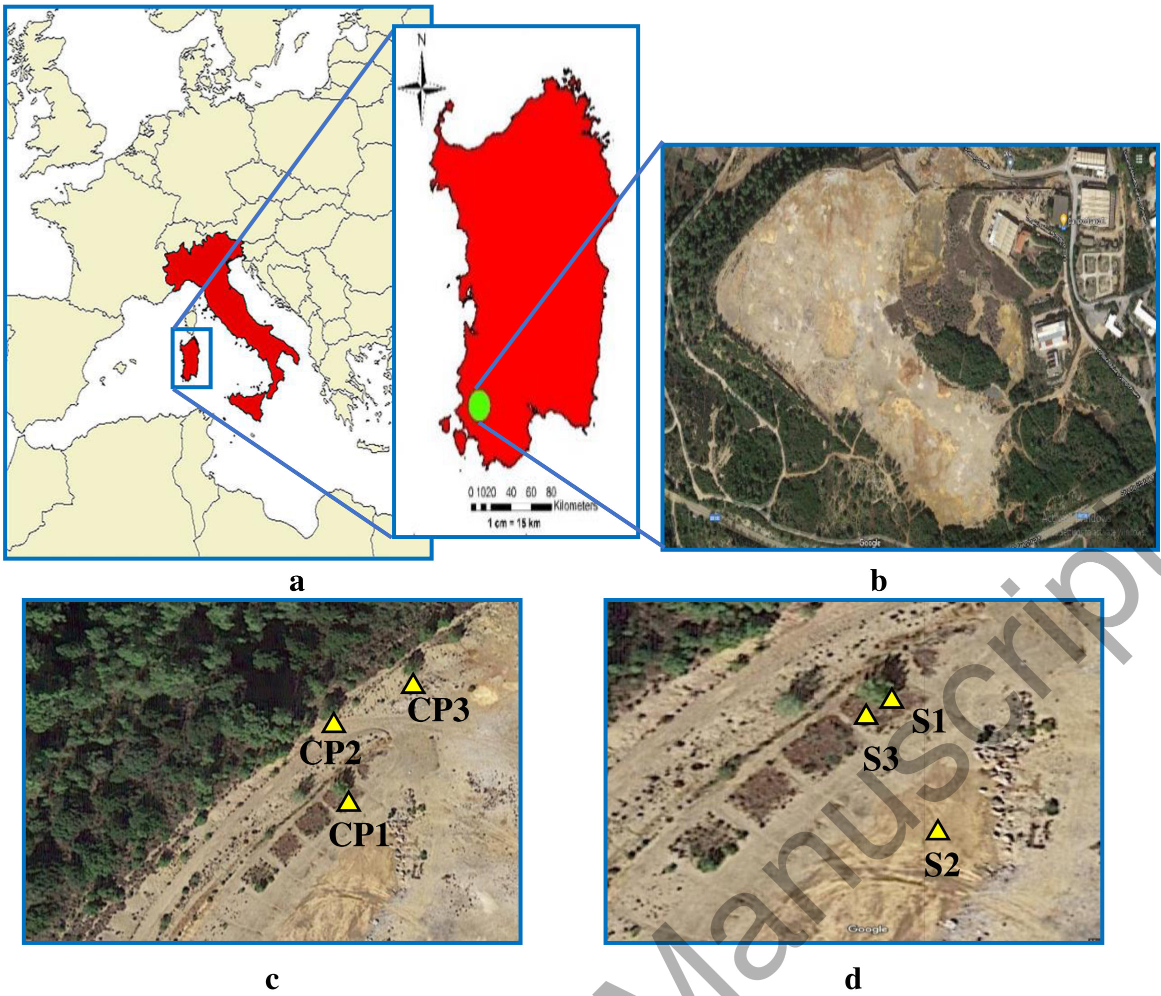

Figure 1. Geo-location maps of the study area; (**a**) Location of Sardinia in the Mediterranean Basin (the green spot indicates the sampling site); (**b**) Campo Pisano tailing dump; (**c**) details of sampling points of the collected plant parts from specimens of *Pinus halepensis* (CP1, CP2 & CP3); (**d**) sampling points of the collected core drilled soil samples (S1, S2, and S3).

2.3. Mineralogical and chemical characterization

All polluted soil sampled in the mine site (the core soil samples and the one collected around the plant roots) were dried for a day at 45°C (in the oven of Binder GmbH, Tuttlingen, Germany), sieved (less than 2 mm), and fine powder ground in an agate mortar. Plant samples (root, bark, and needles) were manually cleaned by shaking, wiping, removing gently the adhesive remaining particles for X-Ray Diffraction (XRD) and Scanning Electron Microscopy (SEM) test. All plants' samples were one day dried in the oven at 45°C and ground (less than 40 μm) using an Ultra Centrifugal grinder (ZM 200, Retsch GmbH) after being carefully washed with distilled water for performing the chemical test.

2.3.1. Mineralogical characterization

XRD analysis was performed on the substrate samples to investigate the mineralogy and on collected *P. halepensis* samples (root, bark, and needles) to investigate the potential presence of detectable minerals. Approximately 100 mg of each powdered substrate and plant samples were used for powder XRD analysis through laboratory θ-2θ equipment (Pan alytical X'Pert Pro) using the X'Celerator detector with Cu $K\alpha_1$ wavelength radiation, operating at 40 kV and 40 mA. The results were processed by software (X'Pert HighScore Plus, Panalytical B.V., Almelo, the Netherlands) to clarify the mineral phases present.

The element distribution and microscopic characteristics of samples have been investigated through Energy Dispersive Spectroscopy (Thermo Scientific UltraDry EDS Detector, Pathfinder, Waltham) and Scanning Electron Microscopy (SEM) imaging (ESEM QUANTA 200, FEI), under low-pressure conditions. Substrate samples, including the bulk soils and rhizospheres solid materials (operationally defined as the soil grains within 2 mm of the roots) as well as samples of plant compartment (root, bark, and needles) after shaking and wiping gently were left to be dried at room temperature for almost a week before conducting the SEM analysis. Moreover, selected barks and needle samples of *P. halepensis* were first examined as fresh and then as dried samples.

2.3.2. Chemical characterization

The chemical characteristics of soil samples were analyzed using the official Italian analytical methods (D.M. 13/09/99) (Barbafieri et al., 1996; Lindsay and Norvell, 1978). Total metal concentration was assessed through the Environmental Protection Agency (EPA) method 3052 (Bacchetta et al., 2012) using triplicate samples (0.5 g) acid digestion (9 ml HNO_3 65% and 3ml HF) by microwave oven (Start D, Milestone, Sorisole, Italy) (see details in Supplementary Materials, S2).

