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TITLE OF THE Ph.D. THESIS

Investigation on Revegetation/ Phytoremediation of Campo Pisano abandoned mine area

Scientific Disciplinary Sectors

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List of publications

The aim of this PhD thesis is to provide the overall framework within which the following three research investigations were carried out. The findings of the first two research investigations have been published in international journals and the third one is currently under submission.

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

   Pegah Kharazian, Gianluigi Bacchetta, Giovanna Cappai, Martina Piredda and Giovanni De Giudici.

   Published at *"Plant biosystems". 2022, 1–14*. (August 8th, 2022) and is available at: https://doi.org/10.1080/11263504.2022.2100502.

2. *"**Pinus halepensis***** in contaminated mining sites: study of the transfer of metals in the plant-soil system using the BCR procedure**"

   Pegah Kharazian, Emilia Fernández-Ondoño, Maria Noelia Jiménez, Manuel Sierra Aragón, Antonio Aguirre-Arcos, Gianluigi Bacchetta, Giovanna Cappai and Giovanni De Giudici.

   Published at *"Toxics" 2022, 10, 12, 728* (November 26th, 2022) and is available at: https://doi.org/10.3390/toxics10120728

3. *"**Ex-situ***** investigation on phytoremediation capability of *****Pinus halepensis***** Mill. a pioneer tree in abandoned mine tailing**"

   Pegah Kharazian, Giovanna Cappai, Maria Enrica Boi, Marco Porceddu, Martina Piredda, Giovanni De Giudici, Gianluigi Bacchetta

   Under submission to the *"International Journal of Phytoremediation*"

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Cagliari, November 2022

Pegah

Abstract

The contaminated mine tailings rich in toxic metals pose serious environmental threats to the surrounding areas. The reclamation of these mine sites is an economically costly and technically complex process. Phytoremediation is one of the most promising techniques for sustainable, cost-effective and eco-friendly remediation of contaminated mine sites. This technique usually avails of the more suitable metal tolerant plant species among those that have best adjusted to the highly contaminated mine site and also well adapted to its climate without interfering with the floristic and vegetation biodiversity of the area. Furthermore, the phytoremediation capability of plant species can be increased through the application of proper soil amendments.

The PhD research moved from the investigation of botanical (identification of the suitable metal tolerant plant), environmental chemistry (evaluation of metal contamination) and geochemical/mineralogical characteristics (assessment of morphological features and mineral composition) in soil and plant. In this regard, *Pinus halepensis* Mill. was selected due to its special characteristics as a pioneer species growing spontaneously on Campo Pisano (CP) mine tailing, a multiple heavy metal contamination site in SW-Sardinia, Italy.

The aim of this PhD thesis is to assess the phytoremediation capability of *P. halepensis* growing spontaneously in Campo Pisano abandoned mine tailing and focuses on:

- Assessing the geochemical and the main mineralogical characteristics of the soil-plant system of *P. halepensis*, a native Sardinian tree species, through X-ray diffraction (XRD) and scanning electron microscopy (SEM) (chapter 4).
- Evaluating Zn, Pb and Cd concentration and its bioavailable fractions (DTPA-extraction) in CP-contaminated substrates (in-depth and around the roots) (chapter 4).
- Evaluating the metal accumulation and translocation behaviour in *P. halepensis* compartments (roots, barks, wood and needles) by calculating the Biological Accumulation Coefficient (BAC), the Translocation (TF) and the Biological Concentration Factors (BCF) (chapter 4).
- Assessing the geochemical forms of Zn, Pb, Cd and their bioavailability through the three-step BCR extraction method in relation to the Physico-chemical and mineralogical characteristics of the soil in-depth and the soil-root system (chapter 5).
- Evaluating the phytostabilization potential of *P. halepensis* seedlings planted under the controlled-greenhouse condition in four different substrates (reference substrate, CP mine tailings, mine tailings amended with compost, mine tailing with their pine and substrates) in order to evaluate:
    - the bioavailability of Zn, Pb and Cd and their accumulation in the roots and the aerial parts of the plant through the evaluation of biological parameters (BCF, BAC, TF).
    - the plant survival, elongation (root and epigean organs) and biomass production.

- the mitigation of metal stress in the plant by assessing the effectiveness of compost organic amendment (chapter 6).

The outcome of XRD and SEM analysis data showed the presence of pyrite, dolomite, calcite, quartz, gypsum, and barite with iron sulfate, and iron oxide in the polluted substrates. Zn ore minerals (smithsonite) and muscovite were mostly detected in the deeper soil layers. Iron sulfate was found mainly in the substrate around the roots as well as on the surface of plant roots. These sulfate and iron phases indicated the presence of some minerals that were discharged in the mine waste and the pyrite dissolution reaction. The chemical analysis showed that Zn was the most abundant metal in the substrate as well as plant tissues. The high Zn (up to 2710 mg kg^{-1}), Pb (735.88 mg kg^{-1}) and Cd (11.02 mg kg^{-1}) accumulation in the root of *P. halepensis* reflects the high metal contamination in soil. These analysis outcomes together with the low values of biological accumulation and translocation indexes (BCF, TF, BAC <1) for all plant tissues indicated 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 potentially eligible for long-term phytostabilization projects in abandoned mine tailing sites (chapter 4).

The results of BCR analysis data showed that the uppermost alkaline-calcareous layers of mine waste were affected by the rich carbonate lithology and mineralogy of CP, which were different from the deeper acidic layers. It also showed that Pb and Zn were less bioavailable for roots and were often found in high percentages in the residual fraction, mainly in deep layers of the compost-amended soil, while Cd was mainly found in the exchangeable BCR fraction. The presence of Zn and Pb ore minerals having low solubility, or the other beneficial effect of rhizosphere interaction processes may cause their resistance to leaching in BCR extraction. The bioavailability of all metals in the first fraction of BCR was positively correlated with the total metal contents in the soil around the plant root (chapter 5).

The outcome of greenhouse investigation declared the potential of *P. halepensis* to tolerate high metal concentrations, and its adaptation to grow and survive in highly contaminated substrates as well as its aptitude to limit metal accumulation and translocation to the aerial parts. It also showed that the addition of compost enhanced the soil organic carbon and nitrogen contents and restricted the metal bioavailability and metal accumulation in the aerial parts of *P. halepensis*, however, it didn't improve the plant survival and growth (chapter 6).

The general results of this PhD study clarified that *P. halepensis* 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 mine tailing sites.

Further investigation on the plant physiological adaptation is advisable to better trace the mineralization process and profit from the phytostabilization capabilities of *Pinus halepensis*. Moreover, additional investigation on the adaptation of *P. halepensis* in co-culture with other plant species as well as when is treated with apt soil amendments may enhance the phytostabilization capabilities of *P. halepensis* to the benefit of reclamation projects in similar mine tailing sites.

Thesis structure

The Thesis is articulated in seven chapters. The first three chapters frame the introduction. Chapters 4, 5 and 6 describe the sequence of progressive activities and in-depth insights gained through each trial. Chapter 7 offers a summary and conclusion of the three experimental activities. Figure 1 schemes out the chapters and structure of the present work.

Chapter 1 briefly summarized the core issues of heavy metals as soil contaminants and describes the main characteristics of the investigated metals (Zn, Pb and Cd) as well as the most important features of mine site remediation techniques. Moreover, it shortly describes the most frequently used soil remediation technologies focusing on the phytoremediation process.

Chapter 2 introduces the study area and presents the geographical, climatological, hydrological and vegetal features of the Campo Pisano mine sites (SW-Sardinia, Italy).

Chapter 3 reviews the available findings on the presence of *Pinus halepensis* Mill. in mine sites and the parameters affecting the phytoremediation capabilities of the plant considering co-culture with other plant species as well as the application of soil amendments in highly contaminated mine sites.

Chapter 4 presents the geochemical and mineralogical investigation of the soil-plant system of *P. halepensis* pioneer tree species growing spontaneously on Campo Pisano mine tailing. It reports further details about metal contents, metal accumulation and translocation on different plant compartments (roots, barks, wood and needles) at the field scale.

Chapter 5 provides a further investigation through the three-step BCR sequential extraction method to shed light on the geochemical forms of available metals for *P. halepensis* in mine tailing in relation to the Physico-chemical and mineralogical characteristics of the soil in depth and the soil-root system.

In Chapter 6 further attention is posed to the *Ex-situ* investigation of *P. halepensis* capability in the phytoremediation process.

Chapter 7 offers a summary of the three experimental activities carried out, concludes the highlighted results of the present work and offers some ideas for further investigation favouring effective phytoremediation and revegetation of similar contaminated mine sites.

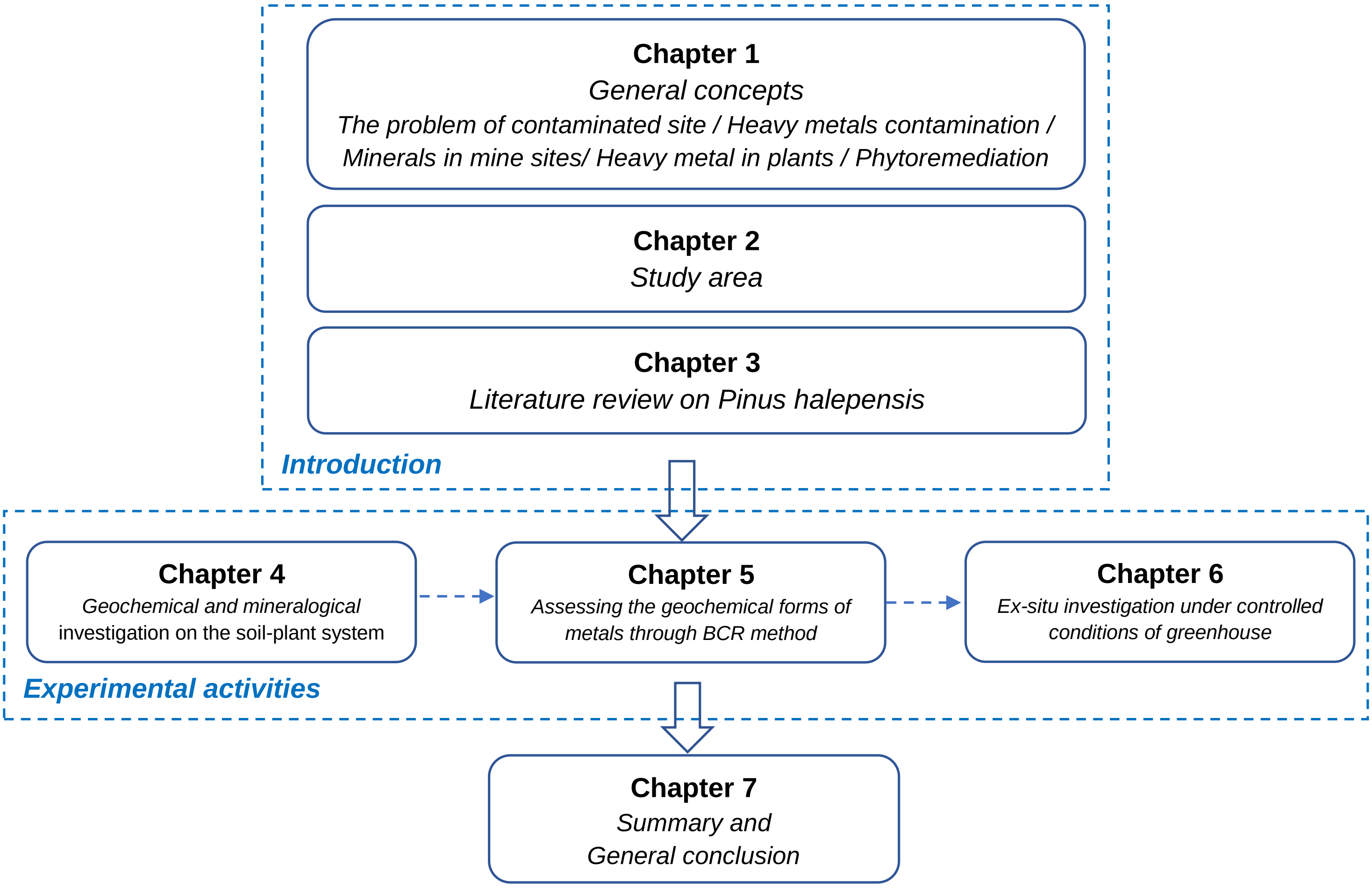


Figure 1. The Scheme of chapters and the structure of the thesis

CHAPTER 1

1. Introduction-General Concepts

1.1. Soil

Soil is a heterogeneous dynamic system involving the interaction among organic and inorganic materials, aqueous and gaseous phases and microorganisms (Yao et al., 2012). The organic materials of soil are derived from the remains materials of plants and animals, while the inorganic materials are formed by physical, chemical and microbiological weathering of bedrock over years. The formation of soil structures is highly dynamic and affected by many interaction factors including climate, geology, topography and biological organisms (Li et al., 2014).

1.1.1. Physical properties of soil

Physical properties of soil deal with soil components and their phases as solids, liquids, and gases. The main physical characteristics of soil are soil texture, soil water and soil structure. The soil texture refers to the soil particles, distribution and the relative percentage of sand, silt and clay particles present in the soil. Soil water fills in the pore spaces and can be affected by the balance between the gravitational water and capillary water forces. The structure of soil refers to the arrangement of soil particles to retain water and nutrient supply in the soil to the plant roots (Pani 2007).

1.1.2. Chemical properties of soil

The main chemical properties of soil are pH, Cation Exchange Capacity (CEC), organic matter, Electrical Conductivity (EC) and colloidal matter. Indeed, metal solubility and availability are strongly influenced by soil pH (Sheoran et al., 2016). Moreover, some metal cations i.e., Cd, Cu, Cr, Fe, Hg, Mn, Pb, and Zn are more soluble and bioavailable in soil solutions at low pH (Ma et al., 2016), in contrast, other anions i.e., As and Se exhibit high mobility at high pH (Kim et al., 2015). In addition, the mobilization and availability of metals in soil are affected by cation exchange capacity and organic matter content (Sheoran et al., 2016). For instance, organic matter is characterized by negatively charged sites that may stabilize metal cations through adsorption reactions, and also may mobilize metal anions by desorption reactions (Bakshi and Banik, 2018).

The main chemical properties of soil that affect the absorption or removal of Zn and Cd in the soil are summarized in table 1.1.

Table 1.1. Summary of the main characteristics of metals and factors that affect the absorption of Zn and Cd in soil (Mahmoudian, 2002)

The main characteristics of metal absorption		
Metals	**factors**	**The effect of factors**
Cd	Increasing pH	Increasing the surface absorption of Cd
	Increasing cation exchange capacity (CEC)	Increasing the surface absorption of Cd (in silicate layers)
	Increase in organic matter (OM)	Increasing the surface absorption of Cd
	Increase in $CaCO_3$	Increasing the surface absorption of Cd
	The competition of Ca, Co, Cr, Ni, Zn and Pb cations	prevent the surface absorption of Cd
Zn	Increasing pH	Increasing the surface absorption of Zn
	Increasing cation exchange capacity (CEC)	Increasing the surface absorption of Zn
	Increasing clay and soil organic matter (OM)	Increasing the surface absorption of Zn
	The competition of Ca^{2+} cations	prevents the surface absorption of Zn^{2+}
	Phosphate	Increases surface absorption of Zn and Fe/Mn-oxides colloids.

1.2. Soil contamination

Soil can be polluted by both organic and inorganic contaminants. The most prominent organic contaminants are petroleum hydrocarbons, polynuclear aromatic hydrocarbons (PAHs), polychlorinated biphenyl (PCBs), chlorinated aromatic compounds, detergents and pesticides. Inorganic contaminants include phosphates, nitrates and heavy metals (i.e. Cd, Cr and Pb), inorganic acid and radionuclides (radioactive substances). However, heavy metals seem to be more important than other contaminations (Guo et al., 2019).

1.2.1. Heavy metals in soil

Metals are an important group of soil contaminants, characterized mostly solid at room temperature, with a high density, a high melting point and a good conductance of electricity and heat. Heavy metals are chemical elements with the following common characteristics:

- atomic numbers of more than 20
- specific mass higher than 5 g/cm^3
- ability to form sulfides
- behave like cations and positively charged ions in an electromagnetic field
- low solubility of their hydrates
- tendency to form complex chemical bonds
- have different oxidation phases depending on the pH and EC conditions (Gaur and Adlholeya, 2004).

Figure 1.1 shows a systematic categorization of contaminants based on the organic and inorganic contents. In this regard, metals (Cd, Pb, Cu, Zn, As) and non-metals (Cyanides ammonium sulfur) are inorganic contaminants (Swartjes, 2011).

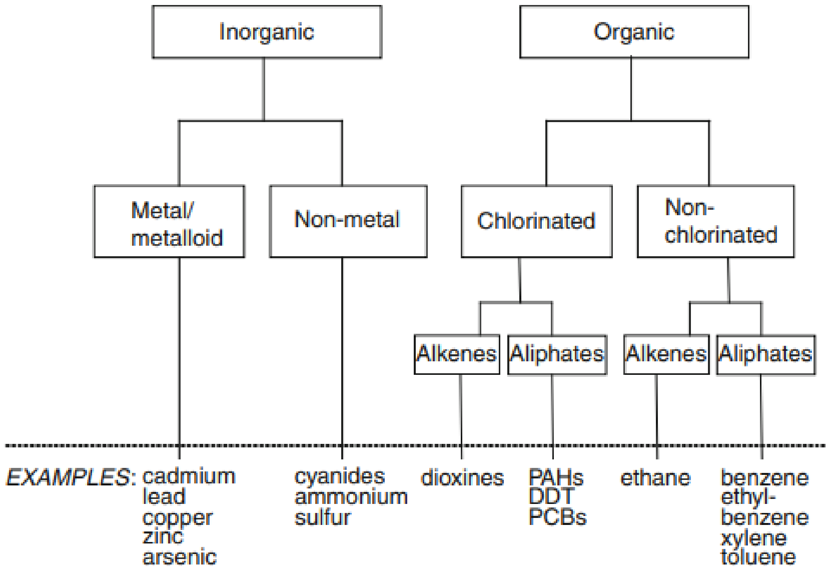


Figure 1.1. Systematic categorization of contaminants (Swartjes, 2011)

The most common heavy metals are Cd, Cr, Cu, Hg, Pb and Zn that have resulted from industrial activities, mining and smelting of metalliferous ores, electroplating, gas exhaust, energy and fuel production, fertilizer and pesticide application (Kabata-Pendias and Pendias,1989; Gaur and Adholeya. 2004). Metals can be originated from both natural and anthropogenic activities (Figure 1.2).

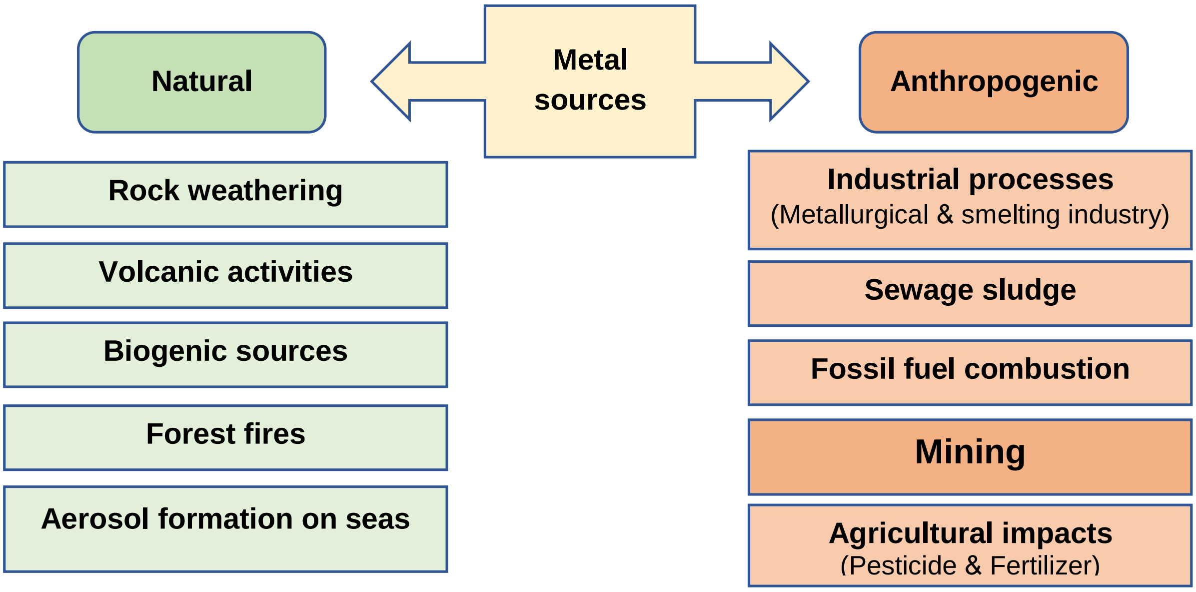
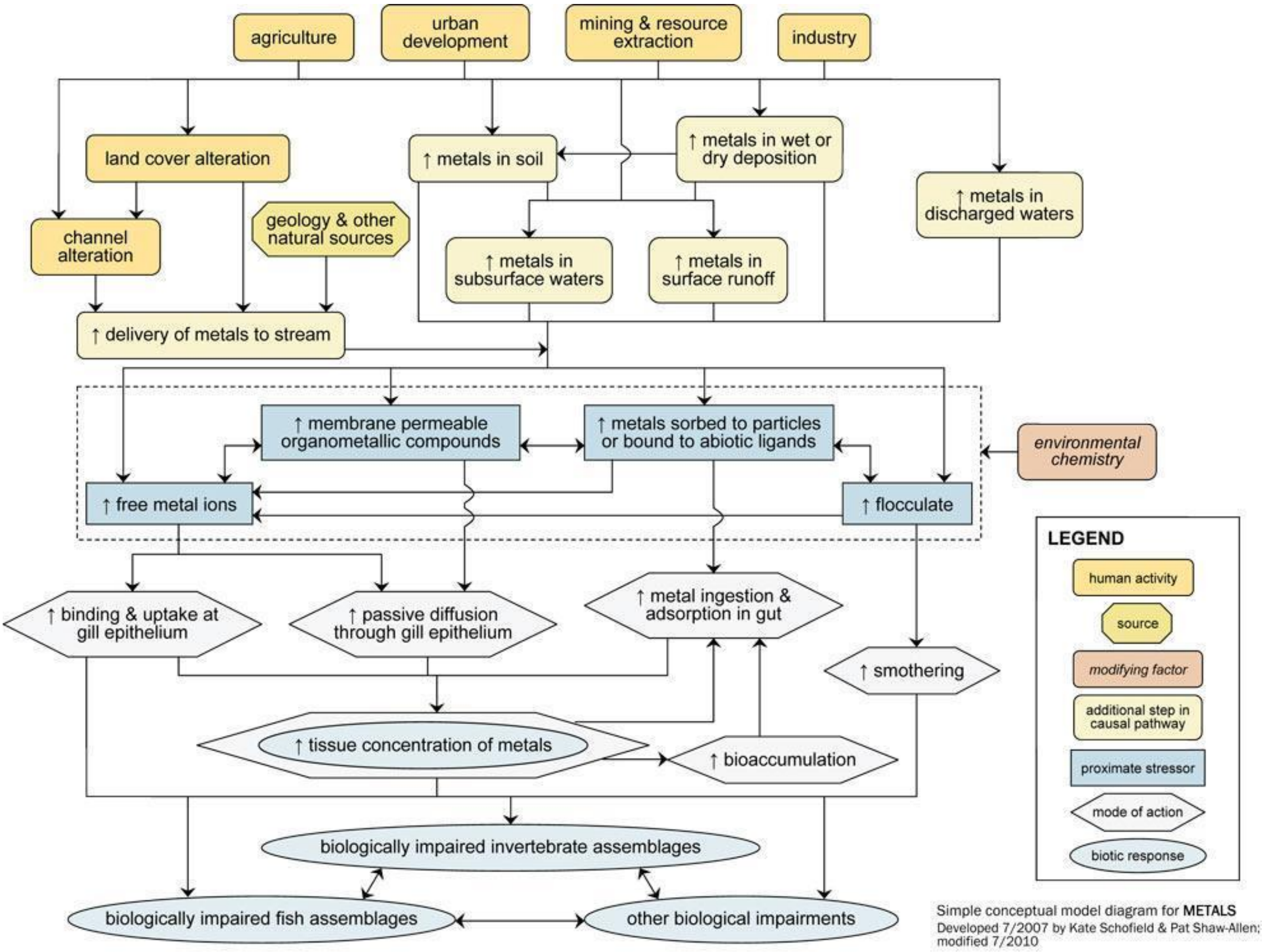


Figure 1.2. Source of metals in contaminated sites (Simiele et al, 2021)

The metal contamination can occur naturally from geological sources and rock weathering (Pal et al. 2010; He et al., 2015) or as a result of industrial, agricultural and mining activities (Figure 1.3).



Simple conceptual model diagram for **METALS**
Developed 7/2007 by Kate Schofield & Pat Shaw-Allen;
modified 7/2010

Figure 1.3. A simple conceptual model diagram for the source of metal contaminants (Kate Schofield and Pat Shaw-Allen, 2010)

Heavy metals derived from anthropogenic activities are mainly present as dissolved forms in soil solution adsorbed on insoluble organic substances or precipitated as pure or mixed solids. On the contrary, metals retained by primary and/or secondary minerals mainly derive from natural activities (i.e. metals from sedimentary rocks) and are more stable than anthropogenic-derived metals (McLean and Bledsoe, 1992; Karczewska, 1996). Metal toxicity depends on the total metal concentration and its availability to the organism (Kamal et al. 2010; Begum et al., 2019).

1.2.2. Metals in abandoned mine areas

The presence of heavy metals in abandoned mine waste is the outcome of natural processes of rock weathering and/ or metals release coming from anthropogenic activities, such as industrial discharge, mine tailings, disposal of wastes/ sludge and wastewater treatment (Lai et al., 2015; Prica et al., 2019). Mining activities have various environmental impacts and produce different wastes in each mineral processing stage, from minerals discovery, extraction and processing to mining closure and remediation of the site (Table 1.2).

Table 1.2. Stages of mineral processing and main related environmental impacts (Lai et al., 2015)

Stages	Process	Impact	Waste / Emission
Extraction	Removal of ore material from a deposit and activities prior to beneficiation	Destruction of natural habitats and landscape	Waste rock piles containing minerals associated with sulfide ores (chalcopyrite, pyrite, calcite, and dolomite)
		Erosion caused by the removal of vegetation	Wastewater from the excavation phase
		Influence on hydrology around the excavated area	Sediment run-off from mining sites. Acid mine drainage
		Soil, water, and air pollution	Wind dispersion of dust and greenhouse gas emissions
Beneficiation	Crushing, grinding, physical and chemical separation	Soil, water, and air pollution	Waste rock and tailings containing high concentrations of metals and minerals, and toxic chemicals
			Wastewater containing dissolved solids and reagents
			Wind dispersion of dust and greenhouse gas emissions
Processing	Smelting and refining of concentrates	Air pollution	Emission of Sulfur dioxide, arsenic, lead, cadmium, and other metals, dust
Closure and remediation	Residue disposal	Contamination of surface, groundwater, and air due to re-entrainment and/or subsequent deposition of particulates	Waste rock and tailings
			Acid mine drainage
			Leaching of pollutants from tailings
			Wind dispersion of dust from tailings

The mine tailings are the waste material of mine activities that remains after metals are extracted from the mineral ore. Tailings consist of ground rock, water and chemical reagents that remain after processing. The composition of mine tailings varies according to the mineralogy of the ore deposit and how the ore is processed.

1.3. The problem of contaminated sites

The problem of contaminated sites is an important controversial issue almost in all countries. Indeed, the contaminated sites pose significant environmental risks to human health, living organisms and ecosystems. Mining activities and mineral processing produce large volumes of waste rock and tailing which are left to weathering processes on site. These processes are subjected to heavy rains, strong winds storm, water runoff and significant soil and wind erosion (Barbafieri et al., 2011; Cao et al., 2004). Thus, large amounts of toxic metals are released into the mine wastes and the surrounding area of abandoned mine sites. Generally, in sulfidic mine wastes, the weathering processes and the oxidation of metal sulfide minerals cause the formation of sulfuric acid and increase the metal mobilization (USEPA. 1994; Lai et al., 2015). The leaching of metals and hazardous compounds and the mobility of soil contaminants (organic and/or inorganic compounds) through runoff and drainage consequently cause secondary sources of pollutants (Candeias et al., 2011). Moreover, this can seriously contaminate a wide range of soil, surface and groundwater even after the mine closure due to the long-term mineralization process in soil (Cao et al., 2008; Cidu et al., 2011). This phenomenon involving among hydrosphere, geosphere, lithosphere and biosphere harms soil, water, wildlife and human health (Li et al., 2014; Nkongolo et al., 2013) and represents an important worldwide environmental problem. (Cao et al., 2008; Bacchetta et al., 2015). Thus, the areas subjected to pollution urge remediation activities.

The number of industrial sites with high levels of contaminants in soil is steadily increasing as reported by the World Health Organization (WHO), the European Center for Environment and Health and the Italian health institute (Istituto Superiore di Sanità, Italy) (Fent, 2003). Even though mining activities are present in almost all countries and the inherent mine tailings may represent potentially high health and environmental risk, there is no comprehensive global register of mineral mine tailings sites. According to the International Resource Panel, IRP (2019) mining of metal ores has increased by 2.7% per year since the 1970s, reaching the current output of 17–20 billion tons /year. The World Tailings[1] Failure Organization estimated cumulative tailings deposits in the world from 1915 to 2019 and reported 29,000–35,000 existing active, inactive and abandoned tailings storage facilities contain approximately 223 billion tonnes (534 billion cubic meters) (IRP, 2019; Figure 1.4).

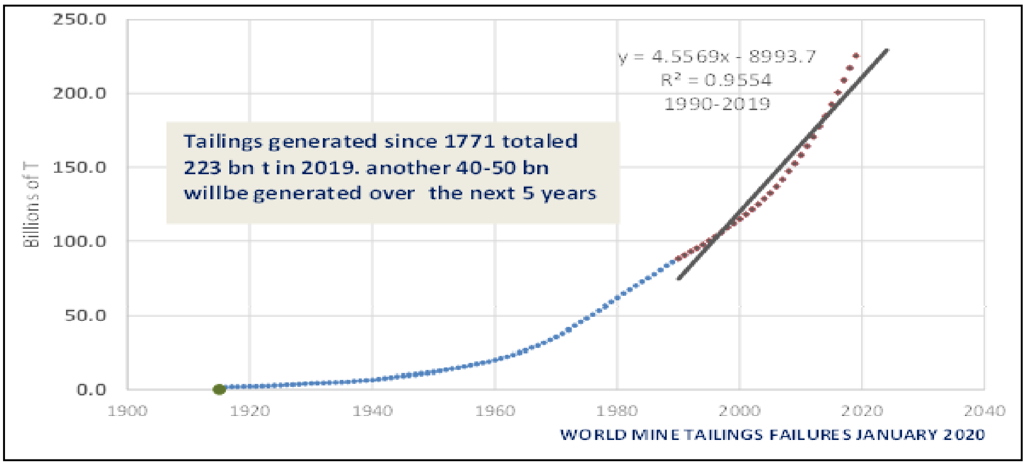


Figure 1.4. Cumulative tailings depositions from 1915–2019 (IRP, 2019)

1.3.1. Contaminated sites in Europe

The report of EU-Horizon (2020) estimated 750 Mt quarry of mine residues per year in 27 EU states[2] which is 30% of the whole waste production. It was noted that 0.3–0.6% of surface land is affected by mine waste disposal in Europe, 0.26% in the USA and 0.34% in Cina (EU-Horizon, 2020). The latest report of the European Environmental Agency (EEA) identified more than 2.5 million potentially contaminated sites (about 342000 sites) in its survey of 39 European countries (45%). Among all identified sites, about 60,000 have been remediated (mostly through “traditional” remediation techniques, in particular, soil excavation and disposal) and 120,000 sites are singled out as in need of certain urgent remediation activities (EEA, 2021). Industrial activities along with mining activities and mine waste tailings are frequently reported to be important sources of contamination. According to EEA. (2021), the total number of EU potentially contaminated sites that

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[1] The World Tailings Failure Organization, state of the world mine tailings portfolio. 2020. Available at: https://worldminetailingsfailures.org/.

[2] Recovery and auto production of base and precious metals are central to green and sustainable development (UE-Horizon 2020).

need remediation will be increased by almost up to 3 million sites by 2025. The reports from investigated European countries indicated that heavy metals (37.3%) are the most frequent soil contaminants in sites (Figure 1.5). According to the LUCAS survey, the concentrations of the ten most dangerous metal contaminants (Cd, Co, Cr, Cu, Hg, Ni, Pb, Sb and Zn) are above the thresholds level in the topsoil of investigated sites of EU countries (Tóth et al., 2016b).

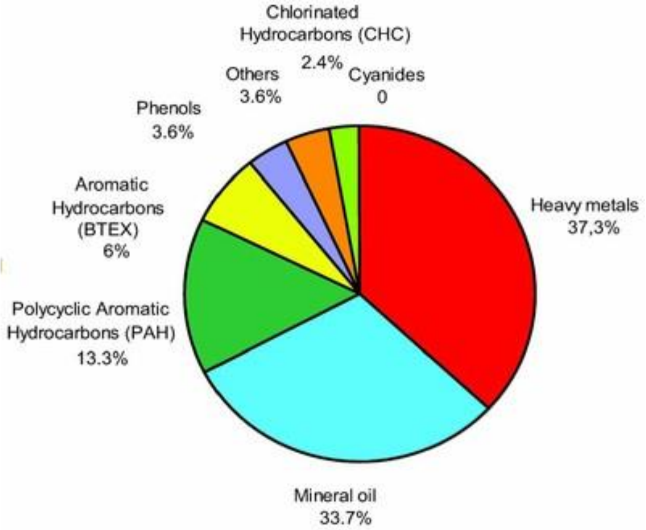


Figure 1.5. Overview of contaminants affecting soil and groundwater in Europe

1.3.2. Contaminated sites in Italy

Italy has a long mining history that begins in pre-Roman times. According to the Italian government agency ISPRA (Istituto Superiore per la Protezione e la Ricerca Ambientale) database, 3015 mines were active during the years between 1870 and 2018 (Figure 1.6). They reported 794 metal ores mines that were present mainly in the Alps, Tuscany, Calabria, and Sardinia. All of them are now abandoned, except 69 non-metallic ores (ISPRA, 2020). Figure 1.6 shows that most of the contaminated Sardinian mine waste storage sites are of metallic and industrial minerals.

The latest report of EU-Horizon (2020) estimated:

- **In Italy**: 50 contaminated sites of national interest (SIN) (spread over 316 towns and villages comprising a population of 7 million inhabitants); > 600 abandoned mine sites which is about 12000 sites potentially claiming for remediation.
- **In Sardinia**: 169 mine sites, 71 Mt of mine residues, heavy metals dispersions in water, soil and air and other industrial (SIN) of different kinds.

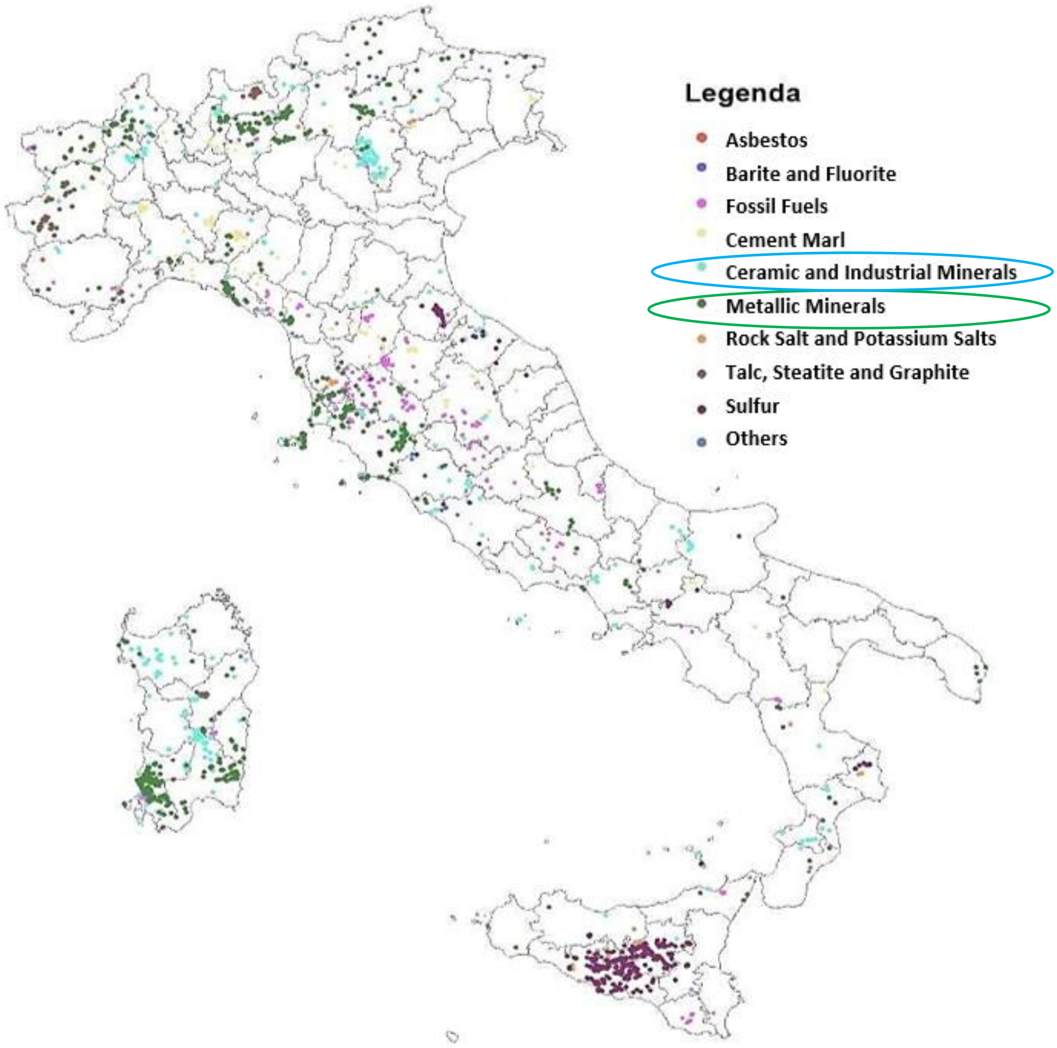


Figure 1.6. The active mining sites in Italy during the years 1870–2018 (ISPRA, 2020)

Under the Italian legislation, ISPRA has been tasked with upgrading every three years the health and environmental risk assessment of 650 mine waste storage facilities registered in 2017 (Table 1.3).

Table 1.3. The number of contaminated mine waste storages considering the health and environmental risks (medium, MH: medium-high and high) in Italian regions

	Ecological-sanitary risks				**Static-structural risks**				
Italian Region	Medium	MH	High	**Total**	M	MH	High	Total	**Total**
Sardinia	73	80	56	**209**					**209**
Lombardia	67	37	24	**128**					**128**
Toscana	46	21	13	**80**				0	**80**
Piemonte	25	21	11	**57**	7			7	**64**
Trento	19	16	0	**35**	4			4	**39**
Bolzano	4	8	0	**12**				0	**12**
Lazio	11	10	0	**21**					**21**
Liguria	13	6	0	**19**	5			5	**24**
Sicilia	19	3	1	**23**	1			1	**24**
Abruzzo	12	0	0	**12**					**12**
Veneto	9	2	2	**13**				0	**13**
Valle d'Aosta	6	4	0	**10**					**10**
Calabria	5	2	0	**7**	1			1	**8**
Friuli Venezia Giulia	0	0	1	**1**	2			2	**3**
Emilia Romagna	0	2	0	**2**				0	**2**
Molise	0	1	0	**1**					**1**
Total	307	213	108	**630**				20	**650**

According to the last available report of ISPRA, (2020), in Sardinia:

- 56 out of 108 mine waste storage facilities are classified as posing high health and ecological risk (52%).
- 80 out of 213 mine waste storage facilities are classified as posing medium-high health and ecological risk (36%) (Figure 1.7).

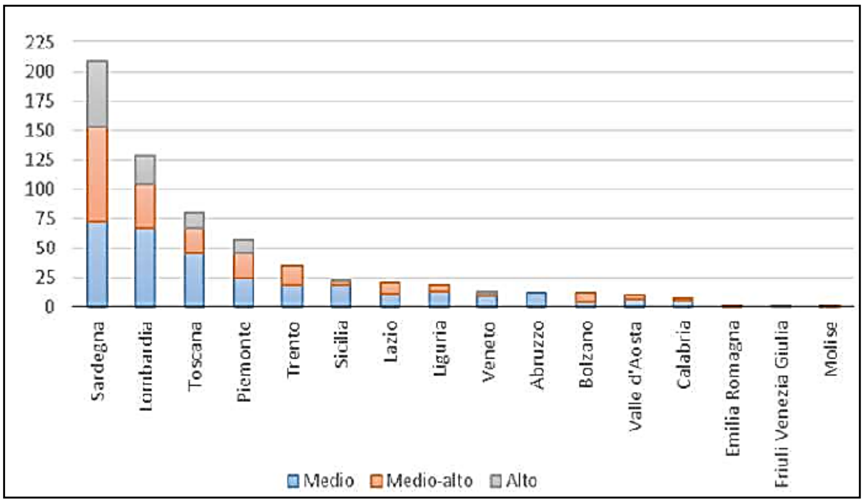


Figure 1.7. The distribution of mine wastes in different Italian regions in terms of health and ecological risks (medium, medium-high and high risk) (ISPRA, 2020)

1.4. The investigated metals of the study (Zn, Pb and Cd)

Zinc (Zn)

Zinc is an element widely discharged into the soil from many industrial, agricultural and mining activities (Liu et al., 2005). Zinc is present in different chemical production (i.e. plastic and pesticides), batteries, automobile equipment, pipes and household devices. Moreover, it is also spread in magmatic and sedimentary rocks and is usually extracted from mineral ores to form Zn-oxide and does not occur naturally in elemental form. Zinc is one of the most mobile metals as soluble compounds in neutral and acidic pH in aquatic environments. It can form carbonate and hydroxide complexes at higher pH values, precipitate in highly polluted soils and co-precipitate with Fe-oxides and Mn-Oxide (Evanko and Dzombak, 1997). The most common Zn minerals are sphalerite (ZnS), smithsonite ($ZnCO_3$), zincite (ZnO), willemite (Zn_2SiO_4) and hemimorphite ($Zn_4Si_2O_7(OH)_2\ H_2O$). Zn ores can contain other metals, such as Pb, Cu and Cd. Zinc is also common as free ions and complex ions in soil. Several factors, such as pH and EC, phosphorous and clay content can control its mobility in soils, (Kabata-Pendias and Krakowiak, 1995). There are two different mechanisms of Zn sorption:

- in acidic media, related to cation exchange sites
- in alkaline media, highly influenced by the organic compounds.

Moreover, Al, Fe and Mn-oxides and hydroxides are considered the most important binding of Zn in soils with the presence of goethite (FeOOH). In acidic soil, the adsorption of Zn^{2+} may result in the mobilization of Zn, whereas, in alkaline soil, organic compounds have an important role in the solubility of Zn. Indeed, Zn-organic complexes are very common in alkaline soil (pH>7).

Zinc is an essential micronutrient for plants and has an important role in different processes of plant metabolisms. It is a component of biosynthesis enzymes (dehydrogenases, proteinases, peptidases, and phosphor-hydrolases) and is involved in the metabolism of carbohydrates, proteins, and phosphates (Lindsay, 1972). The soluble form of Zn is quickly available to plants and its uptake is generally controlled by plant metabolism. Zinc is predominately adsorbed by roots as Zn^{2+}, hydrated Zn and Zn chelated to organic compounds. However, Zn at high concentrations may cause leaf chlorosis and induce a decrease in plant growth, root length and plant height, alteration in metabolism processes and induction of oxidative damage (Moore, 1972).

Lead (Pb)

Lead is a nonessential heavy metal for living organisms that is commonly discharged into the environment from mining and smelting, metalliferous ores, burning of leaded gasoline, municipal sewage and industrial wastes enriched in Pb and paints (Gispert et al. 2003). Lead can be found mainly as lead ore minerals and galena (PbS). Indeed, Pb was extracted from galena by roasting to $PbSO_4$ followed and reduction to Pb using $CaCO_3$ as a flux in mining sites (Nriagu, 1983). Moreover, Pb is present in the acid series of igneous rocks and argillaceous sediments (Kabata Pendias and Pendias, 2011). Lead can be found in the form of elemental lead, Pb-oxide, Pb-hydroxide and lead metal oxyanion complexes. Most of the released Pb into the environment remains in the soil. It can have various geochemical characteristics, but it is predominately associated with sulfur. Lead is commonly present in the environment as Pb^{2+} and Pb^{4+}. The main common mineral forms of Pb are galena (PbS), anglesite (PbSO4), cerussite ($PbCO_3$), minium (Pb_3O_4), pyromorphite ($Pb_5(PO_4)_3Cl$) and mimetesite ($Pb_5(AsO_4)_3Cl$).

It is a toxic element for plants and a carcinogenic compound for humans. The toxicity of Pb generally affects many photosynthesis processes of plants such as water absorption. (Kabata-Pendias and Pendias, 2011). For pH values in the range of 5–9, the speciation of Pb will be dominated by inorganic complexes especially Pb (OH), $PbHCO_3$, $PbCO_3$ and possibly $Pb(CO_3)_2^2$ and $PbHPO_4$. Lead will only be present as a free cation in acidic soils in the absence of organic complexes which may dominate at these low pH values (Cotter-Howells, 1991). The Absorption of Pb in plants will increase in the order: $Pb_3(PO_4)_2 < PbCO_3 < PbSO_4 < Pb(OOCH_3)_2 < PbC_{12} < Pb(NO_3)_2$. The toxicity of Pb causes chlorosis in plants, the reduction of plant growth and root lengths (Ghori et al., 2019). It can inhibit various enzymatic activities and reduce the water content and mineral nutrition in plants (Dubey et al., 2018).

Cadmium (Cd)

Cadmium is one of the most eco-toxic metals and one of the nonessential elements for living organisms (Zhao et al., 2003). It can be found in batteries, pigments, coatings and stabilizers in plastics production or as a result of fertilization with phosphates, mining and smelting activities.

Industrial sites and mine waste tailing dumps are the main sources of Cd pollutants. It is recovered as a byproduct from the mining of sulfide ores of Pb, Zn and Cu. Cadmium usually occurs in the form of CdS or $CdCO_3$ and is mobile in water systems as hydrated ions or as complexes as humic acid and other organic acids. It may form complexes with chloride and sulfate under acidic conditions. Cadmium can be removed from water by precipitation and sorption to mineral surfaces, especially oxide minerals at higher pH (pH >6) (Evanko and Dzombak, 1997). The most common Cd minerals are greenockite (CdS) and monteponite (CdO). Cadmium is often associated with Zn and Pb deposits and it is a host of some minerals i.e. biotite ($K(Mg,Fe)_3AlSi_3O_{10}(F,OH)_2$) , smithsonite ($ZnCO_3$) and sphalerite (ZnS). Indeed, Zn and Cd have similar behavior and ionic structure, with a common affinity to S atoms. Curlik and Forgac. (1996) reported some natural high levels of Cd in soil deriving from the lithogenic origin in alluvional soils derived from pyritized quartzes and from hydrothermally altered pyritized andezine and also like natural Cd anomalies. Cadmium is commonly present in the form of Cd^{2+} and its elementary form can be recognized in clay deposits with a strong affinity to sulfidric groups. This is common in metallothioneins and phytochelatins, which are proteins able to complex metals.