The bioavailable metal content (Zn, Pb, and Cd) was evaluated only for the uppermost samples (S1, S2, and S3) and soil around the roots (CP1, CP2, and CP3) through the analysis of a single extracts method performed by using 0.005 M DTPA solution added to substrate samples (Guan et al., 2011; Barbafieri et al., 1996) (see details in Supplementary Materials, S3). After shaking for half an hour at 180 oscillations/minutes, the mixture was filtered, and the concentration of the extracted metal was measured through the ICP-OES technique (the Agilent 725-ES method). DTPA extraction procedure is described as the most thermodynamically efficient, which prevents the carbonate dissolution and consequently, the release of the bounded metals (Lindsay and Norvell, 1978; Feng et al., 2005).

Metal concentrations of the extracted solutions were analyzed through Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES, Perkin Elmer Optima DV 7000, Waltham) with the wavelengths (nm) of 206.200, 220.353, and 228.802, for Zn, Pb, and Cd, respectively. The precision of the chemical test was evaluated by performing a triplicate sample. Blank solutions and different reference materials were utilized to ensure the reliability of the analytical methods in the analysis of the total metal content of soil samples and plant tissues. The accuracy of the analyzed data was verified by using reference material for soil (GBW-07403-GSS-3) and plant samples (GBW-07603-GSV-2, bush twigs, and leaves) as well the blank solution (the EnviroMAT-pure water high reference solution, EP-H-1150). All standards and blanks were matrix-matched with the samples and reagents.

The chemical bioavailability data was estimated in terms of the percentage of bioavailable fraction as %Bioavailability = Mean value of metal bioavailability in soil / Mean value of metal concentration in soil × 100.

Total carbon and nitrogen contents of the substrate around the roots (CP1, CP2, and CP3) were determined through an element CHN analyzer (Leco CHN-628) calibrated with the reference material (Ore Tailings) (Bacchetta et al., 2015).

The ability of *P. halepensis* to take up metals from the substrate and translocate them to the aboveground organs was calculated separately for each compartment (barks, wood, and needles) by means of the Biological Accumulation Coefficient (BAC), the Translocation (TF) and the Biological Concentration Factors (BCF) (Fellet et al., 2007) calculated as follows (Figure 5):

The BCF determined the metal uptake (mg kg^{-1}) from the soil and calculated as the ratio between the metal content in roots and soil (Fellet et al., 2007):

$$BCF = [M_{Root}] / [M_{Soil}] \quad \text{Eq. (A.1)}$$

The BAC was calculated to estimate metal transferring from the soil to the epigean parts, according to Marchiol et al., 2013:

$$BAC = [M_{Epigean}] / [M_{Soil}] \quad \text{Eq. (A.2)}$$

The TF reported the metal translocation from roots into epigean organs, according to Brunetti et al., 2009:

$$TF = [M_{Epigean}] / [M_{Root}] \quad \text{Eq. (A.3)}$$

where $[M_{Epigean}]$ and $[M_{Root}]$ are metal concentrations in the epigean parts and the roots, respectively.

3. Results

3.1. Mineralogical and chemical characterization

3.1.1. Bulk soil samples

Table 1 shows the results of the XRD analysis performed on the collected bulk soil samples. Dolomite (CaMg $(CO_3)_2$), quartz (SiO_2), and gypsum ($CaSO_4$) are present in all different samples. Pyrite (FeS_2) was detected in the bulk layers, except in the layers of S1 (20-43 cm), S2 (16-27 cm), and S3 (55-65 cm), where goethite [FeO (OH)] was found. Jarosite $[KFe^{3+}_3(SO_4)_2(OH)_6]$ was present in the amended plot layers of S1 (20-43 cm) and S3 (50-65 cm). Smithsonite (Zn CO_3) and muscovite (KAl_2 $[(AlSi_3O_{10})$ (F, $OH)_2]$ were both found in the deeper soils layer of amended plot S3 (65-110 cm) and the not- amended plot S2 (27-103 cm). Moreover, muscovite was also detected in the soil around the roots of CP3. Illite [(K, H_3O) (Al, Mg, Fe)$_2$ (Si, Al)$_4$ O_{10} $(OH)_2$, (H_2O)] was presented in the bulk soil samples S2 (27-103 cm) and S3 (0-55 cm) as well as the soil around the roots of CP1. Brushite (Ca PO_3 $(OH)_2$ H_2O) was found only in the uppermost bulk soil layer S1 (0-20 cm) and CP1. The outcome of soil CHN analysis showed that the highest percentage of total carbon and nitrogen content is 0.96 and 0.74, respectively, in CP1 (Supplementary Materials, S4).

Table 1. Minerals detected in the collected bulk soils samples; the soil from the amended plot where *Pinus halepensis* growing (**S1**), from out of the amended plot with 4-5m distances (**S2**), Inside the amended plot with 1-2 m distance (**S3**), the soil around the plant root located in: the amended plot (**CP1**), out of the amended plot with 3-4 m distance (**CP2**), out of the amended plot with 6-7m distances (**CP3**); and roots (**R**), barks (**B**) and needles (**N**) considered as representative of selected *P. halepensis*

Sample name	Bulk soil Sample	Dolomite	Pyrite	Calcite	Quartz	Silica	Barite	Illite	Smithsonite	Muscovite	Jarosite	Goethite	Gypsum	Brushite	Cellulose	Whewellite	Iron sulphate	Iron oxide
S1	0-20 cm	●	●		●								●	●				
	20-43 cm				●						●	●	●					
S2	0-16 cm	●	●		●								●					
	16-27 cm					●						●	●					
	27-103 cm	●	●	●		●		●	●	●			●					
S3	0-55 cm	●	●		●			●					●					
	55-65 cm				●						●	●	●					
	65-110 cm	●	●		●				●	●			●					
CP1	around the root	●	●	●	●		●	●					●	●				
CP2	around the root	●	●		●		●						●					
CP3	around the root	●	●		●		●			●			●					
R		●			●		●									●	●	●
B						●										●		
N															●	●		

Table 2 reports the mean value of metal concentration (Zn, Pb, and Cd) of collected soil samples. The data shows that in all examined soil samples, Zn is the most abundant metal, followed by Pb. In the core soil drilled samples, the highest Zn and Cd concentration was found in all uppermost soil layers, with the highest values in S2 (16983.5 mg kg^{-1} and 72.97 mg kg^{-1}, respectively). Moreover, in the lower soil layers of the amended plot, Zn and Cd concentrations decreased to 3424.4 mg kg^{-1} and 5.97 mg kg^{-1} in S1 (32-38 cm); and to 4184.88 mg kg^{-1} and 11.39 mg kg^{-1} in S3 (55-66 cm), respectively. However, in the same plots, the detected Pb concentration is higher in the lower soil layers (S1: 4537.57 mg kg^{-1} and S3: 3458.09 mg kg^{-1}). In the soil collected around the roots, the highest concentration of Zn, Pb, and Cd pertains to the non-amended plot CP2 (15299.52 mg kg^{-1}, 4413.29 mg kg^{-1}, 58.54 mg kg$^{-1,}$ respectively).