Braude et al. (1980) have reported that Cd can be absorbed by plants and other soil microorganisms and is more concentrated in the protein fraction of plants that is passively taken up by roots. Cadmium toxicity in plants inhibits the formation of anthocyanin and chlorophyll pigments and decreases cell reproduction. Cadmium toxicity in plants usually retards the growth and affects photosynthesis throughout the many different ways:

(i) Inhibits the enzyme activity of Fe^{3+} reductase that leads to Fe^{2+} deficiency
(ii) reduces the synthesis of chlorophyll
(iii) Inhibits the enzymes involved in CO_2 fixation
(iv) Interferes with uptake, transport and use of Ca, K, P, Mg and water
(v) Reduces the translocation and absorption of nitrate by inhibiting nitrate reductase (Tran and Popova, 2013; Huang et al., 2017).

1.5. The mobility and availability of metals

Generally, the toxic heavy metals present in the mine areas are free metal ions, soluble metal complexes, exchangeable metal ions, organically bound metals, precipitated compounds, such as oxides, sulfide, carbonates and hydroxides, or as constituents of silicate materials (Rathore et al., 2019). The mobility of heavy metals in soil depends on the geochemical form and speciation of the metal. That is hindered by reactions that cause metals to adsorb or precipitate or metal chemistry that tends to keep metals associated with its solid phase (Evanko and Dzombak, 1997). Metals in the soil can act in different ways according to the soil structures. The geochemical behaviour of heavy metals in soils can be described in terms of their distribution between these phases; (**i**) metal ions entering the soil solution e.g. from the chemical weathering of primary minerals may be adsorbed onto the surfaces of clay minerals, organic matter, Fe/Mn-oxides or (**ii**) precipitated to form an inorganic solid. These elements cannot be absorbed by plants in the forms of organic materials complex, but they may be released from binding with organic materials over time and can be less available for plants (Cotter-Howells, 1991).

Metals in soil are available and associated with several fractions:

- in soil solution, as free metal ions and soluble metal complexes
- adsorbed to inorganic soil constituents at ion exchange sites
- bound to soil organic matter
- precipitated as oxides, hydroxides, carbonates
- embedded in the structure of the silicate minerals.

Soil sequential extraction methods are employed to quantify metals associated with different fractions (Tessier et al., 1979).

As an example, Figure 1.8 shows the geochemical behaviour of Pb in soils in terms of the following phases (Cotter-Howells, 1991):

- soil solution
- surfaces of clay minerals and Fe/Mn-oxides
- organic matter
- solid (primary and secondary minerals)

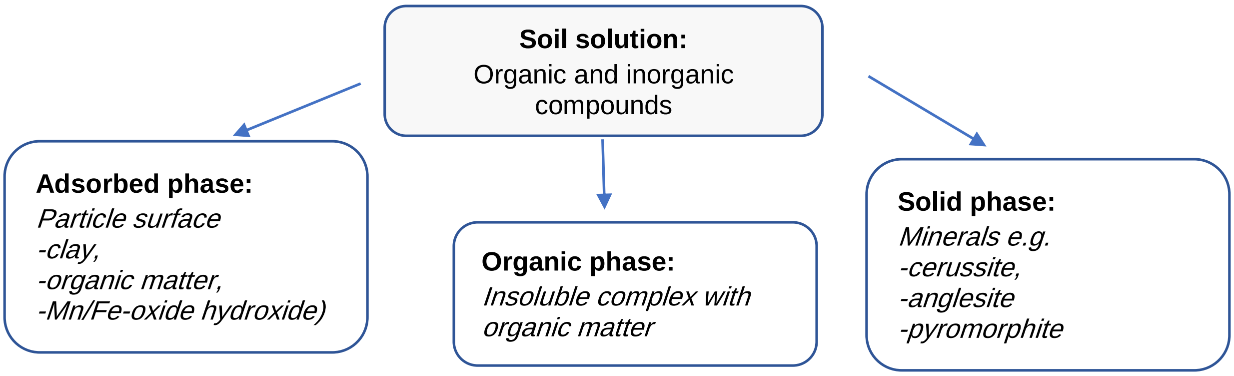


Figure 1.8. Geochemical behaviour of Pb in soil

Since metals can lose electrons, they easily form positive ions (cations) in soils and, hence, produce metallic bonds between metal atoms and non-metal ions. Examples of the latter often found in soils are salts of metals and anions, such as chloride (Cl^-) and carbonate (CO_3^{2-}). The main important heavy metals in soil are Cd, Cu, Pb, Zn, Hg and Se (Swartjes, 2011). Metals can mainly be absorbed by the clay materials or/and combined with the organic materials of the soil and form the complex of carbonate, phosphate, hydroxide and oxide in soil (Figure 1.9).

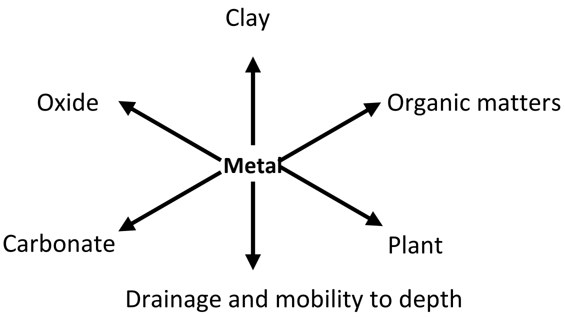


Figure 1.9. The overall view of metal interactions in soil

These soluble forms of metals can be absorbed by plants and can be mobilized into the deep soil layers as well as the underground water. There is an equilibrium state between the liquid and solid phases of the soil, which depends on the soil properties, such as EC, pH, etc. Some elements, such as B, Cu, Co, Mn, Zn, and Fe are essential for the growth and biochemical and metabolic activity of plants, when metal concentration is below the required toxic level for plants. These elements can be stored in different parts of the plants where their concentrations may exceed the limited dose in plants. Indeed, organic matter is characterized by negatively charged sites that may be involved in the adsorption and precipitation reactions of metal cations, as well as desorption and mobilization of metal anions (*e.g.*, arsenic anions) in soil (Bakshi and Banik, 2018) (Figure 1.10).

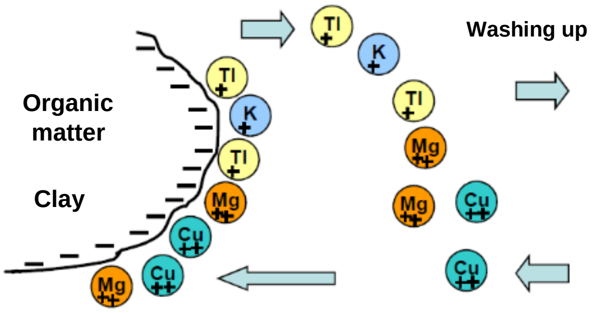


Figure 1.10. The overall interactions between metals and organic matters in soil (by author)

In addition, metals inhibit soil enzyme activity throughout the (**i**) catalytically active groups, (**ii**) denaturation of enzyme conformation and (**iii**) competition with enzyme-substrate (Sun et al., 2007). Moreover, enzymatic activities are indirectly affected by the soil microbial community induced by metals (Shun-hong et al., 2009).

1.6. Minerals and mineralogical conditions in mine sites

Mine wastes are complex poly-phasic systems comprising primary and secondary minerals, aggregates of the original ore deposit, mechanical disaggregating phase and anthropic added substances. The main minerals include sulfides, silicates, oxides, hydroxides, phosphates and carbonates. Generally, sulfidic materials are exposed to an oxidizing environment and together with a series of complex weathering reactions can make the mine waste materials chemically unstable.

The weathering of minerals (dissolution, hydrolysis, oxidation and hydration) processes can be classified as

- acid-producing (i.e., generation of H^+),
- acid buffering (i.e., consumption of H^+) or
- non-acid generation or consuming reaction (i.e., no generation or consumption of H^+).

For example, the degradation of pyrite (FeS_2) is an acid-production reaction, whereas the weathering of calcite ($CaCO_3$) is an acid-buffering process and the dissolution of quartz (SiO_2) does not consume or generate any acid. The balance of the chemical reactions, occurring within a particular mine waste will determine whether the material will produce acid solutions. Different minerals reacting with acidic solutions have different resistance to weathering.

Carbonate can rapidly be dissolved in the acid solution faster than pyrite. Carbonate minerals play an important role in acid-buffering reactions. Minerals such as calcite ($CaCO_3$) and dolomite [CaMg (CO_3)] neutralize acid generation from sulfide oxidation. Calcite is the most important neutralizing agent, because of its common occurrence in a wide range of geological environments and its rapid reaction compared to dolomite. The grain size, texture and the presence of elements in the carbonates may increase or decrease their resistance to weathering (Lottermoser, 2007).

Silicate minerals are abundant in sulfidic wastes and may suggest that waste with silicate has a significant buffering capacity. However, it may not be completely dissolved and the chemical weathering rate of silicate is very slow compared to the production of acid by pyrite oxidation. Silicate minerals are the most common gangue minerals in sulfidic wastes, but they may not dissolve completely and have a very low chemical weathering rate compared to pyrite oxidation.

Pyrite is the dominant metal sulfide phase in ore deposits and its dissolution is the most important process in the formation of acid solutions. However, other sulfide minerals, such as galena (PbS), sphalerite (ZnS) and chalcopyrite ($CuFeS_2$) commonly occur with pyrite evolution and can influence the chemistry of mine tailing site (Lottermoser, 2007).

The sulfide minerals are different in what it concerns acid–producing solutions, reactivity and dissolution products. Pyrite, marcasite (FeS_2), Pyrrhotite ($Fe_{1-X}S$) and mackinawite [$(Fe, Ni)_{1+X}S$)] appear to be the most reactive sulfides and their oxidation generates low pH waters. Other sulfides such as covellite (CuS), millerite (NiS), and galena (PbS) are generally far less reactive than pyrite. This is partly due to

- the greater stability of their crystal structure
- the lack of iron released
- the formation of low solubility minerals such as cerussite ($PbCO_3$) or anglesite ($PbSO_4$) which may encapsulate sulfide such as galena preventing further oxidation.

The appearance of Fe in sulfide minerals is important for sulfide oxidation and acid solution production. High Fe^{2+} sulfides containing mine waste (i.e., pyrite) or sulfides having Fe as a major constituent (i.e., chalcopyrite ($CuFeS_2$), Fe-rich sphalerite) generate significantly high acidity environment. Moreover, Fe^{2+} can be oxidized to Fe^{3+} and generate acid solution through hydrolysis. Hence, sulfide minerals without Fe in their structure (i.e., galena or iron-poor sphalerite) generate low acid solution (Lottermoser, 2007; Narula et al, 2012).

Secondary minerals can be grouped into sulfates, oxides, hydroxides, silicate and carbonates. The type of secondary minerals formed in mine wastes is controlled by the mine waste composition (Lottermoser, 2007). Some of the secondary sulfate and carbonate minerals are poorly soluble, such as barite ($BaSO_4$), anglesite ($PbSO_4$) and cerrusite ($PbCO_3$) that effectively will immobilize alkali earth elements as well as lead.

Secondary sulfate minerals may re-dissolve in water and release their ions back into the solution or may dehydrate to less hydrous or anhydrous compositions. The hydrous Fe^{2+} sulfate may oxidize to Fe^{2+}-Fe^{3+} or Fe^{3+} sulfate salts that are more stable and resistant to re-dissolution. The precipitation of minerals effectively immobilizes elements in acid mine waters and hence provide an important natural attenuation and detoxification mechanism in mine waste (Lottermoser, 2007).

Heavy metal-contaminated soils are often found as a result of oxygen introduction through mining activities. The mobilization of metals is due to sulfuric acid generation caused by oxidation of pyrite and other sulfidic minerals (e.g., ZnS) during mining operations, a process accelerated microbiologically (Banks et al., 1997; Kothe et al., 2005). The complex cycle of reactions during pyrite oxidation is described in a simplified manner with the following reactions (Banks et al., 1997):

1. $2FeS_2 + 2H_2O + 7O_2 \rightarrow 4Fe^{2+} + 4SO_4^{2-} + 4H^+$
   pyrite + water + oxygen ferrous iron + sulfate + acid
2. $4Fe^{2+} + 4H^+ + O^2 \rightarrow 4Fe^{3+} + 2H_2O$
   Ferrous iron + protons (acid) + oxygen ferric iron + water
   [Oxidation of ferrous iron]
3. $FeS_2 + 14\ Fe^{3+} + 8H_2O \rightarrow 15\ Fe^{2+} + 2\ SO_4^{2-} + 16\ H^+$
   pyrite + ferric iron + water ferrous iron + sulfate
   [Further pyrite Oxidation]

and/ or

4. $Fe^{3+} + 3H_2O \rightarrow Fe(OH)_3 + 3H^+$ [hydrolysis]
5. $4FeS_2 + 14H_2O + 15O_2 \rightarrow 4Fe(OH)_3 + 8SO_4^{2-} + 16H^+$
   Pyrite + water + oxygen ferric iron hydroxide + sulfate + acid

The oxidation of pyrite is accelerating the action of iron or sulfide oxidizing bacteria, e.g., *Acidithiobacillus ferrooxidans*, *Leptospirillium ferrooxidans* and *Ferroplasma acidiphilum* or *F. acidarmanus*. The resulting acid and sulfate production, as well as the release of heavy metals are characterizing acid mine drainage and acid rock drainage (Banks et al., 1997; Kothe et al., 2005).

1.7. The standard of soil quality

Soil pollution is associated with the presence of substances capable of inducing potential toxicity toward living organisms, such as humans, animals and plants. Some elements are essential for the growth and the biochemical and metabolic activities of plants at their concentration below the required toxic level. These elements can be toxic in different parts of plants and consequently enter the food cycle in case of their high concentrations exceed the limited dose. “Soil quality” expresses the status of the soil functional ability in the ecosystem, as indicated by its physical, chemical and biological properties (Doran and Zeiss, 2000). However, there are wide discrepancies in defining metal critical limits in soil among different countries and these limits also differ according to the type of soil (urban, agricultural or industrial soil) (Bakshi and Banik, 2018).

1.7.1. The standards of soil quality in the USA

The U.S. Environmental Protection Agency EPA (1996) defines the procedures of the soil quality standards for different contaminants. It set the Soil Screening Levels (SSLs) for various contaminants, defined as the concentrations in soil above which the human health is at risk. Since 2003, EPA have developed the Ecological Soil Screening Levels (Eco-SSLs), or the concentration of 17 metals and 7 organics for 24 contaminants in order to guarantee human health, plants and soil

(EPA, 1996). The Soil Screening Guidance is a tool that the U.S. Environmental Protection Agency (EPA) developed to help standardize and accelerate the evaluation and cleanup of contaminated soils for sites included in the National Priorities List (NPL) with the residential land use in future. This guidance provides a methodology to calculate risk-based, site-specific, soil screening levels (SSLs) for contaminants in soil (Tóth et al., 2016a). This indicator quantifies the progress in the management and remediation of contaminated soil and is very important for policymakers in soil management. In this guidance, "screening" refers to the process of identifying and defining areas, contaminants, and conditions, at a particular site that does not require further Federal attention. Generally, when the concentrations of contaminant fall below SSLs, no further action is warranted under the comprehensive environmental response and when the concentrations of contaminant are equal or exceed SSLs, further study or investigation is required, but not necessarily cleanup is warranted (Tóth et al., 2016a).

Figure 1.11 shows the spectrum of soil contamination and the ranges of risk management responses. The Soil Screening Levels (SSL) identify the lower bound of the spectrum levels below which EPA believes there is no concern under the comprehensive environmental response. Appropriate cleanup goals for a particular site may fall anywhere within this range depending on site-specific conditions.

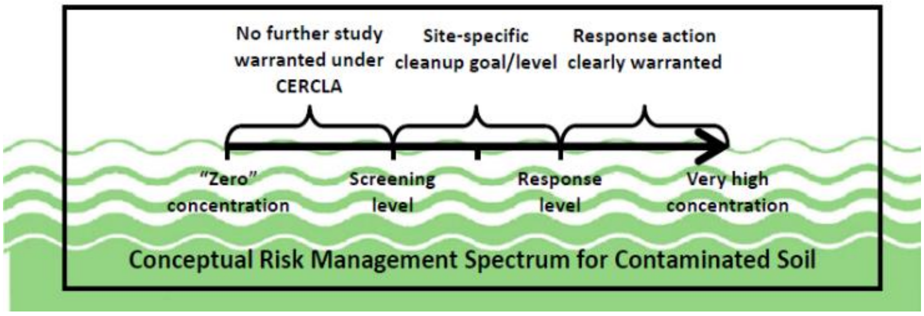


Figure 1.11. Soil screening guidance: the conceptual risk management spectrum for contaminated soil (EPA, 1997)

1.7.2. The standards of soil quality in Europe

European countries have a number of approaches to define the risk levels and the thresholds associated with different concentrations of heavy metal in soil. For example, the standards set in the Finnish legislation for contaminated soil were reported by the Ministry of the Environment Finland (MEF, 2007). The Finnish standard values represent an approximation of mean values for each hazardous element in different national systems in Europe (Awasthi, 2000) and they have been applied in an international context for agricultural soils as well (UNEP, 2013). The lower and higher concentration levels indicate the need for different remediation actions in contaminated soils. Higher concentration levels are defined for industrial and other land uses. The "threshold value" indicates the need for further assessment of the area. If the "guideline value" is exceeded, the area has a contamination level that presents ecological or health risks. Different guideline values are set for industrial (higher guideline value) and for all other land uses (lower guideline value) (Tóth et al., 2016a). With the aim to characterize the European soil contamination according to the Ministry of

Environment of Finland (MEF, 2007), the topsoil samples were classified by metal concentration values using the threshold and guideline standard values into four categories (Figure 1.12):

- Soil samples in the first category have no detectable content or the concentration is below the threshold value set by the MEF
- The concentration of the investigated element in the second category is above the threshold value, but below the lower guideline value
- The third category includes samples in which the concentration of one or more elements exceeds the lower guideline value but is below the higher guideline value
- the fourth category includes samples having concentrations above the higher guideline value.

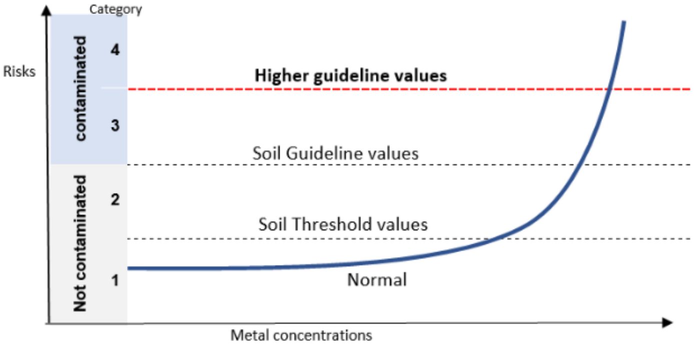


Figure 1.12. Diagram showing the four categories (1–4) for assessment of contaminated soil

Table 1.4 shows the threshold and guideline values for metals in soils based on the ecological risks (e) or health risks (t) according to the Ministry of Environment of Finland (MEF, 2007).

Table 1.4. The threshold and guideline values for metals in soils (MFF, 2007)

Substance (symbol)	Threshold value (mg kg^{-1})	Lower guideline value (mg kg^{-1})	Higher guideline value (mg kg^{-1})
Antimony (Sb)	2	10 (t)	50 (e)
Arsenic (As)	5	50 (e)	100 (e)
Mercury (Hg)	0.5	2 (e)	5 (e)
Cadmium (Cd)	1	10 (e)	20 (e)
Cobalt (Co)	20	100 (e)	250 (e)
Chrome (Cr)	100	200 (e)	300 (e)
Copper (Cu)	100	150 (e)	200 (e)
Lead (Pb)	60	200 (t)	750 (e)
Nickel (Ni)	50	100 (e)	150 (e)
Zinc (Zn)	200	250 (e)	400 (e)
Vanadium (V)	100	150 (e)	250 (e)

The threshold values of some metals are shown in Table 1.5 for residential use and in Table 1.7 for industrial use (Provoost et al, 2006).

Table 1.5. The threshold values of some heavy metals for residential land use in some countries (modified from Provoost et al, 2006)

Contaminant (mg kg^{-1} dm)	Belgium	Netherlands	Germany	France	Sweden	Norway	Great Britain + plant	Great Britain – plant	Canada	Switzerland	U.S.A.
Arsenic	110	55	50	37	15	2	20	20	12	N.A	22
Cadmium	6	12	20	20	0.4	3	8	30	10	20	37
Chromium III	300	380	400	130	120	25	130	200	64	N.A.	100000
Copper	400	190	N.A.	190	100	100	N.A.	N.A.	63	1000	3100
Mercury	15	10	20	7	1	1	8	8	6.6	N.A.	23
Lead	700	530	400	400	80	60	450	450	140	1000	400
Nickel	470	210	140	140	35	50	50	75	50	N.A.	1600
Zinc	1000	720	N.A.	9000	350	100	N.A.	N.A.	200	2000	23000

N.A.: Not applicable

Table 1.6. The threshold values of some heavy metals for industrial land use in some countries (modified from Provoost et al, 2006)

Contaminant (mg kg^{-1} dm)	Belgium	Germany	France	Sweden + ground water	Sweden - ground water	Great Britain	Canada	U.S.A.
Arsenic	300	140	120	15	40	500	12	260
Cadmium	30	60	60	1	12	1400	22	450
Chromium III	800	1000	7000	250	250	5000	87	100000
Copper	800	N.A.	950	200	200	N.A.	91	41000
Mercury	30	80	600	5	7	480	50	310
Lead	2500	2000	2000	300	300	750	600	750
Nickel	700	900	900	150	200	5000	50	20000
Zinc	3000	140	120	700	700	500	360	100000

N.A.: Not applicable

The concentration of some heavy metals in soil and plants are reported in Table 1.7 (Alloway, 1990).

Table 1.7. The concentration of heavy metals in soil and plants (Alloway, 1990)

elements	Soils (µg g^{-1} d.wt.)		Plants		
	Normal range (total)	Concentration (toxic)	Normal range (µg g^{-1} f.wt.)	Concentration (contaminated) (µg g^{-1})	Plant uptake (kg ha^{-1} yr)
Cd	0.01–7	3–8	0.2–0.8	5–30	N.D.
Ni	10–1000	100	0.02–5	10–100	N.D.
Cr	5–1000	75–1000	0.03–15	5–30	N.D.
Co	1–70	25–50	0.05–0.5	15–50	0.0006
Pb	2–100	100–400	0.1–10	30–300	N.D.
Cu	2–100	60–125	4–15	20–100	0.006
Zn	10–300	70−400	8–400	100–400	0.01
Mn	200–2000	1500–3000	15–1000	300–500	0.1
Sn	<5	50	0.2–6.8	60	0.001
Hg	0.02–0.2	0.3–5	0.005–0.5	1–3	N.D.

N.D., Not Detectable; d.wt. Dry weight; f.wt. Fresh weight

Toxic heavy metals, such as Pb, Cd, Hg, Ni, Zn and Cr are mainly present in mine areas with high concentration that often exceed the critical level. Normally, the toxic heavy metals present in mine areas are free metal ions, soluble metal complexes, exchangeable metal ions, organically bound metals, precipitated compounds such as oxides, sulfide, carbonates and hydroxides, or as constituents of silicate materials. Mine solid wastes are the result of mining/ ore extraction, hydrometallurgical and process-related factors influencing the distribution of elements in mining dumps, concentration tailings, effluent treatment residues, metal recovery sludge, smelter slags, smelter flue dust and smelter refining effluent treatment residues. Table 1.8 shows the critical concentration limit of some metals that may be toxic for plant survival (Rathore et al., 2019).