Table 2 reports the bioavailable metal content (percentage) of the uppermost soil samples (S1, S2, and S3) and soil collected around the roots (CP1, CP2, and CP3) shows that the highest Cd and Zn bioavailable fraction percentage (Cd>Zn) was found in S2 (21.02% and 8.07%, respectively). In the amended plot (S3), the bioavailability of lead is remarkably high (31.65%).

Table 2. Metal contents in the bulk soil collected from different depth layers of core samples (S1, S2, and S3) and around the collected *Pinus halepensis* root samples (CP1, CP2, and CP3) grown in mine tailing dumps, and the bioavailable metal content (%) in the uppermost soil (0-20 cm) and the soil around the root samples.

Soil samples	Horizon depth (cm)	Mean value of metal concentration in soil (mg kg^{-1}± standard deviation)			The bioavailable metal content of Zn, Pb & Cd (%)		
		Zn	Pb	Cd	Zn	Pb	Cd
S1	0-20	11818.91±1979.05	1827.15±112.61	67.05±9.61	7.74	3.96	11.83
	20- 28	2615.39±269.09	2235.47±72.68	19.92±2.21			
	32-38	3424.49±102.43	4537.57±277.16	5.97±0.14			
S2	0-16	16983.55±1349.23	3866.24±257.75	72.97±8.58	8.07	4.43	21.02
	16-20	9220.4±579.32	6214±430.80	21.4±1.50			
	20-23	11317.72±448.08	6564.45±192.87	11.91±0.59			
	23-27	6832.22±241.38	4988.42±302.55	12.45±0.34			
	47-50	8654.94±341.65	1419.87±61.64	34.39±5.30			
S3	0-20	10199.2±554.77	1591.42±99.01	55.48±9.86	6.30	31.65	12.26
	20-45	11066.45±757.35	1922.19±77.01	46.46±8.71			
	56-66	4184.88±124.57	3458.09±289.82	11.39±0.45			
CP1	around roots	11588.05±871.81	2904.98±336.55	48.11±13.45	5.68	4.10	13.45
CP2	around roots	15299.52±610.4	4413.29±268.67	58.54±1.51	3.58	5.40	9.51
CP3	around roots	9043.26±739.64	1604.47±128.89	46.11±11.38	7.25	2.38	13.84

3.1.2 *Pinus halepensis root samples*

Results of the mineralogical investigation are shown in Table 1 and Figure 2. The root surface of *P. halepensis* has a different mineral composition than the soil collected around the roots. The XRD analysis of *P. halepensis* root samples found dolomite, quartz, barite, and whewellite (Ca $(C_2O_4)\cdot 2(H_2O)$). Moreover, quartz and barite ($BaSO_4$) are both present mainly in the external part of root samples and the contiguous rhizospheres solid materials. SEM analysis on collected root samples of *Pinus halepensis* allowed us to investigate the particles sticking on the root surface, their morphology, and chemical composition. It shows that dolomite, quartz, and Al-silicates are the main mineral particles embedded and adhering to the root surface samples. SEM analysis did not provide evidence of the presence of calcium phosphate (brushite) on *P. halepensis* root samples, while it was detected only in the uppermost bulk soil layer S1 (0-20 cm) and CP1. It also detected the presence of iron sulphate, iron oxide, as well as several other elements, mainly Al, Si, Zn, and Fe, adhering to the external part of root samples (details in Supplementary Materials, S5).

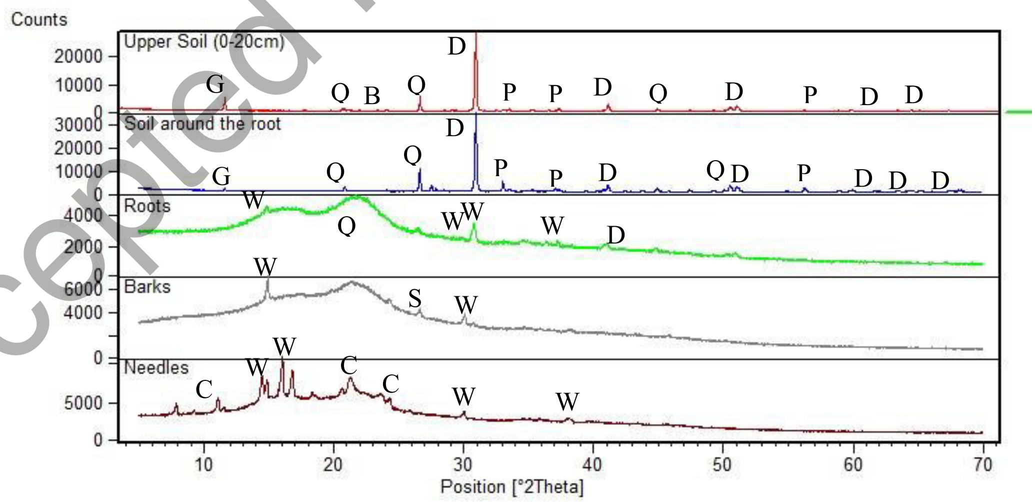

Figure 2. XRD patterns of the uppermost soil layer (0-20 cm) of the core sample, collected soil around the roots samples, roots, barks, and needles samples of *Pinus halepensis* grown in CP mine; Mineral legend: Gypsum (**G**), Quartz (**Q**), Dolomite (**D**), Pyrite (**P**), Brushite (**B**), Cellulose (**C**), Silica (**S**), Whewellite (**W**)

The chemical data of Zn, Pb, and Cd concentration of root samples and the soil collected around the plant root shows that CP2 has the highest concentration of Zn, Pb, and Cd in roots (2710.13 mg kg^{-1}, 735.88 mg kg^{-1}, and 11.02 mg kg^{-1}, respectively) and the collected soil around roots samples (15299.5 mg kg^{-1}, 4413.29 mg kg-1, and 58.5 mg kg^{-1}). It also makes evident that the plant growing in the amended plot (CP1) has the lowest Pb concentration in the root (58.3 mg kg^{-1}) (details in Supplementary Materials, S6 and S7).

3.1.3 *Pinus halepensis bark samples*

The XRD analysis of the bark sample in Figure 2 showed the presence of whewellite (Calcium oxalate) and silica mainly in the external section of selected *P. halepensis* bark samples with no significant variation among the collected bark samples.