Table 1.8. The critical concentration of some heavy metals in plant (Rathore et al., 2019)

Heavy metal	Critical concentration in the plant (µg g^{-1})
Cd	5–10
Cr	1–2
Se	1–10
Ni	20–30
Hg	2–5
Pb	10–20
Cu	15–20
Zn	20–100

1.7.3. The standards of soil quality in Italy

In this thesis, the general criteria for safety measures emergency, remediation and environmental restoration and the acceptable limit for metal concentration in soil were assessed according to the Italian Legislative Decree no. 152/2006 established by the Italian Environmental Protection Policy set for contaminated soil and is in line with the EU Environmental Protection Directive (Guri, 2006). Table 1.9 shows the threshold concentration of contaminants (CSC - Concentrazione Soglia di Contaminazione) in Italian soils according to different uses in private and residential as well as industrial and commercial use. This is the limit above which the site is considered potentially contaminated. In particular, the potentially contaminated sites are those in which one or more concentration values of the polluting substances detected in the environmental matrices are above the CSCs.

Table 1.9. The threshold concentration of contamination in soil and subsoil reported the specific intended use of the sites to be reclaimed

Inorganic compounds	Sites for public green use, private and **residential** (mg kg^{-1})	Sites for commercial use and **Industrial** [mg kg^{-1})
Sb	10	30
As	20	50
Be	2	10
Cd	2	15
Co	20	250
Cr (total)	150	800
Cr (VI)	2	15
Hg	1	5
Ni	120	500
Pb	100	1000
Cu	120	600
Se	3	15
Tl	1	10
V	90	250
Zn	150	1500

If it is ascertained that the CSCs have been exceeded even for just one parameter, it is necessary to proceed with the detailed characterization of the site. Based on the results of the characterisation, the specific site risk analysis procedure is applied to the site for the determination of the risk threshold concentrations (CSR). In the event that the CSR are exceeded, it is necessary to proceed with reclamation or safety measures.

Furthermore, the Italian Legislative Decree n. 99/1992 and Decree n. 75/2010 defined maximum concentration values of heavy metals in sewage sludge and fertilizers for spreading on agricultural soils (see Table 1.10).

Table 1.10. The maximum concentration of metals in fertilizers and sewage sludge for agricultural soil application

	Maximum Allowed Value (mg kg^{-1})	
Total Metals	in **fertilizers** (D.Lgs 75/2010)	in **sewage sludge** for spreading on soils for agricultural (Legislative Decree 99/1992)
Cd	1.5	20
Ni	100	300
Pb	140	750
Cu	230	1000
Zn	500	2500
Hg	1.5	10
Cr (VI)	0.5	–

1.8. Heavy metals in plants

Some metals, such as Zn, Cu, Cr, Ni, Se and Fe are considered to be essential for plant growth and the physiological function of any living organisms at low concentrations, however, these metals may become toxic at high concentrations and their toxicity increases with increasing the total metal contents (Ghani, 2010). For example, some metals, i.e. Cu and Zn, are important enzyme activators, but others, such as As (metalloid), Cd and Hg are extremely poisonous. Table 1.11 shows metals that are classified as (**i**) nutritionally essential, (**ii**) nonessential with a possible beneficial effect, and (**iii**) nonessential with no beneficial effects (Goyer et al., 2004). Moreover, the nonessential elements are potentially toxic to the living organism even at low concentrations, while the essential ones can be toxic at high concentrations (mainly in the form of free ions, oxide and sulfide (Begum et al., 2019).

Table 1.11. Classification of metals based on the health impact characteristics (Goyer et al., 2004)

Nutritional essential metals	Nonessential metals	
	with possible beneficial effects	**with unknown beneficial effects**
Cobalt (Co)	Boron (Bo)	Aluminum (Al)
Chromium (Cr^{3+})	Nickel (Ni)	Antimony (Sb)
Copper (Cu)	Silicon (Si)	Arsenic (As)
Iron (Fe)	Vanadium (V)	Barium (Ba)
Manganese (Mg)		Beryllium (Be)
Molybdenum (Mo)		Cadmium (Cd)
Selenium (Se)		Lead (Pb)
Zinc (Zn)		Mercury (Hg)
		Silver (Ag)
		Strontium (Sr)
		Thallium (Tl)

The defense and tolerance mechanisms of plants can control metal uptake, accumulation and translocation (Mecwan Neha et al., 2018). Based on the type of defense mechanism some plants that are able to grow in the presence of metals are categorized as excluders and accumulators/ hyper-accumulators. Indeed, Co, Mg and Fe are relatively bioavailable metals, while Pb, Cr and Ur are not very bioavailable. Moreover, some metals, such as Cr and Pb can mainly accumulate in the roots (Ernst et al., 1992), Cu appears to remain confined to the root, while Zn and Cd seem to accumulate preferentially in chloroplasts (Van Assche and Clijster, 1990).

1.9. Remediation strategies in metal-contaminated sites

Several strategies have been proposed for the remediation of metal-contaminated soil including (**i**) physical, (**ii**) chemical and (**iii**) biological techniques (Figure 1.13) (Yao et al., 2012). Physical remediation is a simple technique that involves soil replacement and thermal desorption and gives the possibility to reuse the remedied soil (Bakshi and Banik, 2018). Physical and chemical methods: (**i**) require expensive devices, (**ii**) are time-consuming, (**iii**) need intensive labor and (**iv**) have negative environmental impacts (Bolan and Duraisamy, 2003; Guo et al., 2019). On the contrary, phytoremediation strategies are cost-efficient, non-destructive, easy to replicate and long-lasting sustainable processes (Tack and Van-decasteele, 2008; Bonanno, 2013; Angiolini et al., 2005; Tordoff et al., 2000; Wong, 2003). In the present study, attention is given to phytoremediation technology.

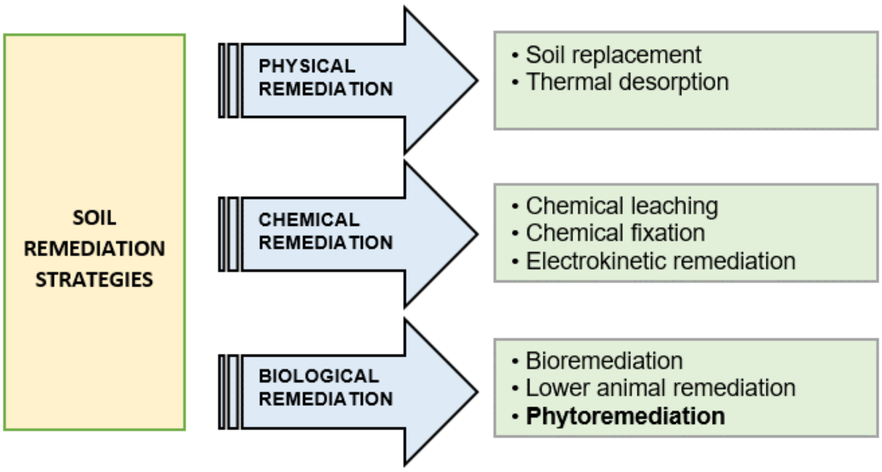


Figure 1.13. Remediation strategies for metal-contaminated soils (by author)

1.9.1. Phytoremediation technologies

The essential elements as well as available metals can be absorbed by the roots of plants. Rhizosphere is the zone of soil surrounding the root system of the plant that is influenced by the biological and chemical activity of the soil-root systems, bacteria and other microorganisms. (Kumar et al., 2007). The phytoremediation technique is based on some natural processes that are involved with plants, including (Bonomo and Sezenna, 2005):

- direct absorption of metals and some organic compounds;
- accumulation or transformation of the same chemical substances through lignification, metabolization, volatilization;
- use of enzymes released by plants to catalyze the degradation of metal-organic compounds;
- release of exudates into the rhizospheres that stimulate microbial activity for the degradation of contaminants.

The advantages of applying phytoremediation technique in contaminated soils are (Bonomo and Sezenna, 2005):

- lower costs compared to the other in-site applications;
- removal of low levels of contamination in very large areas;
- soil erosion control, infiltration and dust emissions control;
- potentially applicable in remote areas and contaminated sites with multiple or mixed contaminants.

The limitation of applying phytoremediation technique in contaminated soils are (Minello, 2010):

- the vegetation growth system;
- the ability of plant roots to penetrate the soil;
- the quantity of available land for the planting process;
- the high dependence on the pedo-climatic conditions.

Figure 1.14 shows different mechanisms of action on phytoremediation techniques as follows (Salt et al., 1998; Pulford and Watson, 2003):

Phytotransformation / Phytodegradation is an effective mechanism to decontaminate moderately contaminated soil. Indeed, the metal-organic complex may transform into simple molecules and accumulate into non-toxic catabolites in plant tissues. Moreover, plants can transform the organic contaminant through different enzymes and internal metabolic processes, degrade them into simple molecules and store them in vacuoles of plant tissues. Metal translocation from root to shoots is mainly controlled by two processes: root pressure and leaf transpiration.

Phytovolatization is the transformation of some metals and organic substances into volatile chemical forms that are released into the atmosphere (Burken and Schnoor, 1997; Banuelos et al., 1997). Metals complexes can be transformed into the plants roots through the rhizospheres. The modified form of contaminants can be translocated inside the plants and released into the atmosphere through transpiration. It allows pollutants to be removed from the contaminated site without concern about disposal and residual management of plants (Pilon and Smits, 2005).

Rhizofiltration is a technology that requires the absorption and translocation of contaminants in solution or the precipitation and the immobilization of metals at the root system. Metal hyperaccumulating plants with a particularly widespread root system are capable of absorbing and concentrating metals in their roots, as well as promoting precipitation (Dushenkov et al., 1995).

Phytoextraction is a suitable technique that some plants can remove a high concentration of pollutants from the soil and accumulate/ translocate within the aerial parts of plants (leaves and stem) (Kumar et al., 1995). This technique is mainly used for the decontamination of industrial discharges, agricultural runoff and acid mine drainage. In order to be suitable for phytoextraction, plants should be tolerant to high metal concentrations in soil, have a high growth rate, biomass production and an extended root system (Hernández-Allica et al., 2008; Favas et al., 2014; Lai et al., 2015).

Phytostabilization is using plants that tolerate high concentrations of metals and immobilize them at the soil-root interface. Plants can limit the transport process, increase the stability of metals and reduce the diffusion effect through root absorption, complexation, precipitation, metal reduction, humification or alteration in the rhizospheres of the chemical-physical characteristics of the substrate. The plants considered for these types of techniques should be tolerant to the soil pollutants, have a high growth rate, provide a dense ground cover and have an extended root system. Moreover, plants must concentrate contaminants as much as possible in roots in comparison to aerial parts (Marques et al., 2009). The stabilization of metals can occur:

- in the rhizospheres: the proteins produced by plants can be released into the rhizospheres through the roots system
- on the cell walls: the proteins directly associated with the root cell walls can bind and stabilize the contaminated outside the root cell
- in root cells: the proteins present on the root membranes can facilitate the transport of the contaminated inside the cell and vacuoles.

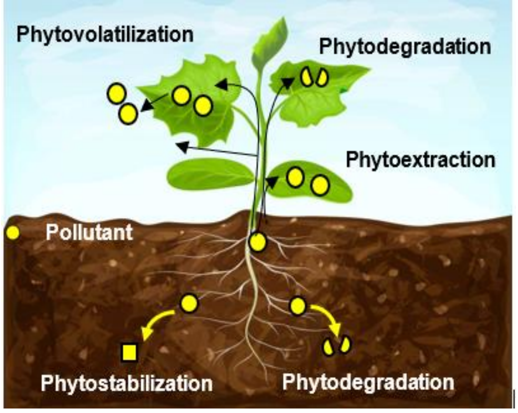


Figure 1.14. Different mechanisms of phytoremediation in plants (by author)

The adaptation strategies can be different depending on the plant species, thus, the plants that grow on polluted soils, according to their ability to absorb and transfer metals to the aerial parts, are divided into three groups:

- **Excluder plants** that restrict metals in their roots and have very low concentrations of metals in the aerial parts by limiting the metal translocation from the roots to the aboveground parts. This happens through different strategies, such as altering the permeability of the cell membrane, adaptations of the cell wall to ion transporter complex and release of chelating agents (Kabata Pendias et al., 2011) (Figure 1.15).

- **Accumulator plants** can actively accumulate metals in the plant tissues, reaching concentrations in their tissues higher than that in the surrounding soil. Metal accumulation depends on the physiological characteristics of plants, the synthesis of chelating molecules or storage in less sensitive parts of plants where metals are present (Zerbi and Marchiol, 2004) (Figure 1.15).

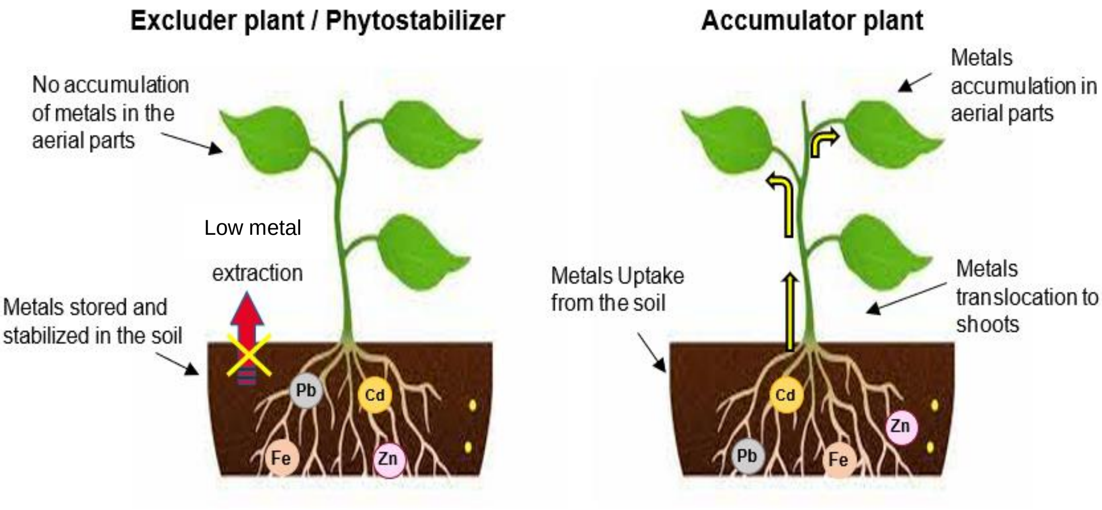


Figure 1.15. Excluder and hyper-accumulator plants (by author)

- **Indicator plants** present root absorption and translocation to the aerial parts that usually reflect the metal concentration in the surrounding soil. Indicators are species that tolerate high concentrations of metals by producing metal-binding compounds or storing metals in non-sensitive parts (Kabata Pendias et al., 2011), but may eventually die off as a result of continuously taking up metals.

Figure 1.16 shows the linear relationship between the concentration of metals in the soil and the aerial parts of plants for an indicator and accumulator plant species (Zerbi and Marchiol, 2004; Minello et al., 2010).

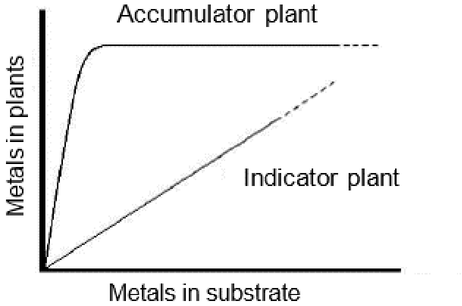


Figure 1.16. Accumulating and Indicator plant behaviour in the presence of metals (Zerbi e Marchiol, 2004)

1.9.2. Phytoremediation in mine-polluted sites

Mine waste contamination has led to the development of different remediation techniques, particularly phytoextraction and phytostabilization. In mine tailings sites, the phytostabilization process mainly exploits the capabilities of plants for reducing the mobility of contaminants and immobilizing and/or stabilizing metals in soil-root substrate through the release of chemical compounds by plant roots (Wong, 2003). This process can be improved through the application of soil amendments capable of inducing the formation of less soluble forms of metals (Barbafieri et al., 2011) and improving the essential elements (N, P, and other nutrients Cr, Cu, Zn) for plant growth (Lebrun et al., 2020). It should be noted that:

- mineralization in the presence of sulfides is negatively correlated to soil pH values (Pratas et al., 2005)
- high levels of sulfides, in particular pyrite (FeS) and arseno-pyrite that are easily weathered, favors the dissolution of toxic elements, allowing higher dispersion and bioavailability (Favas et al., 2014)
- the specific physiological functions of plants affect the chemical composition and plant biominerals (He et al., 2014).

1.10. Mechanisms of metals uptake in soil-plant system

One of the plant mechanisms responsible for metal immobilization into roots is binding metal ions to the extracellular proteins transmembrane (as transporter) in order to transfer into the cell membranes. The transmembrane connects extracellular and intracellular and facilitates the transformation of bound ions into the root cell. It should be noted that from the total amount of ions associated with the root, only the available part is absorbed into the root cells. Indeed, metal ions (i.e., Pb, Zn) will be physically adsorbed and bound to the negatively charged sites (COO^-) of the extracellular part of the cell wall in roots, but they cannot be transferred to the areal parts of plants (excluder). Thus, a significant metal accumulation can be stored and immobilized in the roots (Lasat.

1998). Metals can also be complexed and sequestered in cellular structures (i.e., vacuole) in order to restrict the transformation to the areal parts of plants. Moreover, some plants (excluders) can restrict metal uptake into the roots with special mechanisms (Lasat. 1998; Figure 1.17).

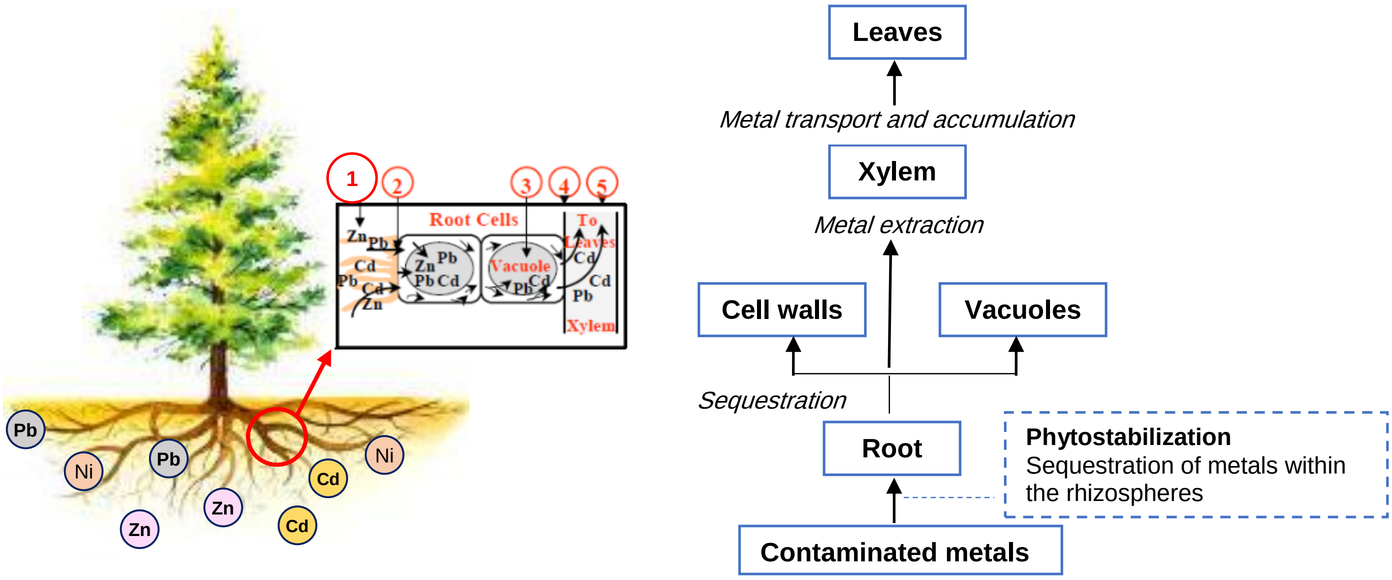


Figure 1.17. Metal uptake and accumulation in plants

1. Metal fraction at the root surface
2. Bioavailable metal moves across the cellular membrane into root cells
3. Metal fraction absorbed into roots and immobilized in the vacuole
4. Intracellular metal moves crosses the cell membranes into root vascular tissue (xylem)
5. Metal translocation from the root to the aerial parts of the plant (Lasat, 1998)

1.11. Factors affecting phytoremediation in mine-polluted sites

Several factors can affect the phytoremediation process in mine tailing sites. The specific Physico-chemical characteristics of abandoned mine wastes (*e.g. metal content, pH, cation exchange capacity, electrical conductivity, total carbon, nitrogen and organic carbon)* can influence plant growth (Prica et al., 2019). Moreover, other factors such as type and concentrations of metals, geochemical characteristics of soil and plant species selection can also affect the success of remediation process (Lai et al., 2015) (Figure 1.18).

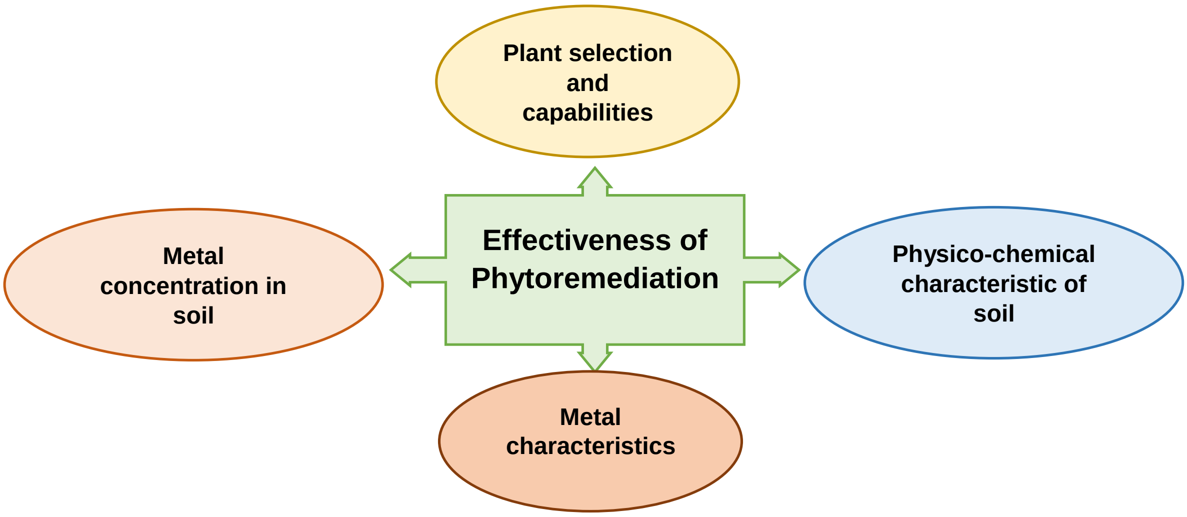


Figure 1.18. Factors that influence phytoremediation (by author)

The success of long-term phytoremediation techniques is highly dependent on the selection of the best-performing plant species (Jiménez et al., 2011). Moreover, the phytoremediation capabilities of metal-tolerant plant species mainly depend on the plant growth rate and the extended root systems (Conesa and Pàrraga-Aguado., 2019; Martínez-Oro et al., 2019) as well as the improvement of soil-plant interrelations/rhizospheres functions (Pàrraga-Aguado et al., 2014c). Generally, the best-performing plant species are the pioneer native ones that grow spontaneously on highly contaminated sites (Cao et al., 2009; Bacchetta et al., 2015; Lai et al., 2015; Barbafieri et al., 2011), adjusted to soil characteristics, well adapted to the local climate and soil conditions, better fit for enduring abiotic environmental stresses (Tamburini et al., 2014; Barbafieri et al., 2011; Favas et al., 2014) and do not interfere with floristic and biodiversity of the local environment (Concas et al., 2015; Cao et al., 2009). They can:

- directly prevent the spread and leaching of contaminants into the soil, sediments, sludge and water (Bacchetta et al., 2015); and
- indirectly limits soil erosion and improve soil properties (Lai et al., 2015; Barbafieri et al., 2011).