Moreover, SEM analysis revealed the presence of particles on the bark surface of *P. halepensis*. Carbonate mineral (i.e., dolomite) (Figure 3b, point 4) and Al-silicates (Figure 3 a, points 1 and 2) were mainly detected together with Zn and Fe in the external part of the bark sample (Figure 3b, points 3 and 4). Fresh and dried bark samples were examined separately through SEM and EDS analyses to better understand the post-mortem effect on the formation of oxalates. Figure 3c point 5 shows in detail the morphological features of whewellite in the collected bark sample of *P. halepensis.* Whewellite was found to be abundant in dried bark samples (Figure 3c). Furthermore, Figure 3a points 1 and 2 clearly show that silica is present in the external part of the barks, while Figure 3b (points 3 and 4) and Figure 3c (points 5 and 6) indicate that Al as well as Ca and Mg are mainly present inside the dried bark sample as a magnesium-aluminum phyllosilicate mineral.

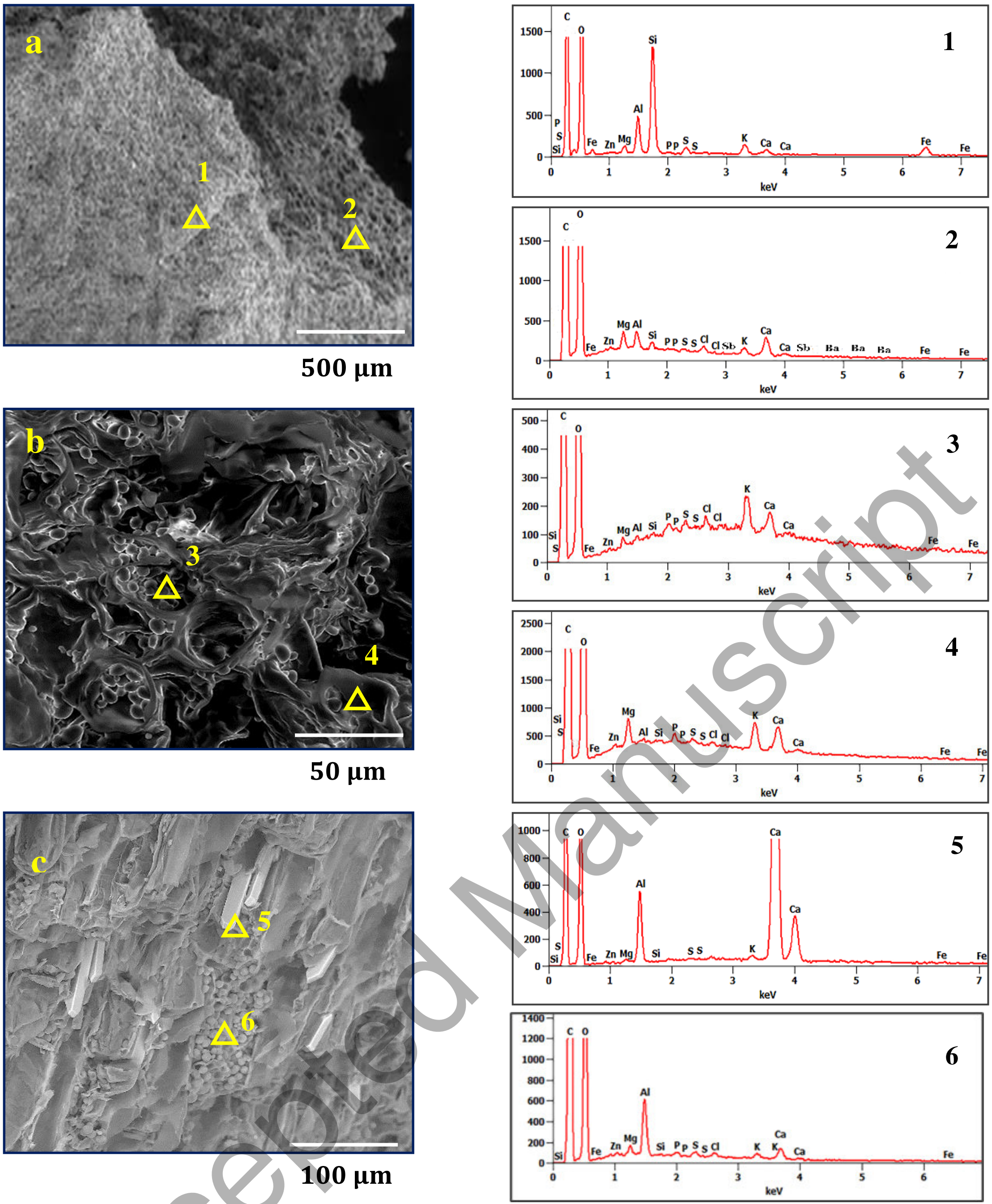

Figure 3. SEM-EDS analysis of selected *Pinus halepensis* bark samples**;** External part of bark **(a)**, inner part of fresh bark **(b)**, and the inner part of dried barksamples **(c)**. The indicated numbers from 1 to 6 are the points where EDS spectra were acquired.

Figure 4 shows that bark samples collected from the main stem of *P. halepensis*, are enriched in Zn, Pb, and Cd, with the highest mean value of Zn and Pb concentration in CP3 and CP2 (583.8 mg kg^{-1} Zn and 582.4 mg kg^{-1} Pb followed by 316.6 mg kg^{-1} Zn and 267.5 mg kg^{-1} Pb, respectively). It also shows that among the main stems, the bark of CP1 in the amended plot has the lowest concentration of Zn, Pb with 118.6 mg kg^{-1}, 60.8 mg kg^{-1}, respectively, and the highest Cd for all collected bark samples (3.4-3.6 mg kg^{-1}).

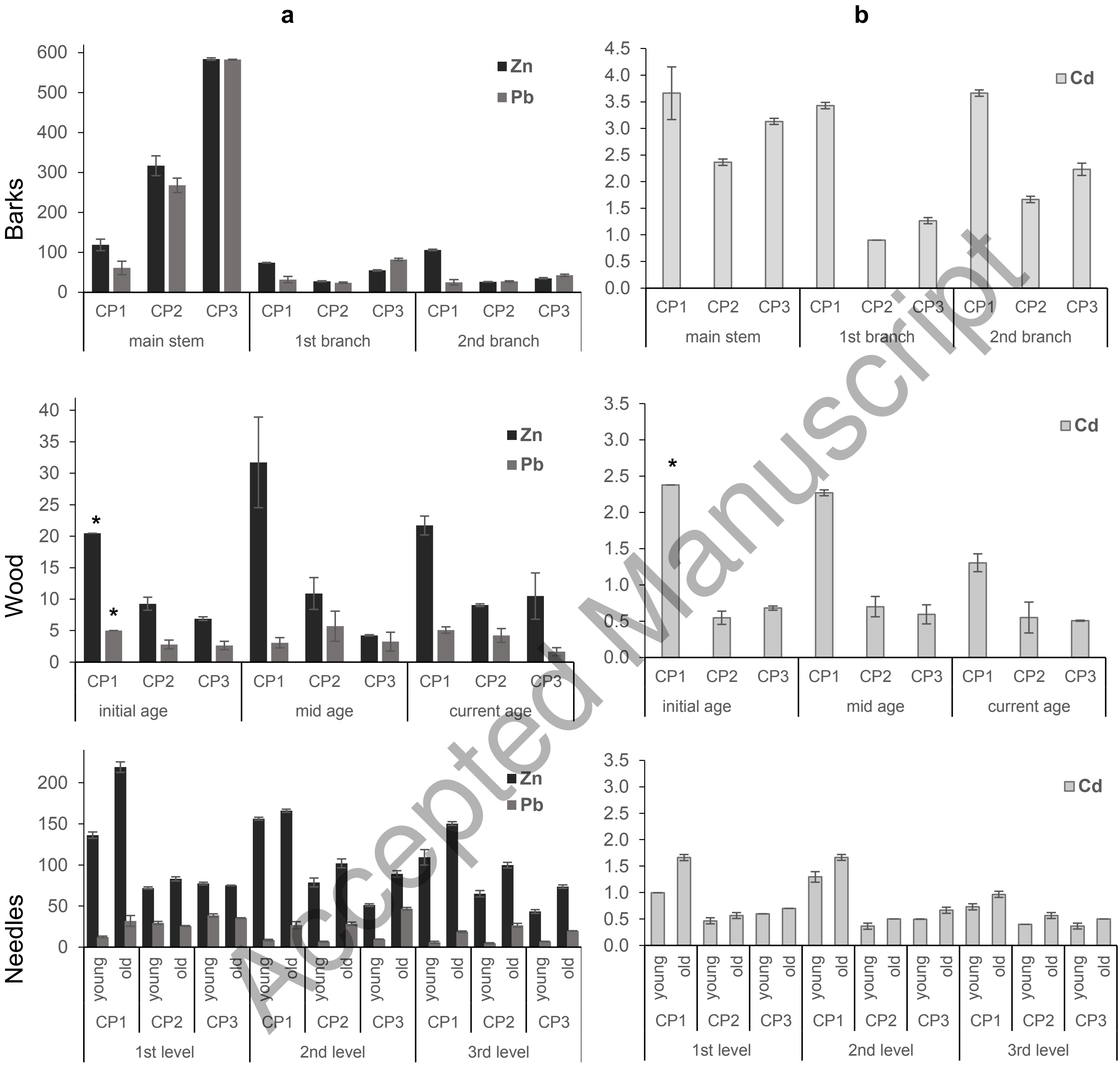

Figure 4. Mean concentration of Zn, Pb (**a**), and Cd (**b**) in *Pinus halepensis* tissues samples (barks, wood, and needles); n=3; the values marked with * indicate the single metal concentration value of the wood sample that had not adequate mass for performing a triplicate analysis.

3.1.4 Pinus halepensis wood samples

Figure 4 reports the metal concentration of the three wood samples collected in different plant growth ages (rings): the initial age (1st year), the mid-age (5th year), and the current age (10th-12th year). The data indicate that CP1 wood samples have the highest Zn, Pb, and Cd concentration in all different ages, with the maximum Zn and Cd concentration in the mid-age (5th year) of plant growth, equal to 31.7 mg kg^{-1} and 2.28 mg kg^{-1}, respectively. Moreover, CP2 and CP3 plants wood, which grow outside the compost-amended plot, do not present comparable differences in their Pb and Cd concentration along the considered life spans.

3.1.5 Pinus halepensis needles samples

The X-Ray diffraction of collected *P. halepensis* needles samples detected cellulose and whewellite with a low degree of crystallinity (see table 1 and Figure 2). The SEM analyses on needle samples show that many resin canals appear to be filled with some compound made of Al, Si, Ca, Fe, S, Zn, Mg, and K (see details in Supplementary Materials, S8 a, points 1 and 2).

Moreover, the inner parts of fresh needles showed a similar composition as the external parts. We noticed that Ca and Al have been detected in the inner part of needles after samples are dried (see details in Supplementary Materials, S8 b, and c), while Si was found in the external part and inside the resin canals. The heavy metal concentration of old and young needles indicated that Zn is the most abundant metal in all collected needle samples (see Figure 4). Needles of CP1 have the highest concentration of Zn and Cd in the old needles of each plant height level compared with needles of CP2 and CP3 plants. Moreover, the old needles picked up from the lower height of *P. halepensis* growing in the amended plot (CP1) have the highest Zn and Cd concentration (219.14 mg kg^{-1} and 1.66 mg kg^{-1}, respectively).

3.2. Biological accumulation parameters

Figure 5 reports the values of three biological accumulation parameters (BCF, BAC, and TF) assessed for aboveground and belowground organs of *P. halepensis* samples.

The BCF and BAC values are consistently very low (<1) for all investigated metals. The BCF values of CP1 and CP3 are very similar for the up-take of Zn (0.07) and Pb (0.02 CP1 and 0.05 CP3) from soil to roots and significantly lower than those of CP2 (0.17 Zn, 0.18 Pb), that was the soil having the highest metal concentration. The calculated BCF for Cd shows values in the amended plot CP1 (0.18) similar to non-amended plots CP2 and CP3 (0.19 and 0.11, respectively). The BAC values were generally in the order Zn>Cd>Pb. BAC related to needles and wood were in the order CP1>CP3>CP2, while BAC related to barks and considering Zn and Pb accumulation was higher for CP3. The metal translocation to the plant epigean organs is generally low (TF<1), especially for wood and needles. TF in needles ranges between 0.03-0.32 Pb, 0.03-0.19 Zn, and 0.04-0.14 Cd, and in wood, it ranges between 0.006-0.08 Pb, 0.004-0.03 Zn, and 0.05-0.24 Cd. Higher values have been measured for barks in CP3, where the TF of Pb was the only value higher than 1 (2.8). The higher BAC and TF values measured in bark samples are likely due to the presence of dust particles tightly adhering to the surface of the bark (see more in the discussion section).