Several reasons promote the use of autochthonous or/and native plants (Cao et al., 2009; Bacchetta et al., 2012, 2015; Concas et al., 2015):

- they are adapted to local climate/adverse conditions and are strong in terms of growth, survival, and reproduction under environmental stress
- their use can preserve the plant diversity of the natural territories
- they help to start the rehabilitation of vegetational dynamics by the improvement of the physical-chemical properties of the substrates
- they help the establishment of a long-term plant canopy on mine wastes with relatively low-cost inputs and limited maintenance. All these points are pieces of a more holistic and sustainable approach to phytoremediation.

CHAPTER 2

2. Study area

2.1. Geographical, geological and climatological characters

The study area is the mine tailing of Campo Pisano (CP), located in the catchment basin of Rio San Giorgio near the town of Iglesias in the Sulcis-Iglesiente region (South-Western Sardinia, Italy) (Figure 2.1 and Figure 2.2). The Sulcis-Iglesiente 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). The climate of the area is temperate during the year with an 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). The dominant wind is from NW (Mistral wind) and SW (sirocco), especially during wintertime is one of the most important morphogenetic agents of the area.

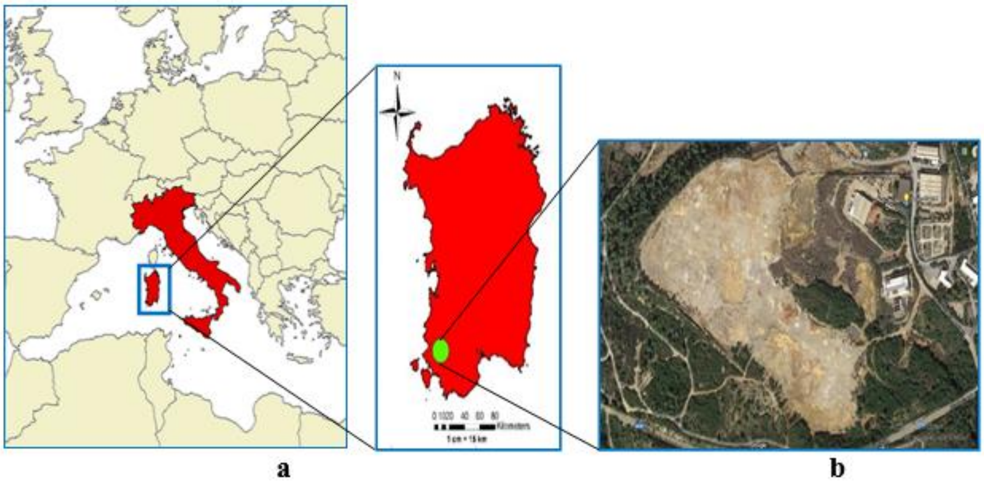


Figure 2.1. Geo-location maps of the study area; (**a**) Location of Sardinia in the Mediterranean Basin (the green spot indicates the sampling site); (**b**) Campo Pisano tailing dump; Geological and ore geological characters

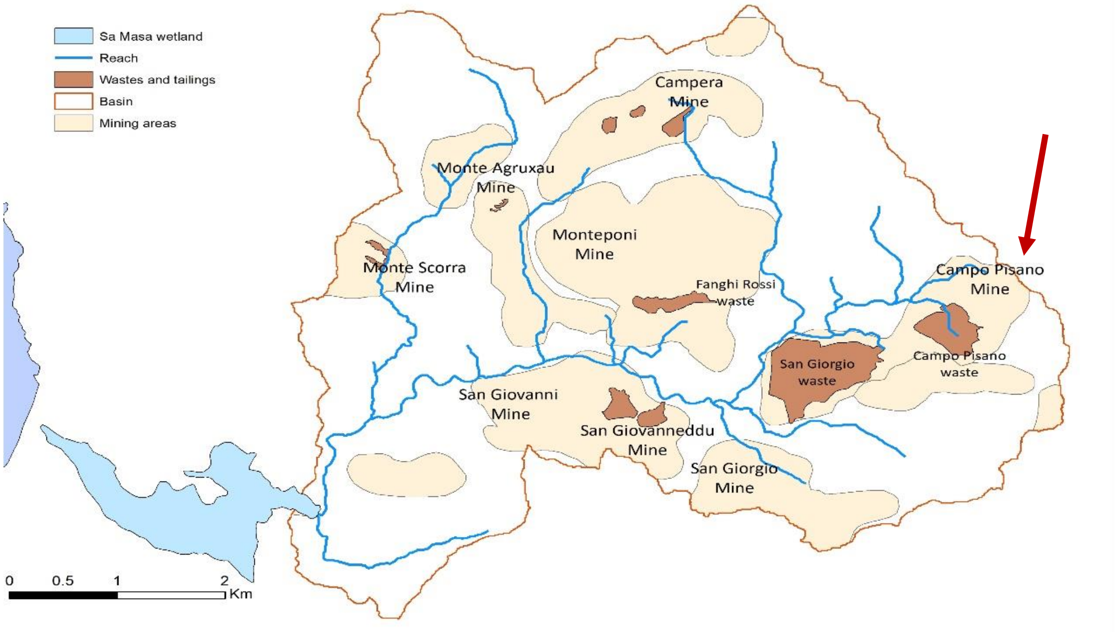


Figure 2.2. Location map of the study area around the catchment basin of Rio San Giorgio, Sulcis-Iglesiente (SW-Sardinia, Italy; Marras, 2020)

The Iglesiente region has a complex geological structure with its earliest formations that date back to the Paleozoic era with the main lithologies ascribable to carbonate rocks (Boni et al., 2003) (Figure 2.3).

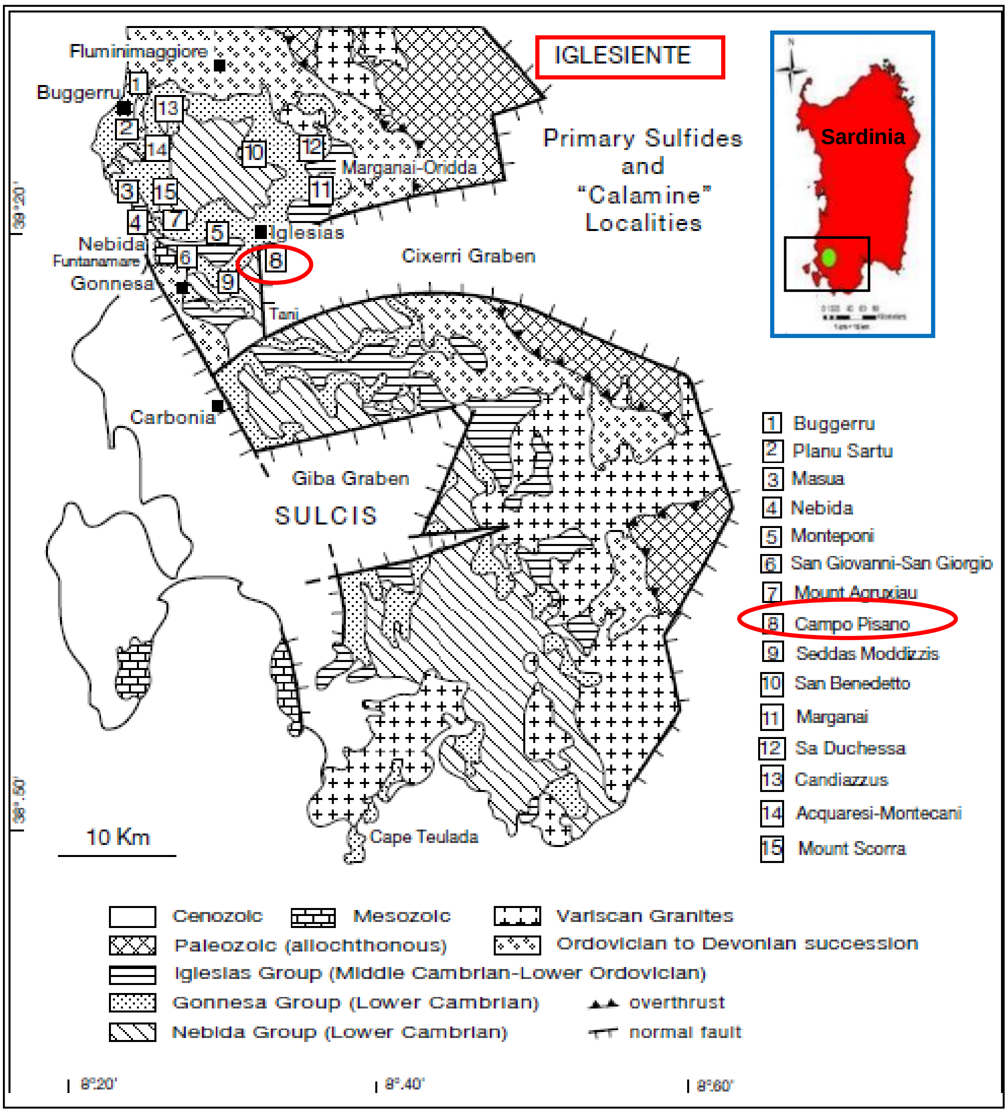


Figure 2.3. Geologic map of southwest Sardinia with the location of the "Calamine" ore bodies; the calamine at Campo Pisano is derived from sedex sulfides (Boni et al, 2003).

The succession of geological description, from the oldest to the most recent reported as follows (IGEA SPA, 2012):

- *Nebida formation*

The Nebila formation is a well-stratified complex made up mainly of subordinate carbonate. The lower layer or "Matoppa consists of clay, siltstone and sandstone. The upper layer or "Punta Manna" is composed of a regular alternation of quartz sandstones with clay-siliceous cement. The top layer is characterized by intercalations of limestone.

- *Gonnesa formation*

It is divided into three parts: striped dolomite, gray dolomite and limestone consisting of Santa Barbara Formation and San Giovanni Formation. The first Formation is made of layered marble and the second one is massive grey marble. Generally, it is composed of fine-grained, non-stratified equigranular rocks.

- *Cabitza formation*

Cabitza Formation is composed of two components in stratigraphic continuity: the nodule clay-calcareous limestone and the Cabitza argilloscists. The nodule limestones are mainly composed of calcite and are characterized by a lenticel and thin bed texture with a thickness varying from a few millimeters to centimeters; the limestone elements are separated by thin siricit-clay.

The Campo Pisano area stands between the lithological formations of the metalliferous and the shales from geological formations that date back to the Paleozoic, from schist, clay-schist and siltstones up to dolomites, limestone dolomites and gray dolomites interspersed with limestone nodule.

The formation of the ore deposits is related to the geological events in Sardinia. These deposits are the results of seven metallogenetic periods from the Cambrian to the Quaternary subjected to different minerogenesis processes. Three sequences are recognized and listed from the most ancient to recent in the following:

- the deposition from sin-sedimentary to epigenetic made of Pb, Zn, Fe and Ag sulfides linked to the Cambrian carbonate platform
- the deposition of sulfides mixed with Fe-oxides linked to the Ordovician's volcanic activity (Upper Ordovician-Silurian)
- the mineralization connected to the leucogranitic plutons formed during the Ercinic Orogeny (Carboniferous-Permian) (IGEA SPA, 2012).

The most abundant mineralizations are present into the Gonnesa formation as Metaliferous ring and are mainly consist of galena, pyrite and barite associated to other metals, such as Cd, As, Hg, Co (Bechstädt and Boni, 1994; Boni et al., 1999). Moreover, the Zn-Pb mineralizations are recognizable in the Cabitza Formation, Monte Argentu and in Portixeddu ones. During the postercinic period, the ores were subjected to two phenomena that resulted in the special characteristic of this mineralization:

- variations in the water table which modified the redox conditions (Boni et al., 2003)
- karst which opens several cavities where rainfall modified the chemistry of the original mineralization (Padalino et al., 1972). The Karst processes mobilized metals that is already presented and concentrated in the same rocks, forming columnar bodies of remarkable dimensions.

2.2. Hydrogeological characters

Campo Pisano area presents the following hydrogeological complexes starting with the oldest:

- The under terrigenous complex

Identified with the Nebida formation of the lower Cambric. The permeability of this complex is very limited due to the low porosity in CP soil texture. The limited circulation of water depends on the presence of carbonate interlayers that only in a few cases flow rates reach higher than one litre per second. The low infiltration index of the subsoil water favors the surface runoff, which can assume a variable torrential character flows according to rainfall.

- The ancient carbonate complex

Represents the main aquifer that is characterized by the presence of lithotypes typical of metalliferous, striped dolomite, gray dolomite and limestone with limited permeability by cracking in the basal part. The permeability tends to increase towards limestone layers due to their high fracturing and karst indexes. The underground water circulation is, in this case, closely linked to the size of the fractures and their state of clogging. In fact, runoff is a phenomenon that occurs episodically and for a limited time. It happens on the occasion of important meteoric events during which the percentage of receptivity of the rocks, which is always considerable, is exceeded. Furthermore, since the Campo Pisano hydrogeological watersheds do not coincide with its hydrographic watersheds, the presence of carbonate hydro structures that extend into neighbouring hydrographic basins allows possible water exchanges between them.

2.3. Vegetation cover characters

The vegetation in the Sulcis-Iglesiente district (SW-Sardinia) includes several endemic or rare species that have been confined to such habitats by millennial mining activity. The extreme edaphic specialization of these species reflects with the unusual heterogeneity of substrata, therefore creating a system of plant communities that largely contributes to the environmental diversity of this part of the island (Angiolini et al., 2005). The area hosts some exclusive taxa that are the result of the local evolution under the selective pressure of edaphic conditions. The mining dumps of the area are covered by several endemic or rare species mainly shrublands and woodlands where the interspecific competition disfavours the pioneer species of the *Ptilostemono casabonae-Euphorbion cupanii* and subordinated syntaxa.

The phytosociological study of Sulcis-Iglesiente distinct presented by Angiolini et al., 2005 recognized eight associations, distinguished by the cluster analysis in two main vegetation groups including:

a) hemicrypto-chamaephytic vegetation

Six associations are belonging to this group described as:

1. *Helichryso tyrrhenici- Dianthetum sardoi* ass. nova occurring on gross-grained, hard-sloping dumps deriving from the geologic unit of Gonnesa;
2. *Coincyo recurvatae- Helichrysetum tyrrhenici* ass. nova colonizing dumps made of metamorphitic flakes and finer particles;
3. *Resedo luteolae-Limonietum merxmuelleri* ass. nova colonizing small-grained mining dumps with high concentrations of sulfides and heavy metals;

4. *Ptilostemono casabonae-Iberidetum integerrimae* ass. nova replacing the previous one on older and more consolidated dumps, where the concentration of phytotoxic elements was reduced by the meteoric agents;
5. *Epipactidetum tremolsii* ass. nova colonizing muddy deposits originating from ferrous clay; Euphorbio cupanii-Santolinetum insularis Angiolini and Bacchetta (2003), occurring on very old, stabilized dumps. All these associations can be ascribed to the class *Scrophulario-Helichrysetea* Brullo, Scelsi and Spampinato (1998).
6. Within this class, a new Sardo-Corsican alliance, *Ptilostemono casabonae-Euphorbion cupanii* all. nov. is proposed, differentiated by a pool of rare or endemic species.

Limonium merxmuelleri is likely to be a neoendemic closely related to *Limonium sulcitanum* ARRIGONI, that probably reached the mining districts from the coastal shipping terminals and further evolution was triggered by the edaphic conditions of mining dumps. *Iberis integerrima*, *Dianthus sardous*, *Coincya monensis* subsp. *Recurvate* and *Anagallis monellii* still have their primary habitats in rocky sites, where they take part to pioneer communities belonging to the classes Asplenietea trichomanis (BR.-BL. in MEIER and BR.-BL. 1934) Oberdorfer 1977 and Carici- Genistetea lobelia KLEIN 1972. *Santolina corsica*, *Helichrysum microphyllum* subsp. tyrrhenicum, *Ptilostemon casabonae*, *Echium anchusoides* and *Euphorbia cupanii* are mainly spread on mining dumps and roadsides.

b) nanophanerophytic garigues vegetation

Two other associations have been recognized as:

- *Dorycnio suffruticosi-Genistetum corsicae* ass. nova, found on abandoned, well-consolidated mining dumps, with an upper layer altered by pedogenetic processes;
- *Polygalo sardoae-Linetum muelleri* ass. nova, occurring in the vicinities of the mines on steep rocky slopes made of Paleozoic metalliferous limestones.

Both of them can be ascribed to the alliance Teucrion mari GAMISANS and MURACCIOLE 1985, grouping the Sardo-Corsican aspects of Cisto-Lavanduletea BR.- BL. in BR.-BL., MOLINIER and WAGNER 1940. Two taxa are new for science: *Dianthus sardous* Bacchetta, Brullo, Casti and Giusso, sp. nova and *Helichrysum microphyllum* (WILLD.) CAMB. subsp. tyrrhenicum Bacchetta, Brullo and Giusso, subsp. nova.

The Campo Pisano mine area is characterized by the presence of typical plants species that are well-adapted to this highly metal-concentrated environment, commonly made of terophite can be recognized on muddy/clay substrate of mine tailing substrates (i.e., *Centaurium erythraea* Rafn. e *Bellium bellidioides* L.) and on gravel substrates (i.e., *Jasione montana* L. and *Rumex bucephalophorus* L.). Moreover, there are many plant species that are endemic in Sardinia and Corse, such as *Echium anchusoides* Bacch., Brullo & Selvi, *Euphorbia cupanii* Bertol ex Moris, *Iberis integerrima* Moris, *Limonium merxmulleri* Erben, *Ptilostemon casabonae* (L.) Greuter, *Scrophularia canina* L. subsp. *bicolor* (Sm.) Greuter. The CP plant species are: *Quercus ilex* L., *Chamaerops* *humilis* L., *Pistacia lentiscus* L., *Tamus communis* L., *Smilax aspera* L., *Rubia peregrina* L., *Asparagus acutifolius* L. *Prasium majus* L. it should be noted that the woody and shrubby plant species are more abundant than the herbaceous ones. Figure 2.4 shows the plant formation, the vegetation canopy and the substrate in some part of Campo Pisano area that are subjected to mine activities.

Figure 2.4. Plant formations and substrate in Campo Pisano

2.4. Mediterranean metal-tolerate plants for phytoremediation in CP mine tailing

The findings of Mediterranean plants species that are suitable for the phytoremediation process refer mainly to grasses, half-shrubs, and shrubs species, such as *Dittrichia viscosa* L. (Greuter), *Cistus salviifolius* L. (Jimènez et al., 2005), *Pistacia lentiscus* L. and *Scrophularia canina* L. subsp. *bicolor* (Sm.) Greuter (Jimènez et al., 2005, 2014; Lai et al., 2015), *Euphorbia cupanii* Bertol. ex Moris (Jimènez et al., 2005; Medas et al., 2015 and 2017), *Phragmites australis* (Cav.) Trin. ex Steud and *Juncus acutus* L. (De Giudici et al., 2015; Medas et al., 2017), but few available findings are on the use of trees despite their great potential for long-term stabilization of mining sites (Disante et al., 2010; Conesa and Pàrraga-Aguado, 2019).

The floristic, physiognomic and structural diversity of the plant communities reflects the ecological diversity present in the mining environment. Figure 2.5 shows some endemic plant species (Except *P. halepensis*) that can be considered for phytoremediation and revegetation processes in Campo Pisano mine tailing site.

Scrophularia canina L. subsp. *bicolor* (Sm.) Greuter.

Dittrichia viscosa (L.) Greuter

Limonium merxmuelleri Erben *

Euphorbia pithyusa subsp. *cupanii* (Guss. ex Bertol.) Radcl. Sm. *

Helichrysum microphyllum (Willd.) Camb. subsp. *tyrrhenicum* Bacch. et al.

Echium anchusoides Bacch. et al.

Iberis integerrima Moris

Pistacia lentiscus L.

Pinus halepensis Mill.*

Phragmites australis

Bellium bellidioides L.

Juncus acutus

Figure 2.5. Plant species considered to be candidates for phytoremediation project in Campo Pisano; * indicates plant species which may be considered for more in-depth study

The available findings on the phytoremediation capabilities of Mediterranean woody tree species refer to:

- **willow and poplar** plant species, that have been proposed for phytoremediation of metal-contaminated mine sites seem to be very sensitive to metal accumulation, especially at the seedling stage. (Adriano, 2001; Disante et al., 2010; Marmiroli et al., 2011; Nandillon et al., 2019)
- ***Pinus contorta*** reported that it was tolerant to high concentrations of SO_4^{2-} (up to 3,000 mg L^{-1} of SO_4^{2-}) in water solutions, even though the plant was sensitive at the seedling stage (Renault et al., 1998; Pàrraga-Aguado et al., 2014c).
- ***Pinus halepensis*** ability for
  - reforestation of the Mediterranean degraded soil due to it is well adapted to the poor quality of soil, high temperatures and limited irregular rainfall (Pérez-Piqueres et al., 2018; Pàrraga-Aguado et al., 2014c)
  - pursuing soil retention, preventing/ contrasting soil erosion (as windbreakers near the coast) of Mediterranean barren slopes (Mauri et al., 2016)
  - revegetating the barren and fire-prone arid areas due to the plant ability to oblige seed germination by releasing seeds out of their serotinous cones at high Mediterranean temperatures and during a fire (Pausas & Vallejo 1999; Pesaresi et al., 2017).

2.5. Sampling site in Campo Pisano mine tailing dump

In this study, soil and plant were collected from southwest Sardinia, an Italian mine area with a rich history of mine activities that have left large quantities of mine wastes in dumps and flotation tailings. Several pilot projects have been carried out focused on the rich plant diversity of the areas in order to identify the most suitable autochthonous plant species possessing the best phytoremediation capabilities to be exploited (Bacchetta et al., 2018; Medas et al., 2015 and 2019).

During the years 2008 and 2010, a pilot scale phytoremediation study was carried out 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* (Sm.) Greuter] and their responses to different soil amendments (Lai et al., 2015; Boi et al., 2020). An investigated site area of about 300 m^2 was subdivided into ten different experimental plots to apply various soil amendments (Figure 2.6 and Figure 2.8). 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 a total content of 45 mg kg^{-1} Pb and 235 mg kg^{-1} Zn (Bacchetta et al., 2015).

Figure 2.6. Campo Pisano site (in 2008)

Figure 2.7. CP site with native spontaneous vegetation (in 2019)

The sampling site of this work is in the same experimental site and its surrounding area where the pioneer tree species *Pinus halepensis* grows spontaneously (Figure 2.7 and Figure 2.9).

Figure 2.8. The amended experimental CP Plot area (in 2009)

Figure 2.9. The same experimental CP Plot area with native spontaneous vegetation (in 2020)

For assessing the phytoremediation capabilities of *P. halepensis*, samples were collected according to the aim of the investigation and will be described in the following related chapters.

CHAPTER 3

3. Pinus halepensis **Mill.** (Literature review)

3.1 *Pinus halepensis* characteristics in the Mediterranean region

Pinus halepensis Mill. (*Aleppo Pine)* is a circum-Mediterranean tree species and is mainly present in the coastal zones of the western side of the basin (Tutin et al., 1993; Fady et al., 2003; Mauri et al., 2016; Farjon 2017; Pesaresi et al., 2017; Pignatti 2017–2019). In the Mediterranean Basin, the forests dominated by this species cover more than 3.5 million ha. Even though the name of plant species derives from the Syrian town of Aleppo, the plants are mainly concentrated in Western Mediterranean, while they are more scattered in the Eastern side (Pesaresi et al. 2017) (Figure 3.1 and Figure 3.2).