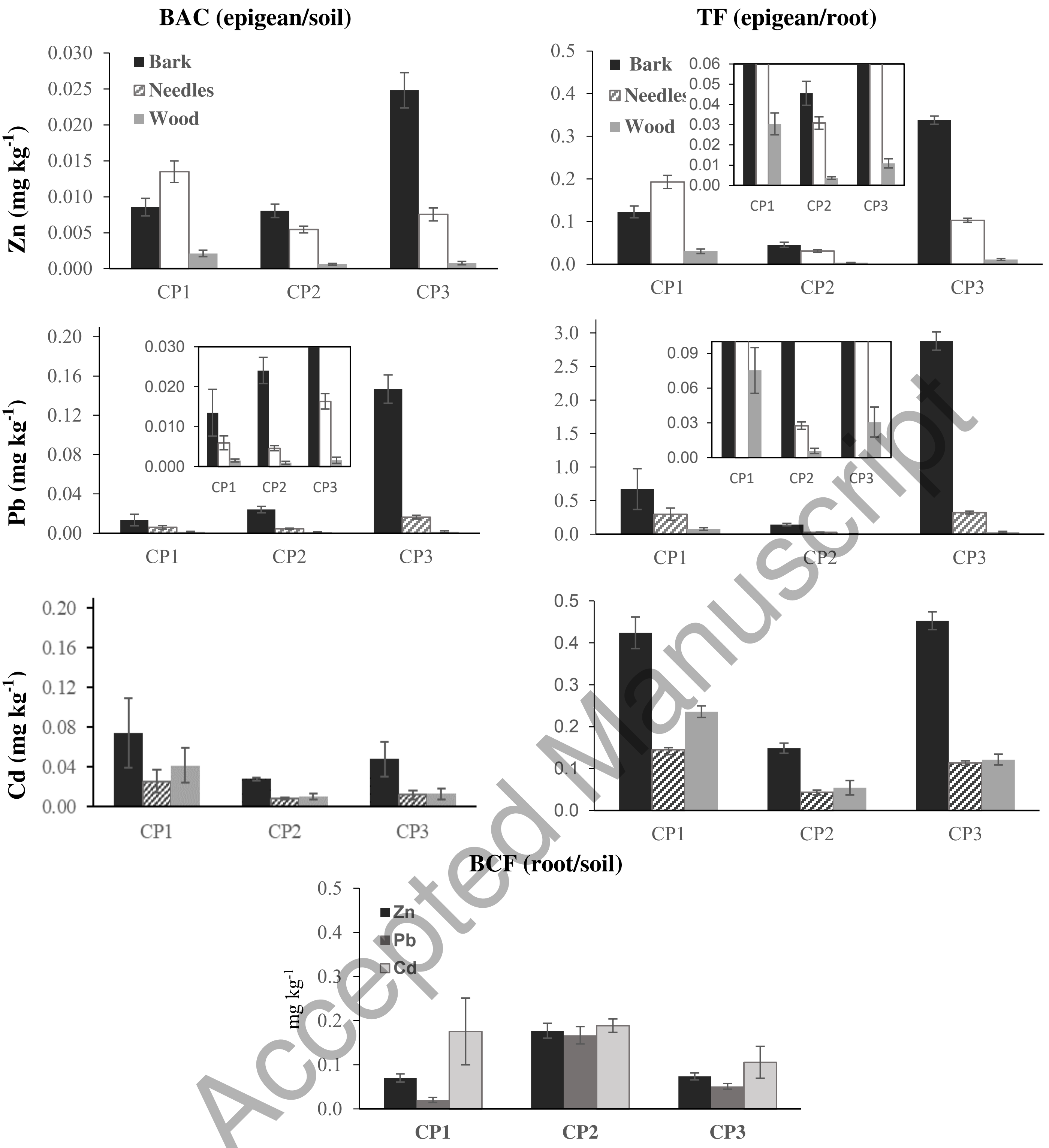

Figure 5. Biological accumulation coefficients (BAC, TF, and BCF) calculated from the data in table 2 and Figure 5

4. Discussion

4.1 Metal accumulation and translocation

The metal contents that exceed the critical concentration, may limit the soil functions and plant growth. The metal concentration tolerance for plants to grow was reported between 20-100 mg kg^{-1} Zn, 10-20 mg kg^{-1} Pb and 5-10 mg kg^{-1} Cd (Rathore et al., 2019).

The findings of this study (see table 2) demonstrate that the range of soil-heavy-metal contents (9043.26 - 15299.52 mg kg^{-1} Zn and 1604.47- 4413.29 mg kg^{-1} Pb and 46.11- 58.54 mg kg^{-1} Cd) is significantly above the maximum threshold limits required by Italian laws for the industrial site (Zn 1500 mg kg^{-1}, Pb 1000 mg kg^{-1}, Cd 15 mg kg^{-1}) (D.lgs.152/2006; Guri, 2006). Moreover, concentrations measured in root samples for Zn (664.65 - 2710.13 mg kg^{-1}) and Pb (58.39-735.88 mg kg^{-1}), was above the reported phytotoxic levels (Rathore et al., 2019; Kabata-Pendias and Pendias, 1992), indicating *P. halepensis* abilities to naturally grow and develop the tolerance mechanisms to survive in contaminated mine sites without showing any visual symptoms of metal toxicity, such as chlorosis or necrosis.

Figure 4 shows that the metal concentrations in the plant compartments generally follow the order barks>needles>wood. The only exceptions are Zn in CP1, in which needles concentration is higher than in barks, and Cd in CP1, in which wood concentration is higher than that in needles. Particularly, high Zn and Pb concentrations were assessed in CP3 barks. However, it should be noted that the bark behavior most probably reflects the effect of wind transported dust particles into the surrounding areas, which can influence the outermost surface of dead plant tissues and it will be increased over time by plant growth (Chiarantini et al., 2016; Sawidis et al., 2011; Párraga-Aguado et al., 2013). For instance, dolomite and Sb sulphide (Figure 3a, point 2) detected only in external surfaces of barks probably derive from particles transported by wind. Therefore, the Pb detected on barks may not be only the result of uptake and translocation through plant roots. Indeed, the direct path introducing metals to be accumulated in barks and wood, the roots capacity to uptake metals from soils (Sheppard and Funk, 1975), and the physiological behavior of plants for metal mobility in the xylem and phloem (Robitaille, 1981) are all inherent with likely unknown issues and need more investigation (Rodríguez et al., 2018; Chiarantini et al., 2016).

It should be noticed that different factors such as soil metal content (Witte et al., 2004) or radial mobility in the plant woody stem (Nabais et al., 1999) may limit the metal accumulation in wood (Watmough and Hutchinson, 2003; Rodríguez et al., 2018).

Literature provides little data on the metal concentration of *P. halepensis* needles growing in polluted sites (see table 3). Among available literature, the findings of Disante et al. (2010) reveals that *P. halepensis* has less capability to accumulate Zn in epigean organs in comparison to other different woody species tested, such as *Quercus suber* L., *Pinus pinea* L., *Pinus pinaster* Aiton, *Tetraclinis articulata* (Vahl) Mast., and *Rhamnus alaternus* L. According to their findings, the old needles of *Pinus pinaster* accumulated up to 217 μg g^{-1} Zn. However, the maximum Zn concentration was reported in roots samples of *P. halepensis* (4358 μg g^{-1}) among the mentioned woody plants species. Moreover, Sun et al. (2009) reported Pb concentrations ranging from 1.5 to 20 mg kg^{-1} in pine needles of industrial areas in China. The Zn and Pb values measured in this study are in the ranges of those obtained by them.