Figure 3.1. Distribution map of *Pinus halepensis* in the Mediterranean basin (Mauri et al., 2016)

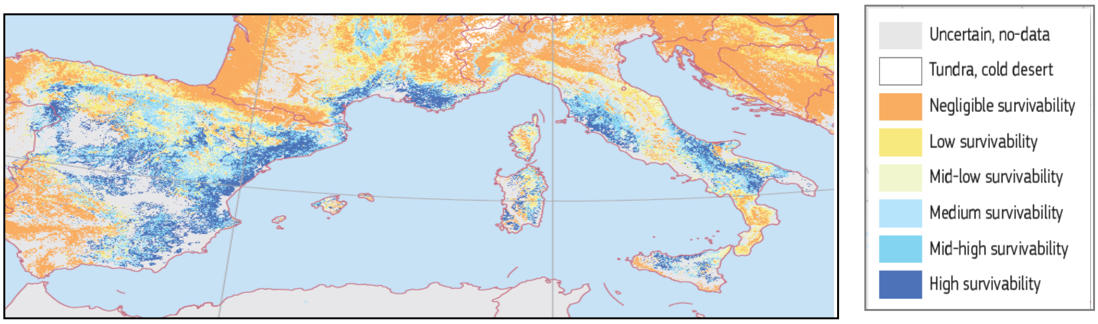


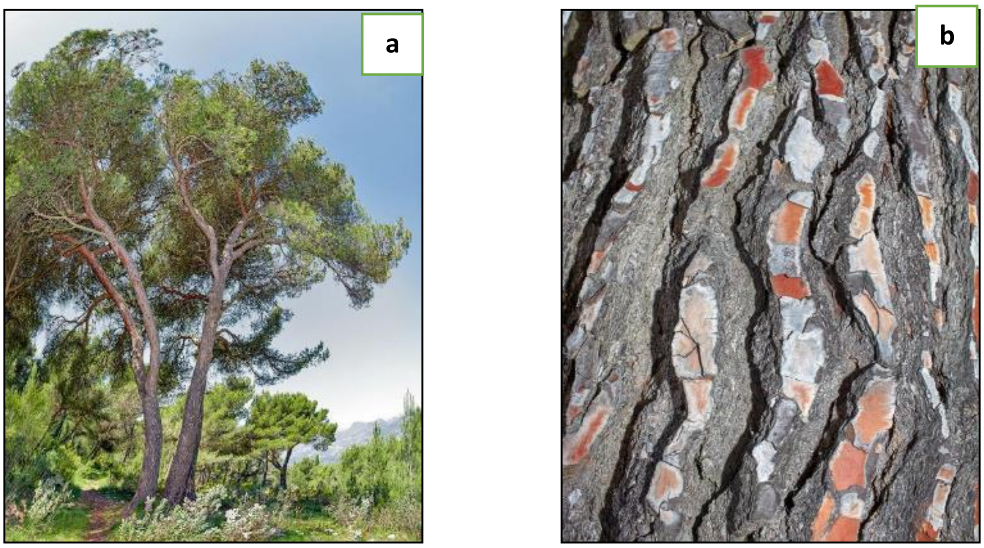
Figure 3.2. Estimating the maximum habitat suitability for *Pinus halepensis* (Mauri et al., 2016)

In Italy, *P. halepensis* is considered a native species in many regions, as well as the islands of Sicily and Sardinia (Bartolucci et al., 2018, 2020). In Sardinia, the natural distribution of *P. halepensis* is restricted to the south-western part of the island, in the Sulcitano-Iglesiente sector (Island of San Pietro, the area between Porto Pino and Capo Teulada) and at the extreme north-western border of the Island of Sant'Antioco (Calvia et al., 2022; see Figure 3.3).

Figure 3.3. Map of Sardinia showing the distribution of the three pine species on the island. **A** Blue: *Pinus halepensis* area; **B** green: *P. pinaster* area; **C** purple: *P. pinea* area (Calvia et al., 2022)

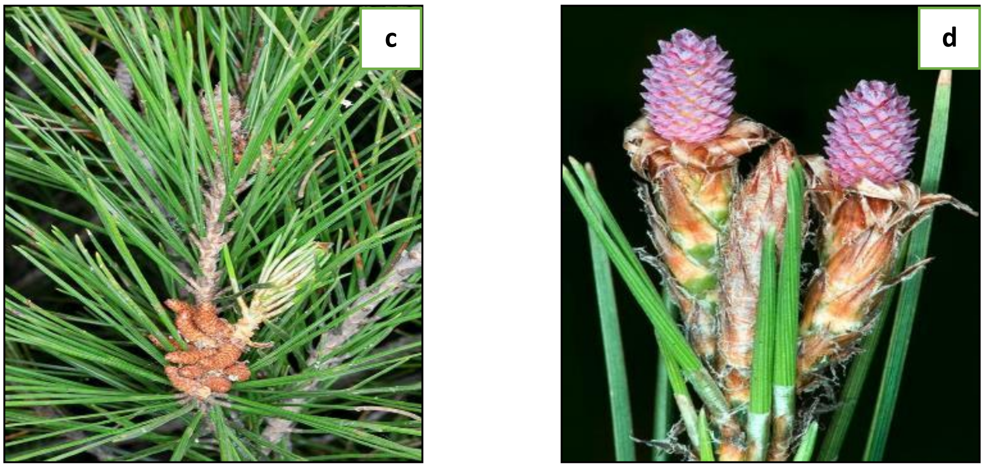
P. halepensis can grow spontaneously in different substrates often in marls, limestone (Barbéro et al., 1998; Mauri et al., 2016, Farjon 2017) and low fertile soil at the altitudinal range from sea level up to 1700 m a.s.l. in Morocco (Farjon, 2017). It is a drought-tolerant coniferous species and grows also at lower altitudes, in the thermo- and meso-Mediterranean zone and its habitat ranges from the lower arid or semiarid to humid bio-climate favouring minimum temperatures of 2 and 10°C (Mauri et al., 2016). It is a thermophilous and xerophilous plant that grows in areas with annual average rainfall between 350 and 700 mm (Barbéro et al. 1998; Mauri et al., 2016) and tolerates low temperatures between 2 and 10°C (Querejeta et al., 2008; Pérez-Piqueres et al., 2018).

Moreover, it has been frequently noted for its ability to tolerate high amounts of metals (Pulford and Watson, 2003; Conesa and Pàrraga-Aguado, 2019), low nutrient requirements, moderate tolerance to salinity (Pàrraga-Aguado et al., 2014c) and for the restoration of degraded soil (Querejeta et al., 2008; Pàrraga-Aguado et al., 2013), as well as low fertile soils in mine areas (Sardans et al., 2005). These special characteristics of *Pinus halepensis*, together with its lateral and higher root systems (Martínez-Oro et al., 2019; Mauri et al., 2016) make it to be a good candidate for the phytoremediation process (Figure 3.4). To our knowledge, little attention has been devoted to the geochemical and mineralogical investigation as well as element speciation in the soil-root system of *P. halepensis.*



Pinus halepensis Mill.

Details of the trunk of *P. halepensis.*



The needles of *P. halepensis* are in two branches with the male cones

The female cones of *P. halepensis*

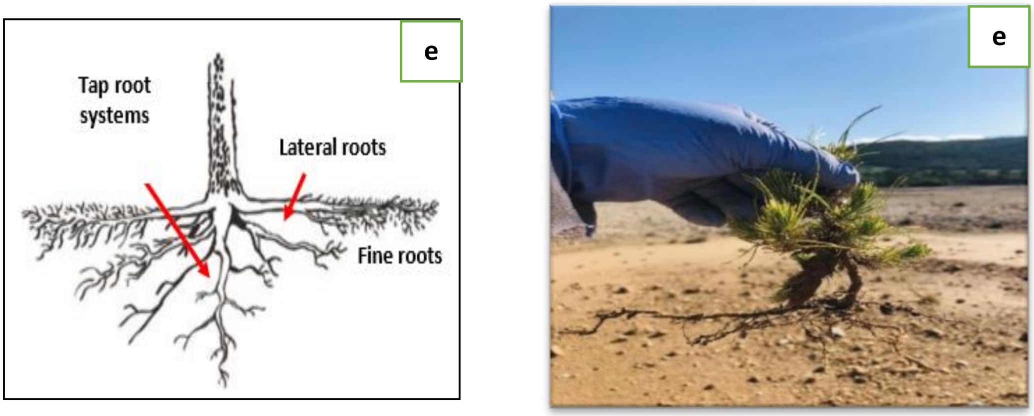


Figure 3.4. *Pinus halepensis* (a); barks (b); needles (c); cones (d); root system (e)

3.2. *Pinus halepensis* capability in metal uptake

3.2.1. P. halepensis interaction with Zn

Zinc at low levels (e.g., 20–100 μgg^{-1} dry weight in leaves) is required for plant enzymatic activity, maintenance of biomembrane integrity, carbohydrate metabolism and protein synthesis in plant metabolism. However, at higher levels can become toxic and its toxicity limitation depends on plant species, genotype and growth stage (Disante et al., 2010).

The findings of Disante et al. (2010) indicated that Zn bioaccumulation is low in the aboveground of *Pinus halepensis* in comparison to the other different woody species tested, such as *Quercus suber*, *Pinus pinea*, *Pinus pinaster*, *Tetraclinis articulata* (Vahl) Mast., *Rhamnus alaternus* L. and *Quercus suber*. In particular, they reported that;

- the accumulation of Zn in hydroponic culture was significantly higher in roots (with 4358 µg g^{-1}) than in shoots. Furthermore, the total Zn content in roots showed a non-linear response to Zn availability.
- The growth of roots was more sensitive to Zn than shoot.
- The Foliar and root biomass decreased in response to increasing Zn content.
- Zinc inhibited root elongation and root biomass.
- In co-culture with the above-mentioned plants, the growth rate of the main root was reduced in response to Zn above 10–20 µm.

Moreover, Favas et al. (2014) reported that in other species of pine, such as *Pinus pinaster,* the young needles and stems accumulated high content of Cu and Ni, whereas the older needles accumulate more As, Fe, Zn, Pb and W in the Vale das Gatas mines (Northern Portugal).

3.2.2. P. halepensis interaction with Pb

The findings of Al-Subu (2002) reported the use of decayed needles of *P. halepensis* alone or in combination with Cypress *(Cupressus sempervirens*) for the removal of Pb from polluted water. This is introduced as a cost-effective method compared to other methods such as precipitation, ion exchange, oxidation, cementation and activated carbon. Moreover, their findings showed that

- the amount of Pb removal were highly affected by pH value (the optimum performance at neutral pH).
- the presence of some ions, such as Cu^{2+}, Ag^{+} and Cd^{2+} can negatively influence on Pb removals, while the presence of other ions, such as Mg^{2+} and Na^{+} showed a positive effect.
- lead removal increased with increasing the Pb ions concentrations and the *P. halepensis* needles employed.
- The efficiency of *P. halepensis* needles in removing Pb increased in combination with *cypress (Cupressus sempervirens)* leaves.

3.2.3. P. halepensis interaction with Cd and other elements

The study carried out by Párraga-Aguado et al. (2013) and (2014b) on the needles of *P. halepensis* in the Cartagena-La Mining district (Southern Spain) indicated high variability of metals contents (e.g. Cd, As, Zn and Mn). They found that:

- the concentration of Mn and Zn detected in needles of *Pinus halepensis* growing in mine tailing (40–100 mg kg^{-1} and 25–55 mg kg^{-1}, respectively) was lower than the ones detected in the not-contaminated forest (up to 320 mg kg^{-1} Mn, 80–130 mg kg^{-1} Zn).

- the precipitation of some minerals, such as gypsum (calcium sulfate) and calcite (calcium carbonate) could decrease the availability of As.
- the concentrations of As and Mn in the one-year-old needles of *P. halepensis* growing in mine tailing were detected 4–5 fold lower and the concentration of Fe, Ni and Zn were 2-fold lower than those detected outside of mine areas.
- the woody stems of *P. halepensis* in mine tailings showed high content of Cu, Fe and Ni.
- Cu concentration in stems was1.5-fold higher than needles, whereas Fe concentration in stems was more than 1.6-fold higher than needles.

3.3 Enhancement of phytoremediation capability in *P. halepensis*

Although phytoremediation proved to be a cost-effective and efficient technology in the contaminated mine sites, it depends on the plant growth to deliver its desired outcomes. Thus, it is essential to enhance the soil fertility (Kumar Awasthi et al., 2019), microbiological activity (Pàrraga-Aguado et al., 2014c), the rhizospheres systems and plant growth (Favas et al., 2014) in order to make this technique economically long-term feasible and minimize the overall reclamation span time. The literature findings show that organic amendments can effectively improve the required essential nutrients of soil (C, N, P, K) and soil microbial populations (Lebrun et al., 2019). It also can improve the soil cation exchange capacity (CEC) and enhance the soil stability, which directly affects the existence of soil minerals (Nejad and Jung., 2017), as well as the mobility and/or availability of metals to plants by changing soil acidity in a wide range of the toxic metal polluted sites (Garau et al., 2007). Moreover, the findings of Prica et al. (2019) indicated that the applications of organic amendments can improve the soil chemical, biological and physical properties, reduce the metals mobility and facilitate the growth of some plant species. It can also enhance the phytoremediation process through a multi-components interaction system in polluted soils (Pàrraga-Aguado et al., 2014a) (see Figure 3.5).

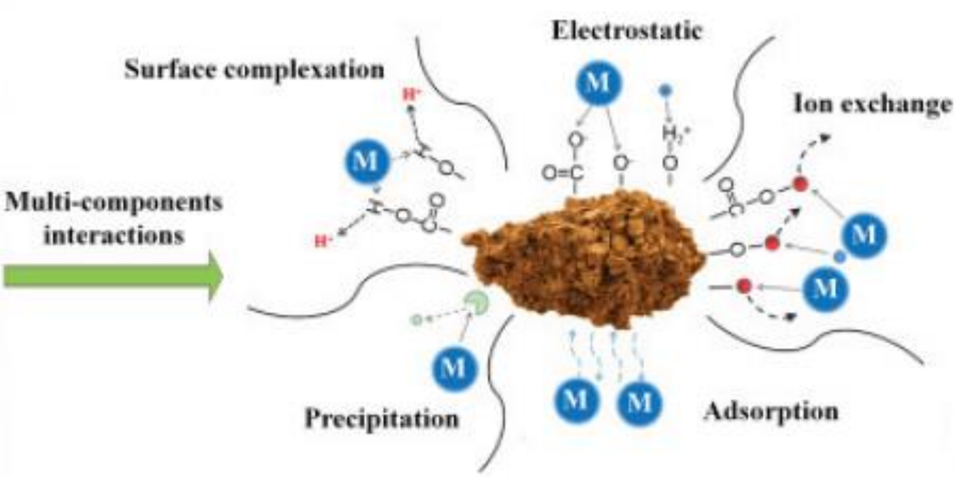


Figure 3.5. Mechanism of multi-component interactions in the enhancement of phytoextraction (Pàrraga-Aguado et al., 2014a)

- Carbon amendment

The investigation on the rhizospheres of *P. halepensis* indicated that is possible to foster pine afforestation capability in low salinity and sulfate soil through the enhancement of soil properties such as an increase of soil Carbon contents (Maestre et al. 2003), soil organic matter content, soil fertility and microbial activity. The investigation found that even a slight change in the amount of soil organic matter can induce a lower amount of mineralization occurrence during the compost amendment application. In fact, the loss of organic matter (C and N) or C/N (i.e., the relative accumulation of nitrogen where most carbon of the organic input is lost as CO_2 in compost transformation in soil) is a typical tendency of any humification process that happens as a result of the oxidation in soil (Román et al., 2003).

- Nitrogen amendments

The study carried out by Pàrraga-Aguado et al. (2014a) on sulfides mines of (Southeast Spain) showed that nitrogen amendments improve the soil properties and induce changes in *P. halepensis* rhizospheres through a significant impact on soil microbiology. They detected that the nitrogen values in the areal parts of *P. halepensis* growing on mine tailing were much lower than in the non-contaminated soils. Indeed, the low values of nitrogen reflected the existence of mycorrhizal bacteria associations in nitrogen-limiting soils. On the other hand, the low values of nitrogen (8%) detected in the needles of *P. halepensis* growing in sulfides mine tailing indicated that plant may use different sources of nitrogen which may affect the microbial populations in rhizospheres and access to organic nutrient sources (Parraga-Aguad et al., 2014a).

- Phosphorous amendments

Phosphorus is known to be an important soil factor in pine growth (Sardans et al. 2005). Conroy et al. (1988) reported that soil phosphorous deficiency decreases plant photosynthetic rates. Moreover, the low phosphorous availability in soil and low phosphorus accumulation in the areal part of plants limit the growth and biomass production of plant (Pàrraga-Aguado et al., 2014c). Another study carried out by Sardans et al. (2005) on *P. halepensis* growing in the mine site (South-east of Spain) reported a lower concentration of phosphorous in needles, below the minimum threshold (500 mg kg^{-1}) compared to its concentration in the non-metal-polluted site. Moreover, Pàrraga-Aguado et al. (2014b) reported that phosphorus fertilization can be considered a promising optimizer option for plant growth and for favoring the enhancement of the phytoremediation process in mine tailing areas.

3.4. The effect of organic matter on phytoremediation in mine tailing

It should be noted that the loss of soil organic matter (C and N) happens as a result of mineralization, a typical tendency of any oxidation process in mine waste sites (Román et al., 2003). Moreover, the addition of soil organic amendments can effectively improve the C/N ratio, the physical, chemical and biological properties of soil and facilitate plant growth. It also can affect the soil and plant interactions and enhance the phytoremediation capabilities of some plants species utilized for remediation projects in abandoned mines tailings, particularly at the seedling stage when plants are very sensitive to metal toxicity (Disante et al., 2010; Lai et al., 2015; Conesa and Pàrraga-Aguado., 2019). However, it should be considered that the metal mobilization depends on the specific plant mechanisms to uptake metals (Neagoe et al., 2012).

The effectiveness of organic amendment in soil depends on the type of organic matter, soil pH, soil depth and the competition with metal compounds:

- **type of organic matter**: according to the findings of Zhang (2010), the addition of phosphate can reduce Pb leaching because of the phosphate competition with dissolved organic carbon. However, the highest amount of *phosphorous* addition to soil resulted in the leaching of Cu, Cd, and Zn in organic forms (Neagoe et al., 2012).
- **Soil pH:** the mineral surface hydroxyl functional groups can effectively absorb metals at pH 6 and 8, while organic matter can be absorbed at lower pH because of some functional groups, especially carboxylic groups. Another field experiment showed that interaction with sewage sludge increased metal mobility (e.g. Cu, Ni, and Pb), especially at high pH (Neagoe et al., 2012). In fact, mobility of contaminants can be reduced by surface adsorption in roots as well as their precipitation in rhizospheres by induced changes in pH (Prasad. 2004; Domínguez et al., 2009; Favas et al., 2014).
- **Soil depth:** Neagoe et al. (2012) reported that deep soil organic matter can have a very long "*residence time*", which offers a large potential for metal retention. Their findings indicated that the leaching was correlated with large dissolved organic carbon concentrations induced by sulfide oxidation. Moreover, the sewage sludge amendment made in the experimental mine tailing material led to an initial large leaching of Al, Cu, Zn, Ni, and Pb in deep soil layers. The investigation carried out by Dudal et al. (2005) on deep layers of polluted soil (in 2 m depth), showed a fivefold decrease of the soluble organic matter concentration in the constant concentration of Cu, Fe, and Mn. Moreover, they noticed that only a small part of the organic carbon was responsible for metal binding and strong rainfalls were able to transport only a small amount of metal-binding organic matter to the deeper layers of soil. Moreover, the concentrations of metals (e.g. Cu, Cr, Ni and Cd) in the water transferred from the organic-rich forest ecosystem soil layer to the deep layers were significantly correlated with dissolved organic carbon (except for Zn) at 20 cm depth of soil and at 90 cm depth where there was no significant correlation (Huang et al., 2010; Neagoe et al., 2012).

3.5. The application of compost on the phytoremediation capability of *P. halepensis*

To our knowledge, few investigations have been carried out on the effectiveness of soil amendments to enhance the phytoremediation ability of *Pinus halepensis* in the mine tailings. The available findings are as follows:

- the findings of Martinez-Oro et al. (2017) conducted in the south of Spain (Sierra Tejeda, Almijaray Alhama Natural Park) showed that the compost made of chipped branches of Aleppo pine (*Pinus halepensis)* in combination with other mulch composts (e.g. straw mulch) can reduce metal transplant stress and improve the plant growth and survival after two years, however, the mulch compost treatments showed no significant improvement on carbon content, pH and electrical conductivity after two years experiment (Hueso-González et al., 2014, 2016).

- the findings of Pérez-Piqueres et al. (2018) showed that the addition of vermi-compost as a root-growth promoter in combination with composts (sewage sludge compost, meat bone and meal, municipal solid waste compost, ovine manure, ground olive stone and coconut fiber) can benefit the cultivation of *P. halepensis* in the Mediterranean forest region. In fact, this amendment increased soil organic matter, enhancing soil nutritional and structural conditions. Furthermore, it could substitute mineral fertilizers and substrates.

- According to Conesa and Pàrraga-Aguado (2019), urban compost amendment is very effective in the phytoremediation of *P. halepensis* in highly metal-contaminated mine sites. Their findings showed that compost made a significant increase in plant biomass (shoot/root ratios) and induced low metal translocation (Cu, Pb and Zn) into the shoots. Nonetheless, the amendment caused the redistribution of Cd into the pine tree shoots, probably due to its metal transport which is associated with increased transpiration. Their findings showed a significant increase of Cd concentrations in the aerial parts (stems> branches and leaves) of *P. halepensis* growing on highly contaminated soil amended with the urban compost. It was also reported the increase in soil fertility and biomass production of plant, whereas the metal concentrations in the areal parts of the plant were decreased with a significant negative correlation between Cd and Zn concentrations.

- An experimental investigation, carried out by Larchevêque et al. (2006) in Mediterranean silty-clay soils in the southern province of Marseille (France), found that organic compost amendment (local municipal sewage sludge, green wastes and pine barks*)* could significantly improve *P. halepensis* growth, soil fertility, total N content, available P and K concentrations (up to 5 times) and total content of Cu and Zn in soil. Furthermore, the investigation also found that:
    - the concentration of P, Cu and Zn was increased by the addition of amendment with a negative potential effect on plant length growth.
    - total Zn and Cu concentrations and their bioavailability in soil decreased after 2.5 years of amendment application with no significant foliar metal absorption on seedlings due to the deep metals leaching.

- the finding of Tlustos et al. (2007) and Conesa and Pàrraga-Aguado. (2019) showed that soil organic amendments can favor the immobilization of metals (i.e., Pb and Cu) in *P. halepensis* roots. Furthermore, it reduced Pb concentration in the branches and at the same time, it limited the transportation of Mn and Zn to the plant shoots.

3.6. The applications of compost amendment to improve the phytoremediation capability of some plant species

The application of organic amendments, such as biosolids, compost (municipal /urban wastes), biochar (hardwood biomass) and compost + biochar is usually an economical and cost-effective approach (Ming et al., 2016; Garau et al., 2007; Martínez-Oro et al., 2019). The most widely used organic amendment for the reclamation of contaminated soil is urban waste compost, which is rich in nutrients and can play an important role in enhancing soil fertility, reducing metal availability and can be effective in long-term phytostabilization processes of metal mining sites (Lai et al., 2015; Conesa and Pàrraga-Aguado., 2019).

Another widely used organic amendment for the reclamation of contaminated soil is Biochar, a solid carbon-rich material made up of pyrolyzed plant biomass or animal wastes (Lebrun et al., 2020) composed of carbon (between 29% and 90%), hydrogen, oxygen, nitrogen and sulfur and traces of other elements (Antonangelo and Zhang., 2019). Applications of Biochar proved to be effective in increasing the sequestration of carbon and influencing CO_2 emissions (Antonangelo and Zhang, 2019) which improves the Physico-chemical properties of soil and plant growth (Simiele et al., 2020). In contaminated mine waste, the absorption capacity of biochar can be exploited for stabilizing metals and reducing the bioavailability, mobility and solubility of metals, such as Cu, Ni, Cd, Pb, Cd and Zn (Lebrun et al., 2019). However, biochar nutrient availability is sometimes low, and it can be improved by its combination with other amendments, such as compost in the reclamation of multi-contaminated mine sites (Lebrun et al., 2019).

The following presents the available findings on the effectiveness of compost amendment in mine tailing sites for improving the phytostabilization capabilities of some plants species:

- In a phytostabilization experiment carried out in mine tailings dumps of Campo Pisano (Southwestern Sardinia, Italy), compost proved to be the best amendment for the long-term plant growth of *Pistacia lentiscus*, in comparison with other different soil amendments, such as chemical fertilizer and zeolites. Moreover, the Zn and Pb bioavailable fractions were reduced with the application of all amendments and *Pistacia lentiscus* appears to be the most suitable species for phytostabilization and revegetation due to its resistance to metals and high phytomass production (Bacchetta et al., 2012).

- In a phytostabilization experiment conducted in the abandoned Pb and Zn mine area of the province of Biscay (Basque Country, Spain), the application of combined organic amendments (*e.g. cow slurry, poultry manure and paper mill sludge mixed with poultry manure)* with *Festuca rubra L. species* proved to be effective in increasing plant biomass production and reducing the bioavailable fraction of Pb and Zn in soils (Boi et al., 2019).

- Moameri et al. (2019) evaluated the effectiveness of soil amendments compounding nano-silica with municipal solid waste compost (2%) for enhancing the phytoremediation capabilities of *Secale montanum* plant species in Pb and Cd contaminated mine sites (Factoryin Zanjan, Iran).

According to their findings, these compounded soil amendments, in addition, to improve plant growth and soil fertility, enhanced the accumulation /immobilization of Pb and Cd accumulation in the roots of *Secale montanum* (with a maximum amount of 533.6 and 208.6 mg kg^{-1}, respectively) than in shoots (with 290 and 181.6 mg kg^{-1}, respectively) due to rhizospheres activities that caused Pb and Cd more soluble (Moameri et al., 2019).

- According to Carbonell et al. (2011), municipal solid waste compost can enhance biomass production in *maize* and increase the concentration and bioavailability of the Cd, Pb, and Zn in the soil at the same time.
- The findings of Vatehova et al. (2012) showed that municipal solid waste compost in combination with Nano-SiO_2 improved seed coat resistance and the nutrition accessibility of *Zea mays. L*. In addition, Silicon (Si) enhanced the photosynthesis and translocation of Cd into the aboveground parts of *Brassica napus.* In this regard, it is worth noticing that Nano-Si is more effective than common Si in limiting Pb translocation from their roots to above-ground organs and decreases the toxic effects of lead on rice cultivars growth (Liu et al., 2015; Moameri et al., 2019).
- According to Sharifian et al. (2014), the growth parameters of *Calendula officinalis* and *Bellis perennis* (e.g. plant height, shoots and roots weight and chlorophyll content) could be improved through the applications of soil amendments with 25–50% municipal solid waste compost.
- Smolinska et al. (2015), reported the application of green waste compost in the enhancement of the Hg translocation from roots to shoots of *Lepidium sativum L*. Moreover, compost alleviated the leaching of Hg in both neutral and acidic soils.

3.7. Enhancement of phytoremediation capability of *P. halepensis* in co-culture with some plant species

The findings declared that the co-cultivation of metal tolerate plants can trigger a combination of different geochemical processes within rhizospheres and plant roots and can influence the overall metal availability, accumulation and translocation in plants (Martinez-Oro et al., 2017). Thus, the co-cultivation of plants can play an important role in successful phyto-revegetation in Mediterranean mine soils (Martínez-Oro et al., 2019; Pàrraga-Aguado et al., 2014a; Domínguez et al., 2016).

Co-cultivation of *P. halepensis* and *Piptatherum miliaceum* (pioneer grass species)

The findings of Martínez-Oro et al. (2019) showed that the combination of planting *P. halepensis* and the pioneer grass species, *Piptatherum miliaceum* (with and without the municipal organics compost amendment), seems to have highly contributed to enhancing the soil stabilization process in mine tailings of Cartagena-La Union mining district (southeast Spain). Indeed, the fast-growing ability of *Piptatherum miliaceum* can play an important role in primary ecological succession and improve soil fertility and soil surface protection. Then, *P. halepensis* could assure the long-lasting development of soil fertility without interfering with the nutrient or water resources, due to the different root depths of the two plant species. According to their findings, the co-cultivation of *P. halepensis* (tree species) with *Piptatherum miliaceum* (grass species) can

- negatively affect the growth and the total biomass of *P. halepensis*
- decrease the nitrogen content in the needles of *P. halepensis* (N/P<10) in neutral pH tailings waste
- increase significantly the Cd translocation into the areal parts of *P. halepensis*
- increase the ratio of C/N in the needles of *P. halepensis,* even in the absence of compost amendment.

These outcomes indicated that the low competitive ability of *P. halepensis* for nitrogen uptake in co-culture with *Piptatherum miliaceum* may constrain the usefulness of the co-cultivation of these two plant species as an effective phytoremediation technique in contaminated mine tailings. The addition of N/P/K fertilizer can be advisable in order to improve the phytoremediation ability of plants. Moreover, the addition of soil organic amendment *(municipal organic waste*) in the monoculture treatment may enhance the growth of *P. halepensis* biomass and, at the same time, increase the metal concentrations in its needles. In fact, Zn accumulation in the needles of *P. halepensis* growing in the contaminated mine sites was about 1.5-fold higher than the one detected on non-amendment soils.

The findings pointed out a significant decrease in biomass production of *P. halepensis* in the co-culture with *Piptatherum miliaceum* of similar size and age due to their intra-specific competition and different abilities in nutrient acquisition. This may happen due to the plants interaction at the initial stages of growth when both root systems compete within the same soil depth. Moreover, *P. halepensis* in relation to *P. miliaceum* resulted in a low competitive ability for nutrient acquisition, especially for nitrogen. Accordingly, it was suggested that the phytoremediation techniques centered on the co-cultivation of *P. halepensis* with *P. miliaceum* may not be among the best option for the reclamation of semiarid mine tailings unless the negative effects of plants competition are alleviated by the addition of N-P amendments (Martinez-Oro et al., 2017).

Co-cultivation of *Pinus halepensis* and *Pistacia lentiscus*

Doni et al. (2017) reported the efficiency of the organic compost amendment may be influenced by local climate changes. In detail, they compared the effect of compost amendment (commercial green compost) on the co-culture of *P. halepensis* with *Pistacia lentiscus* in two degraded sites having different climates. One site was located in South Italy (with an average annual temperature of 16.6 °C and rainfall of 462 mm) and the other in North Italy (with an average annual temperature of 13 °C and rainfall between 600–800 mm). The outcome of the comparison mainly declared that the efficiency of organic compost amendment was higher in the North of Italy than in the south with the largest improvement in the physico-chemical and biological properties in the related degraded soils (Doni et al., 2017). Furthermore, they found that the application of organic matter compost could increase the carbon and humic content of the soil due to the mutual interactions that took place in rhizospheres between the organic matter compost and soil microbial and biochemical metabolism. These interactions can activate the mineralization processes and reduce plant oxidative stress by reducing enzyme activities in the needles of both *P. halepensis* and *Pistacia lentiscus.* According to their findings, the co-culture of *P. halepensis* with *Pistacia lentiscus* even without organic amendment was able to stimulate the soil properties of degraded soil sites in South Italy, but was not able to promote the carbon sequestration in soil (Doni et al., 2017).

The other experiment was conducted by Román et al. (2003) on the effect of the urban waste amendment (the remained of ferromagnetic, glass, plastic, paper and paperboard) on *P. halepensis* co-cultured with *Pistacia lentiscus* in highly degraded gypsiferous and Calcic Regosols soil (mainly gypsum and calcite) in Valdemingomez (southeast of Spain). They reported that this compost amendment could affect the topsoil by increasing (up to 72%) the physical parameters, soil stability and the growth of seedlings as well as the under-topsoil layer (10–20 cm) by increasing the same parameters up to 245%. However, this type of compost amendment increased the percentage of bioavailable fraction for Mn, Zn, and Cu and reduced other parameters, such as the organic matter, C/N ratio, Na and K content, the humic acid to fulvic acid ratio and the lipid content due to the probable leaching (Román et al., 2003).

3.8. Conclusion

The reclamation of abandoned mine tailings is, generally, a controversial issue. Phytoremediation proved to be sustainable, cost effective and eco-environmental friendly technology for the remediation of abandoned mine sites. The more effective way to lower the "*overall reclamation span time*" of *in-situ* phytoremediation processes is increasing the efficiency of phytoremediation process by the selection of suitable plant species and the application of appropriate organic amendments and/or microbial additions.

The available literature findings of this chapter focused on the outcomes of interactions between the long-term phytoremediation capability of *P. halepensis* and the applications of compost amendments as well as its co-culture with other plants species in different phytoremediation projects. In this regard, careful monitoring of the trace elements in soil and plant is required to assess the capability of co-cultured plant species as well as *P. halepensis* and the effect of organic amendments on the phytoremediation and mineralization process in different soil depths and in different parts of the plan. This can help to better understand the interaction/ potential of co-cultured plants and the role of organic amendments on the improvement of the plant-soil system, the total concentration, translocation and bioavailability of metals in mine tailings.

The following chapters (4, 5 and 6) describe the sequence of progressive activities in-depth insights gained through each trial.

In ANNEXs are reported the main summarized findings reported in this review chapter.

ANNEX 3.1. Plants species in phytoremediation experiment with soil amendments in mine contaminated soil

Plant species	Mine	Location	Soil amendments	Effects	Reference
ryegrass plant (Lolium perenne)	Tar Creek mining district	Oklahoma, Kansas, and Missouri	coconut shell and Rice straw biochar	- enhanced Cd immobilization - reduction of Cd concentration - increased of soil biological properties - increased plant biomass by 1.35–2.38 times - increased soil pH	Li et al. (2019) Antonangelo and Zhang. (2019) Zhang et al. (2017)
			poultry litter–derived biochars and switchgrass (*Panicum virgatum)*	immobilized Zn, Pb, and Cd in shoots	Antonangelo and Zhang. (2019) Neuberger et al. (2008)
			biochar produced at high temperatures (700°C)	decreased Pb phytoavailability	Ahmad et al. (2016) Antonangelo and Zhang. (2019)
Brassica napus	Riotinto mine	Spain	biochar applications *(rabbit manure biochars at 450º C)*	increased soil quality and biomass production of plant Reduced the metal amount (e.g. As, Cu, Co, Cr, Se and Pb) Reduced TF for Co, Cr, Cd, Cu, Ni, Zn, Pb and As.	Gasco et al. (2019)
Pistacia lentiscus	Campo Pisano	Sardinia, Italy	compost	reduced the bioavailable metal fraction (for Zn and Pb)	Bacchetta et al. (2012) Boi et al. (2019)
Festuca rubra L.	Biscay	Spain	organic amendments (e.g. cow slurry, poultry manure and paper mill sludge mixed with poultry manure)	increased plant biomass production reduced the bioavailability of Pb and Zn in soils	Boi et al. (2019)
Secale montanum	National Lead & Zinc Factoryin Zanjan	Iran	nano-silica with municipal solid waste compost 2%	improved plant growth and soil fertility enhancement of extraction and remediation factor in root accumulation of Lead and Cadmium in shoots	Moameri and Abbasi Khalaki. (2019)
			Municipal solid waste compost with Nano-SiO_2	improved seed coat resistance Improved the nutrition accessibility of plant	Vatehova et al. (2012)
				More effective in limiting Pb transportation from roots to the above-ground parts of plant enhanced effectively soil stabilization for Pb	Moameri and Abbasi Khalaki. (2019)
Calendula officinalis and Bellis perennis	National Lead & Zinc Factoryin Zanjan	Iran	municipal solid waste compost (green waste compost)	Improved shoots and roots weight, chlorophyll content and plant height	Sharifian et al. (2014) Moameri and Abbasi Khalaki. (2019)
Lepidium sativum L.				enhancement of the Hg translocation from roots to shoots	Smolinska et al. (2015) Moameri and Abbasi Khalaki. (2019)
Salix viminalis	Pontgibaud (Auvergne-Rhone-Alpes) mine	France	Iron-based amendments (Iron sulfate)	stabilized and immobilized metal anion such as Arsenic	Lebrun et al. (2020) Simiele et al. (2020)
				negatively affected plant growth increased soil acidity, metals phytoavailability and accumulation/toxicity in root system	Lebrun et al. (2020, 2019)
Populus euramericana, Salix purpurea and Salix viminalis,	Abbaretz mine (Loire-Atlantique, Pays de la Loire) mine	France	Biochar amendments (plant biomass or animal wastes pyrolyzed with Iron-based amendments (iron sulfate)	improved the physico-chemical properties of mining polluted soil with Arsenic reduced metal concentrations and metal uptake in plant no positive effect on plant growth	Simiele et al. (2020)
Populus nigra L.	Loire valley mine	France	compost (garden soil) and biochar	decreased metal concentrations enhanced effectively soil stabilization for Pb	Nandillon et al. (2019)

ANNEX 3.2. The mutual interexchange and interactions between *Pinus halepensis* and the pioneer tailings colonizer plants co-cultivated with the application of municipal organics compost amendments

	Plant species co-cultured	**Mine**	**Location**	**Soil amendments**	**Effects**	**Reference**
Pinus halepensis	*Piptatherum miliaceum*	mine tailings of Cartagena-La Union Mining District	Southeast of Spain	municipal organic waste compost	enhanced soil stabilization process in combination together	Martínez-Oro et al. (2017, 2019)
					fast growing ability and rapid soil vegetation, soil fertility improvement by *Piptatherum miliaceum*	
					negatively affected as the decrease of total biomass of *Pinus halepensis*	
					induced severe N deficiency in pine leaves	
					Increased the C/N ratio of *Pinups halepensis* leaves	
					Increased Zn and Mn concentration in *Pinus Halepensis* leaves	
	Pinus miliaceum	mine tailings of Cartagena-La Union Mining District	Southeast of Spain	municipal organic waste compost	competitive abilities for nutrient acquisition especially for nitrogen	Martinez-Oro et al. (2017)
	Pistacia lentiscus	South (the Metapontino area, Matera, Basilicata region) North (the Faenza area, Ravenna, Emilia Romagna region)	Italy	organic compost amendment (commercial green compost)	improvement of chemical-physical and biological properties of related degraded soils in North of Italy	Doni et al. (2017)
					an effective technique for soil restoration/ soil rehabilitation	
					increased the carbon and humic content of the soil	
					activated and fostered the mineralization processes	
					reduced plant oxidative stress in the leaves of both plants	
		Valdemingo´mez mine area	Southeast of Spain	composted urban waste amendment (the remained of ferro-magnetics, glass, plastic, paper, paperboard)	improved soil physical parameters, soil stability, seedling growth, soil hydraulic conductivity	Román et al. (2003)
					reduced 5% of erosive processes	
					increased the concentration of available Mn, Zn, and Cu in *Pinus halepensis*	
					reduced C/N ratio, Na and K concentrations, the humic acid to fulvic acid ratio and the lipid content	
					Pistacia lentiscus performed better than *Pinus halepensis* in phytoremediation and revegetation in gypsum and calcite semiarid soils	

CHAPTER 4

Experimental activities

4. Geochemical and mineralogical investigation on the soil-plant system

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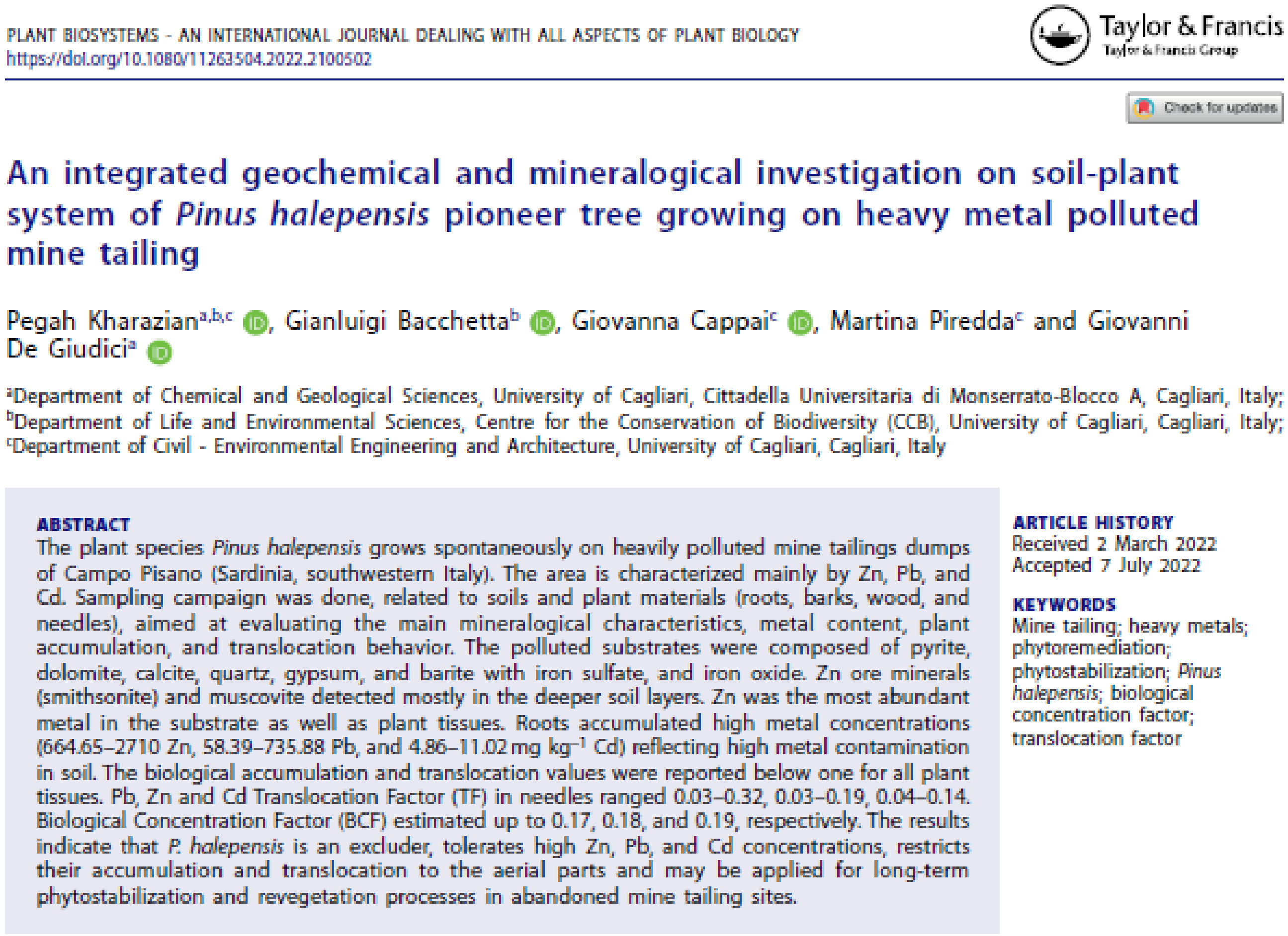

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An integrated geochemical and mineralogical investigation on soil-plant system of *Pinus halepensis* pioneer tree growing on heavy metal polluted mine tailing

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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 sulfate, 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. The 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.

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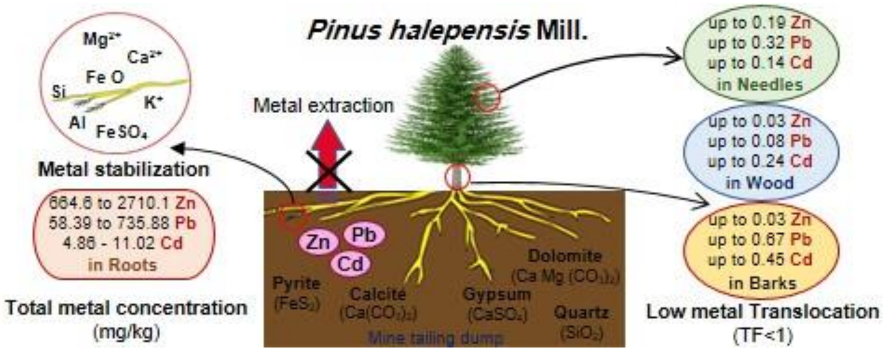


4.1. Introduction

The dispersal processes of mine wastes pose a significant threat to the surrounding environment and cause an adverse impact on soil, water, wildlife and 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 sulfides 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 tolerates 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 *Pinus 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.

4.2. Materials and Methods

4.2.1. 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., 2020a). Mineralization consists of pre-Variscan sulfides (Zn and Pb sulfide), and non-sulfide 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., 2020b; Aversa et al., 2002). The most presented Zn-bearing minerals are smithsonite ($Zn\,CO_3$), hemimorphite [$Zn_4\,Si_2\,O_7\,(OH)_2\,(H_2O)$], 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* (Sm.) Greuter] and their responses to different soil amendments (Lai et al., 2015; Boi et al., 2020b). 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.

4.2.2. 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 4.1c and table 4.1).

It should be noted that metal concentrations of CP mine wastes are highly heterogeneous due to the different extraction methods applied for mine exploitation activities. Moreover, the surface layer of the CP area is characterized by a carbonate lithology rich in alkaline waste materials (coming from the Monteponi area). This was loaded/ discharged into the CP waste dump to reduce the acid mine drainage and to avoid the direct contact of air and water with the acid and pyrite-rich tailings. Therefore, according to these specific characteristics of the CP site and the aim of the research, sampling was done from three different points of CP where *P. halepensis* grows.

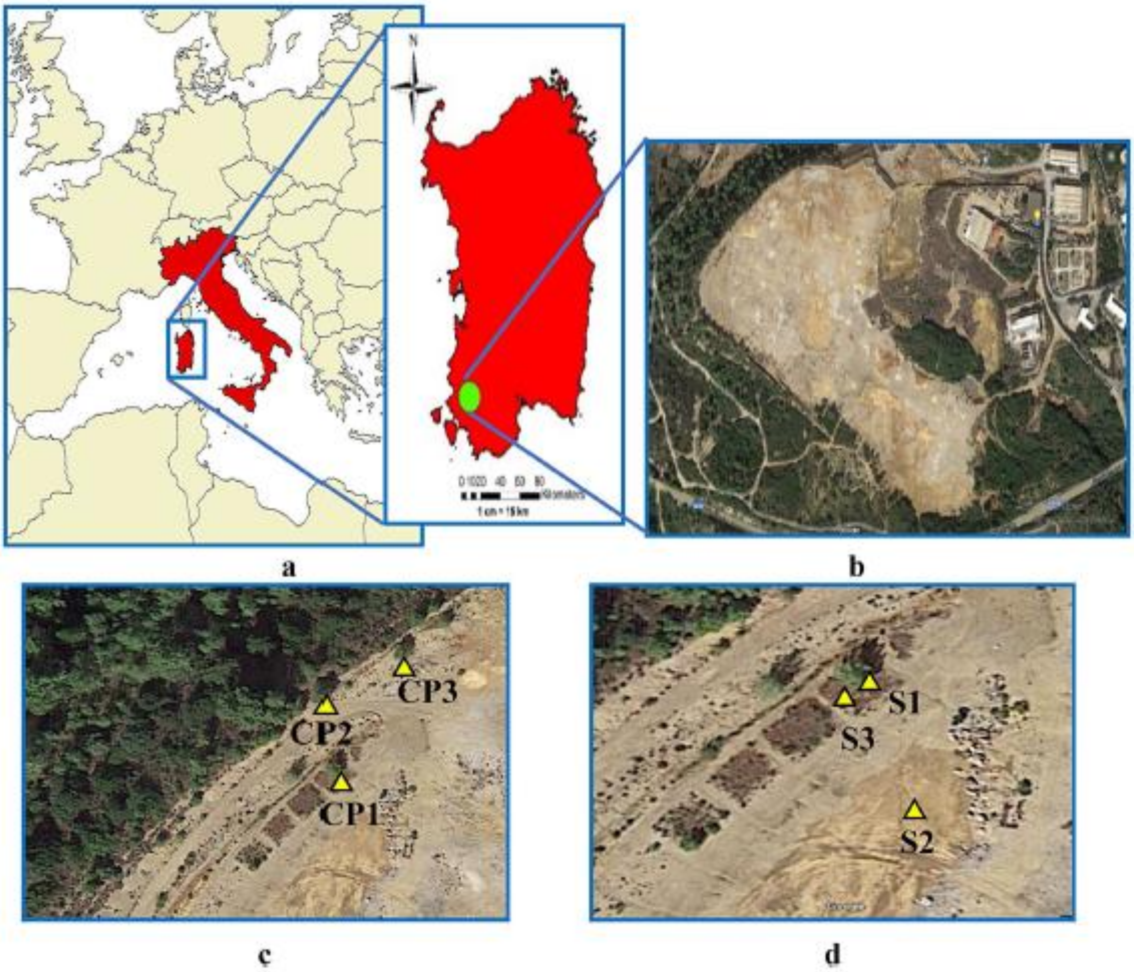


Figure 4.1. Geo-location maps of the study area; (**a**) Location of Sardinia in the Mediterranean Basin (the green spot indicates the sampling site); (**b**) Campo Pisano tailing dump; (**c**) details of sampling points of the collected plant parts from specimens of *Pinus halepensis* (CP1, CP2 & CP3); (**d**) sampling points of the collected core drilled soil samples (S1, S2, and S3).