Another study carried out in Turkey by Cicek and Koparal. (2004) reported metal concentrations up to 222 mg kg^{-1} for Zn, 55 mg kg^{-1} for Pb, and 7 mg kg^{-1} for Cd in needles of *Pinus nigra* J.F.Arnold grown in the polluted soils around a Thermal Power plant. Their Pb and Cd concentration data are comparatively higher than those detected in this study. Párraga-Aguado et al. (2013, 2014a and 2014b) reported 80 - 130 mg kg^{-1} Zn concentration in needles of *P. halepensis* growing in the mine tailings of Cartagena-La Union (Southern Spain), which is lower in comparison to the result found in the present study (see table 3).

Studies carried out by Conesa and Pàrraga-Aguado. (2019) on *P. halepensis* transplanted into compost amended pots show the BAC values are low for Zn and increased significantly for Cd in the aerial compartments (stems, branches, and needles) compared to the non-amended pots except Pb for barks. The BAC for Zn found in our study (<1) is consistent with their findings.

Table 3. Compilation of the Zn, Pb, and Cd concentration data in the Campo Pisano *Pinus halepensis* and other literature data of the same plant species; n.r. indicated not reported data; [a] indicate the mean values of all collected samples of each plant, regardless of its plant age and levels; [b] old needles; [c] young needles; number after ± is standard deviation.

samples		metal concentration in *Pinus halepensis* (mg kg^{-1}) (Campo Pisano study area)			metal concentration in *Pinus halepensis* (mg kg^{-1}) (Available Literature review)			References
		Zn	Pb	Cd	Zn	Pb	Cd	
Soil	Max	15299.52 ± 610.4	4413.29 ± 268.67	58.54 ± 1.51	9500	6800	47	Párraga-Aguado et al. (2013)
	Min	9043.26 ± 739.64	1604.47 ± 128.89	46.11 ± 11.38	4300	4600	23	
					8100 ± 650	5000 ± 490	<10	Párraga-Aguado et al. (2014a)
	Max				784	401	21.7	Cicek and Koparal (2004)
	Min				18	15	1.4	
Root	Max	2710.13 ± 139.10	735.88 ± 36.27	11.02 ± 0.58	n.r.	n.r.	n.r.	
	Min	664.65 ± 12.04	58.39 ± 7.45	4.86 ± 0.06				
Bark[a]	Max	224.50 ± 2.03	235.55 ± 2.33	3.58 ± 0.2	n.r.	n.r.	n.r.	
	Min	99.39 ± 5.65	39.22 ±10.44	1.64 ± 0.04				
Wood	Max	24.52 ± 1.32	4.39 ± 0.51	1.99 ± 0.05	127±10	67 ± 7.11	1.3 ± 0.14	Párraga-Aguado et al. (2014a)
	Min	7.22 ± 1.19	2.51 ± 0.46	0.58 ± 0.04				
Needle[a]	Max	156.27 ± 8.15	26.22 ± 3.64	1.22 ± 0.08	130	20	0.4	Párraga-Aguado et al. (2013)
	Min	68.36 ± 3.86	17.39 ± 2.37	0.48 ± 0.20	80	4	0.1	
	Max	219.14 ± 6.36[b] 156.38 ±1.81[c]	46.48 ±1.37[b] 38.68 ± 1.95[c]	1.66±0.06[b] 1.30±0.10[c]	200 100	50 10	n.r.	
	Min	73.69 ± 2.02[b] 43.45 ± 2.19[c]	18.78±0.80[b] 6.92 ±0.20[c]	0.50±0.0003[b] 0.37± 0.06[c]	153 ± 15[b] 79 ± 5[c]	13.7 ± 2.0[b] 8 ± 1.15[c]	0.16 ± 0.03[b] 0.15 ± 0.02[c]	
					127	12	0.38	Párraga-Aguado et al. (2014b)
	Max				222.4	55	7.23	Cicek and Koparal (2004)
	Min				107	0.1	0.1	

Table 4. Compilation of the literature Zn and Pb data for some investigated plant species in the Campo Pisano mine area

	Total Zn concentration					Zn Biological parameters			Total Pb concentration				Pb Biological parameters			
Plants species	Soil	Mobile	Root	Epigean		TF (leaves / roots)	BAC (leaves / soil)	BCF (root / soil)	Soil	Mobile	Root	Epigean	TF (leaves / roots)	BAC (leaves / soil)	BCF (root / soil)	References
Pistacia lentiscus	8436[a]	456	449	152[b]		0.3385	0.018	0.0532	1587[a]	34	29	10[b]	0.3448	0.006	0.0183	Concas et al. (2015)
	13780	1051	4000	515[c]		n.r.	0.0374		3391	550	1500	144[c]	n.r.	0.0425		Bacchetta et al. (2015)
	13893	1271		483		n.r.	0.0348		3711	696		104	n.r.	0.0280		
	15100	6300		124		n.r.	0.0082		71000	28000		402	n.r.	0.0057		
	10744[a]	1738		248[d]		n.r.	0.0231		2632[a]	210		30	n.r.	0.0114		Lai et al. (2015) Bacchetta et al. (2012)
Scrophularia canina subsp. *bicolor*	10520	1482				n.r.	n.r.		2426	234			n.r.	n.r.		Lai et al. (2015)
			190	600		n.r.	n.r.		n.r.	n.r.	20	68.3	n.r.	n.r.		Bacchetta et al. (2012)
Helichrysum microphyllum subsp. *tyrrhenicum*	24900	-	3290	3080		0.93	0.12	0.13	5000	-	680	1020	1.5	0.20.14	0.14	Lai et al. (2015) Boi et al.(2020)
Pinus halepensis	11588.05[a]	658.34[a]	810.14[a]	156.26[b]		0.19[a]	0.01 [a]	0.07[a]	2904.98 [a]	119.10[a]	58.39[a]	17.39[b]	0.30[a]	0.006[a]	0.02[a]	This study

All data are reported in mg kg^{-1}

n.r. indicated not reported data

[a] CP experimental plot with Compost

[b] in plant leaves

[c] in plants shoots

[d] Average of data in the period May 2008- May 2010

The data of this study can be complemented by those previous studies conducted by Bacchetta et al. (2012 and 2015), Lai et al. (2015) and Boi et al. (2020) reported on different plant species i.e. *Pistacia lentiscus*, *Helichrysum microphyllum* Cambess. subsp. *tyrrhenicum* Bacch., Brullo, and Giusso *and Scrophularia canina* subsp. *bicolor* growing in contaminated CP mine tailing site to determine Zn and Pb content (see Table 4).

The results show that Zn content in the leaves of *P. lentiscus* was similar to our study plant, and the corresponding BAC values were also similar. Conversely, Zn and Pb values in the roots of *P. halepensis* are twice higher than those reported for *P. lentiscus* by Concas et al. (2015). However, the BAC values are similar to those in this study and comparable to the calculated data for *H. microphyllum* subsp. *tyrrhenicum* by Lai et al. (2015) and Boi et al. (2020) in the CP experimental plot amended with compost. Moreover, their data reported a Zn and Pb concentration in roots and leaves much higher than those of our study, along with a higher Pb TF (1.5) and Zn TF (0.9) in *H. microphyllum* subsp. *tyrrhenicum* leaves. It must be stressed that the highest metal concentration assessed in plant tissues may be related to the extremely high Zn and Pb content of the soil (up to twice more than our soil data). Due to the higher Pb Translocation Factor (TF>1), *H. microphyllum* subsp. *tyrrhenicum* should behave as an accumulator for Pb as well as being considered as a plant species to be used in phytostabilization. Comparison with the data obtained by Bacchetta et al. (2012) and Lai et al. (2015), reveals that Zn and Pb are lower in roots but higher in leaves of *Scrophularia canina* subsp. *bicolor* than *P. halepensis*.

The calculated biological parameters (BAC, TF <1) reflect an approximation for describing the ability of plants to restrict the translocation of metals from roots to aerial plant parts. Therefore, due to the low capability to accumulate the investigated metals into the epigean organs, and according to what has already been reported by Brunetti et al. (2009) and Fellet et al. (2007), *P. halepensis* with TF< 1 can be considered as a pioneer species potentially suitable as an excluder plant for phytostabilization in a Zn, Pb, and Cd rich mine waste materials.

4.2 The mineralogical investigation

The XRD data in table 1 confirms the mineralogical composition of the soil sample that indicates the primary mineral of tailing, metal sulphide, and carbonate (i.e., pyrite, calcite, and dolomite) with quartz. The secondary minerals (i.e., Sulphate and iron oxy-hydroxides) such as jarosite-goethite are chemical compounds derived by geochemical and biogeochemical processes involving plants, roots, soil, and minerals. The detected minerals are the same as the previous mineralogical studies of the CP area performed by De Giudici et al. (2015). Jarosite is referred to as the iron sulphate formed during the pyrite dissolution process (Jerz and Rimstidt, 2003). Thus, the absence of pyrite and the presence of jarosite mainly on the soil around roots and the upper soil layers of S1 (20-43cm) and S3 (55-65cm) that were both taken from the amended plot may be due to the dissolution of pyrite on the root surface, the mineralization process induced by plants roots or could be due to any weathering process that may have occurred. The tolerance of pine species to high soil SO_4^{2-} concentrations has also been demonstrated by Renault et al. (1998), who found that *Pinus contorta* Douglas ex Loudon seedlings can grow in water solutions containing up to 3,000 mg L^{-1} of SO_4^{2-} (Pàrraga-Aguado et al., 2014c).

In the deeper layers of bulk soil (S2 and S3), smithsonite was detected. The Zn-bearing minerals were not detected by XRD in the bulk soil around roots samples. EDS analysis revealed the presence of Fe and Zn. Moreover, Zn bearing (bio)minerals were also observed in the other investigation carried on roots of *Pistacia lentiscus*, *Phragmites australis* (Cav.) Trin. ex Steud (De Giudici et al., 2015; Medas et al., 2017), *Euphorbia cupanii* Bertol. ex Moris and *Juncus acutus* L. (Medas et al., 2015 and 2017) grown on Campo Pisano area. Their presence, detectable only by using ultrasensitive techniques, was interpreted as a plant physiological survival strategy for limiting the bioavailability of Zn and other ions (Caldelas et al., 2017).

In addition, all collected root samples show the remarkable presence of dolomite and quartz as residual grains not completely dissolved by plant physiological activities which were also observed by Medas et al. (2015). Dolomite is present due to the discharge of alkaline materials (rich in dolomite) coming mostly from Monteponi mine, into the CP mine waste dump, which was done to reduce the acid mine drainage (Cidu et al., 2009; Bacchetta et al., 2015).

SEM investigation on root samples shows layers rich in iron oxide and iron sulphate with mineral grains coating on root surface constituted of barite, Fe, Zn, and Al silicate. In roots samples of *P. halepensis*, where soluble Ca sulphate minerals are abundant in soil due to the dissolution of pyrite and Ca carbonate minerals, plants can store Ca and Mg in crystalline phases to form dolomite which can also precipitate to form calcium oxalate (whewellite).

Ca was detected through SEM analysis in plant tissues (i.e., roots, barks, and needles) where calcium oxalate crystal (whewellite) formation is also abundant in roots as well as plant needles and barks. The role and the formation of Ca Ox crystal can be attributed to different physiological functions. Its formation responds to many plant species' physiological needs, where it plays the role of heavy metals detoxification mechanism (Franceschi and Nakata., 2005) or probably as plants protection/defense mechanism against herbivores in dried condition and/or it may alleviate the toxicity of Al and other metals in plants (Kopittke et al., 2017; Hodson et al., 1995). However, it should be noticed that the formation of calcium oxalate in our study particularly occurred in the post mort process of plants tissues once the plant samples were dried which can also be developed by plants tissue dehydration/ drying (i.e. barks) (see Figure 3c, point 5).

5. Conclusion

This study was carried out for identifying the phytoremediation capabilities of *P. halepensis* growing spontaneously in the heavily polluted mine tailing dump of Campo Pisano (CP) in South-Western Sardinia, Italy. Different soil and plant compartments samples were analyzed for detecting their metal contents. High contents of Zn, Pb, and Cd were found in *P. halepensis* roots samples concerning the bioavailable metals in soils.

The assessed metal concentration and calculated biological indexes show that *P. halepensis* is characterized by low metal translocation and consequently low accumulation into the plant epigean organs. Consequently, the plant proven capability to grow spontaneously in highly metal polluted mine sites and to colonize them, together with the low biological factors (BCF, TF, BAC <1) indicate that the pioneer woody plant species *P. halepensis* as an excluder plant with low Zn, Pb, and Cd accumulation and translocation capacity in the aerial parts, is potentially eligible for phytostabilization projects. Indeed, the limited metal translocation is a positive factor to minimize the chances of metal transfer to the trophic chain.

The results showed that the high concentration of Zn, Pb, and Cd detected into *P. halepensis* roots mirror the high metals contamination, which confirms the metal tolerance capability of *P. halepensis* growing spontaneously in metal-contaminated substrates. From the results, we may conclude that pyrite, calcite, dolomite, iron sulphate, iron oxide, and jarosite are present in the soil substrate around the roots as well as on the surface of plant roots. These sulphate and iron phases indicated that some minerals present in the material discharged in the mine waste, notable pyrite, undergo dissolution. It is not clear if the velocity of the pyrite dissolution reaction is fastened by rhizospheres processes. Further investigations will be beneficial for acquiring a better understanding of *P. halepensis'* adaptation and survival rate on polluted substrates as well as for profiting from its phytostabilization capabilities in similar Mediterranean mine tailing sites.

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