Table 4.1. *Pinus halepensis* and soil sampling sites, location and the number of collected samples; **SC:** soil core drilled samples, SR: soil around root, R: Root, B: Bark, W: core drilled wood, and N: Needles

Campo Pisano Sampling points	**Distance from *P. halepensis* in the amended plot**	**Code**	**Type and nº of samples**						**Coordinates (WGS 84)**
			SC	**SR**	**R**	**B**	**W**	**N**	
In the amended plot	--	CP1	-	1	1	3	1	6	N 39°17'48.2", E 8°31'54.2"
Out of the amended plot	3–4 m	CP2	-	1	1	3	1	6	N 39°17'48.7", E 8°31'54.0"
Out of the amended plot	6–7 m	CP3	-	1	1	3	1	6	N 39°17'49.0", E 8°31'54.6"
In the amended plot	50cm	S1	1	-	-	-	-	-	N 39°17'48.2", E 8°31'54.1"
Out of the amended plot	4–5 m	S2	1	-	-	-	-	-	N 39°17'47.7", E 8°31'54.4"
In the amended plot	1–2 m	S3	1	-	-	-	-	-	N 39°17'48.1", E 8°31'54.1"

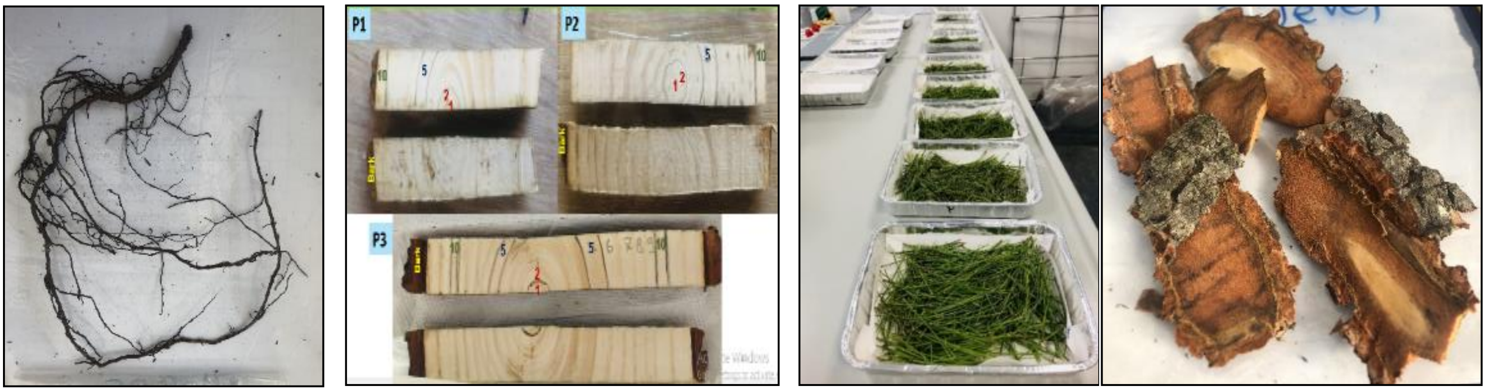
The samples of each *P. halepensis* consist of terranea and epigean organs: **(i)** plant roots (R) **(ii)** six young and old needles (N) of the first level, second level and third level of each plant height; **(iii)** three bark samples (B) from the main stem, the first branch and the second branch 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 (1st year), **(ii)** middle age (5 years), and **(iii)** current age (10th or 12th 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 4.1c); **(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 4.1d).

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 4.1d and Figure 4.2 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 4.1).

Figure 4.2. Campo Pisano experimental plot location where *Pinus halepensis* grown

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), homogenized 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 (see more in Figure 4.3 and Figure 4.4).



Root Wood Needles Barks

Figure 4.3. Collected samples (roots, woods, needles and barks) of *Pinus halepensis*

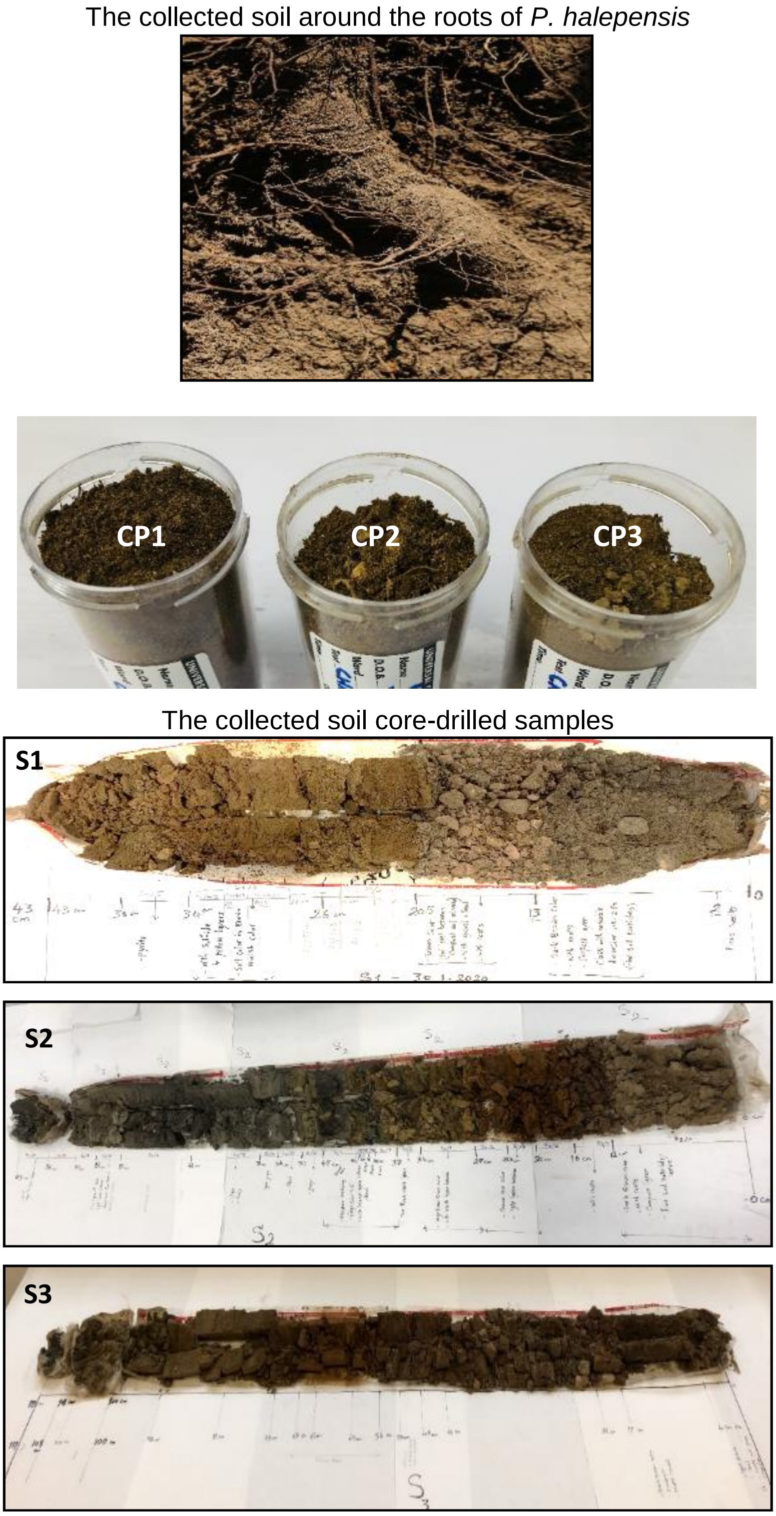


Figure 4.4. Collected soil samples; soil around the roots (CP1. CP2 and CP3); Soil in depths (S1, S2 and S3)

Mineralogical and chemical characterization

Figure 4.5 summarized the mineralogical and chemical lab activities carried out for the collected soil and plant specimens.

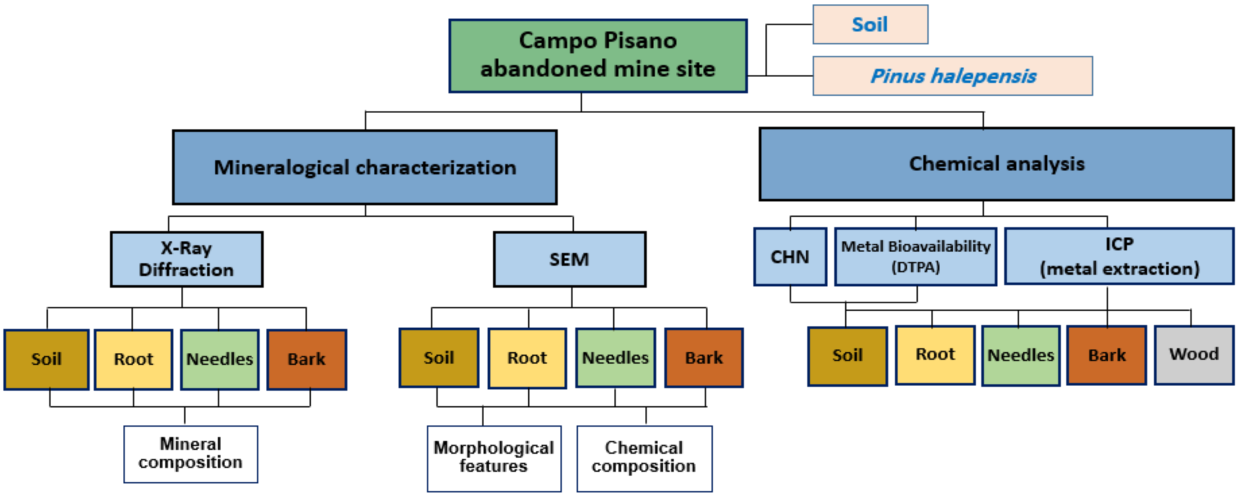


Figure 4.5. Summary of the Lab activities

4.2.3. 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.

4.2.4. 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).

Elemental characterization of the soil was carried out on 0.5 g digested in 9 ml of concentrated HNO_3 and 3ml of HF. Digestion was carried out through the laboratory microwave system (Start D, Milestone, Sorisole, Italy, Advanced Microwave Digestion System, Milestone) at the temperature of approximately 180 ± 5 ºC in approximately 5.5 minutes and remain at the same temperature for 9.5

minutes. After cooling, the vessel contents were filtered, and the solution was diluted up to 50 ml final volume using distilled water. Zn, Cd, and Pb concentrations were determined by ICP-OES. Elemental characterization of the plants was carried out on 0.5 g. A high-purity mixture of 5 ml of Milli-Q water (<0.1 mS/cm), 1 ml of H_2O_2, and 9 ml of HNO_3, was added to the plant samples into microwave vessels. Samples were processed together with a reference (EP-H-1150, SCP Science, Quebec, QC, Canada) and blank GBW-07603-GSV-2 (bush twigs, and leaves) materials prepared with the same mixture. Acid digestion was performed by the microwave Start D, Milestone, Sorisole, Italy, Advanced Microwave Digestion System, Milestone reaching at the temperature of approximately 180 ± 5 ºC in approximately 5.5 minutes and remain at the same temperature for 9.5 minutes. After cooling, the mixture was filtered (0.4 mm), and the solution was made up to 50 ml final volume using distilled water. Zn, Cd, and Pb concentrations were determined by ICP-OES).

The total amount of metal concentrations in all collected substrates were calculated in three repetitions for each sample. Then after, the values of three replication of one composite sample were analytically checked for the quality of analysis according to the aim of the research.

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). As required by the methodology, 10 grams of selected soil samples weighed into a conical flask with 100 ml of 0.005 M of Diethylene Triamine penta-Acetic Acid (DTPA) solution (1.96 g of DTPA, 14.92 g of triethanolamine and 1.47 g of $CaCl_2.2H_2O$ and then let to dissolve in 1000 ml of ultra-pure water in the final solution adjusted to pH~7.3). The flask was shaken for half an hour at 180 oscillations/minute and the mixture was filtered. The metal concentration of final solution was measured with 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):

$$BCF = [M_{Root}] / [M_{Soil}] \tag{1}$$

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

$$BAC = [M_{Epigean}] / [M_{Soil}] \tag{2}$$

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

$$TF = [M_{Epigean}] / [M_{Root}] \tag{3}$$

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

4.3. Results

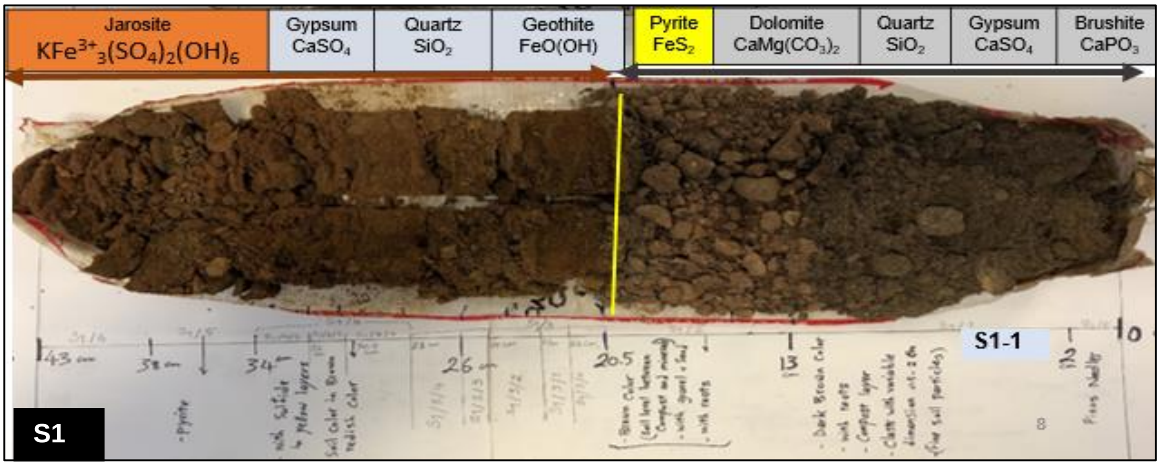
4.3.1. Mineralogical and chemical characterization

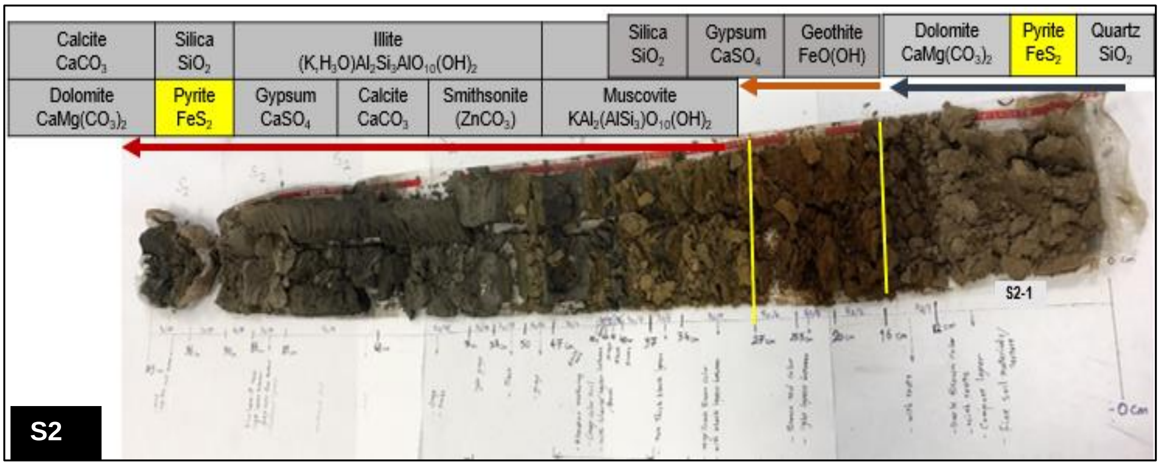
Bulk soil samples

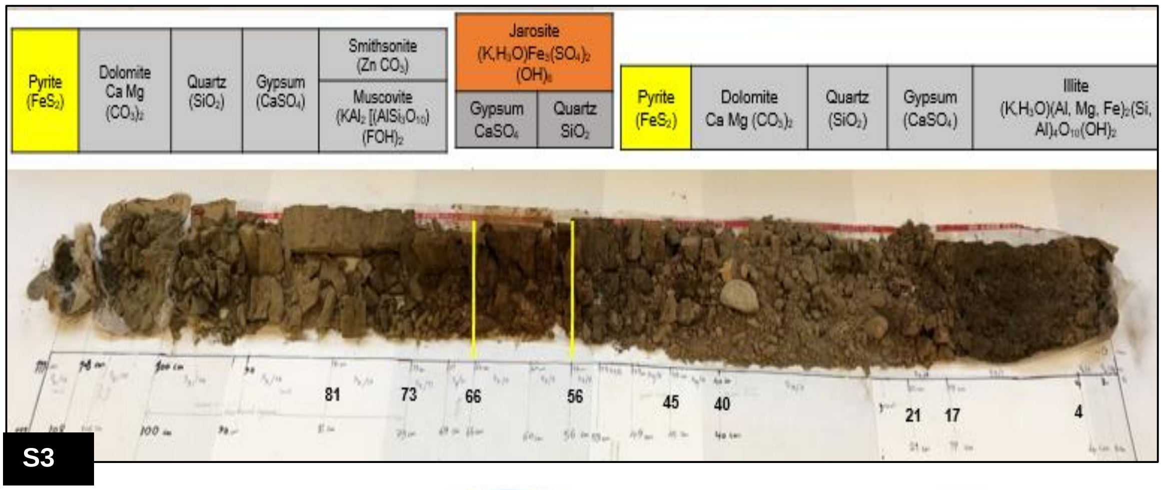
Table 4.2 shows the results of the XRD analysis performed on the collected bulk soil samples. Dolomite ($CaMg(CO_3)_2$), quartz (SiO_2), and gypsum ($CaSO_4$) are present in all different samples. Pyrite (FeS_2) was detected in the bulk layers, except in the layers of S1 (20–43 cm), S2 (16–27 cm), and 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 ($ZnCO_3$) 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 [$(K, H_3O)(Al, Mg, Fe)_2(Si, Al)_4O_{10}(OH)_2, (H_2O)$] 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 ($CaPO_3(OH)_2H_2O$) was found only in the uppermost bulk soil layer S1 (0–20 cm) and CP1 (see more in Figure 4.6).

Table 4.2. 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 sulfate	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															●	●		







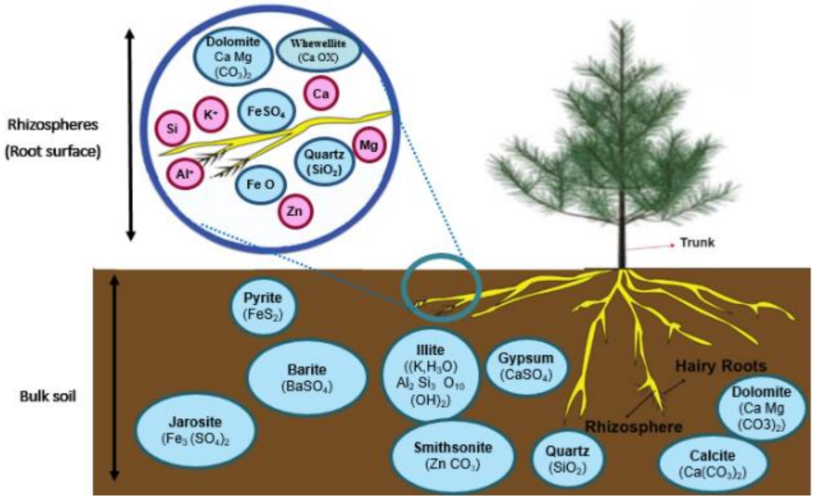


Figure 4.6. An overview of detected minerals of bulk soil and root samples

The outcome of soil CHN analysis showed that the highest percentage of total carbon and nitrogen content is 9.96 and 0.74, respectively, in CP1 (Table 4.3).

Table 4.3. The result of CHN analysis of total Carbon, Hydrogen and Nitrogen in the collected bulk soils around *Pinus halepensis* root samples in the amended plot (CP1); out of amended plot: with 4-5m distances (CP2), and with 6–7 m distances out of plot (CP3) (mean value± SD)

	Soil samples	**C%**	**N%**	**H%**
CP1	5–10 cm around roots	9.96 ±0.04	0.74 ±0.04	0.36 ±0.002
CP2	5–10 cm around roots	5.65 ±0.1	0.27 ±0.02	0.13 ±0.001
CP3	5–10 cm around roots	8.98 ±0.1	0.51 ±0.02	0.34 ±0.03

Table 4.4 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 4.4 reports also 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 4.4. 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

Pinus halepensis root samples

The results of the mineralogical investigation are shown in Table 4.2 and Figure 4.7. 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.

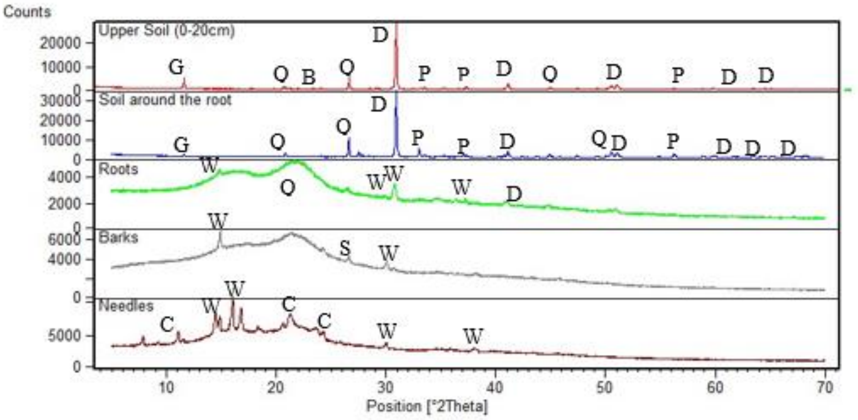


Figure 4.7. XRD patterns of the uppermost soil layer (0–20 cm) of the core sample, collected soil around the roots samples, roots, barks, and needles samples of *Pinus halepensis* grown in CP mine; Mineral legend: Gypsum (**G**), Quartz (**Q**), Dolomite (**D**), Pyrite (**P**), Brushite (**B**), Cellulose (**C**), Silica (**S**), Whewellite (**W**)

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 sulfate, iron oxide, as well as several other elements, mainly Al, Si, Zn, and Fe, adhering to the external part of root samples (Figure 4.8).

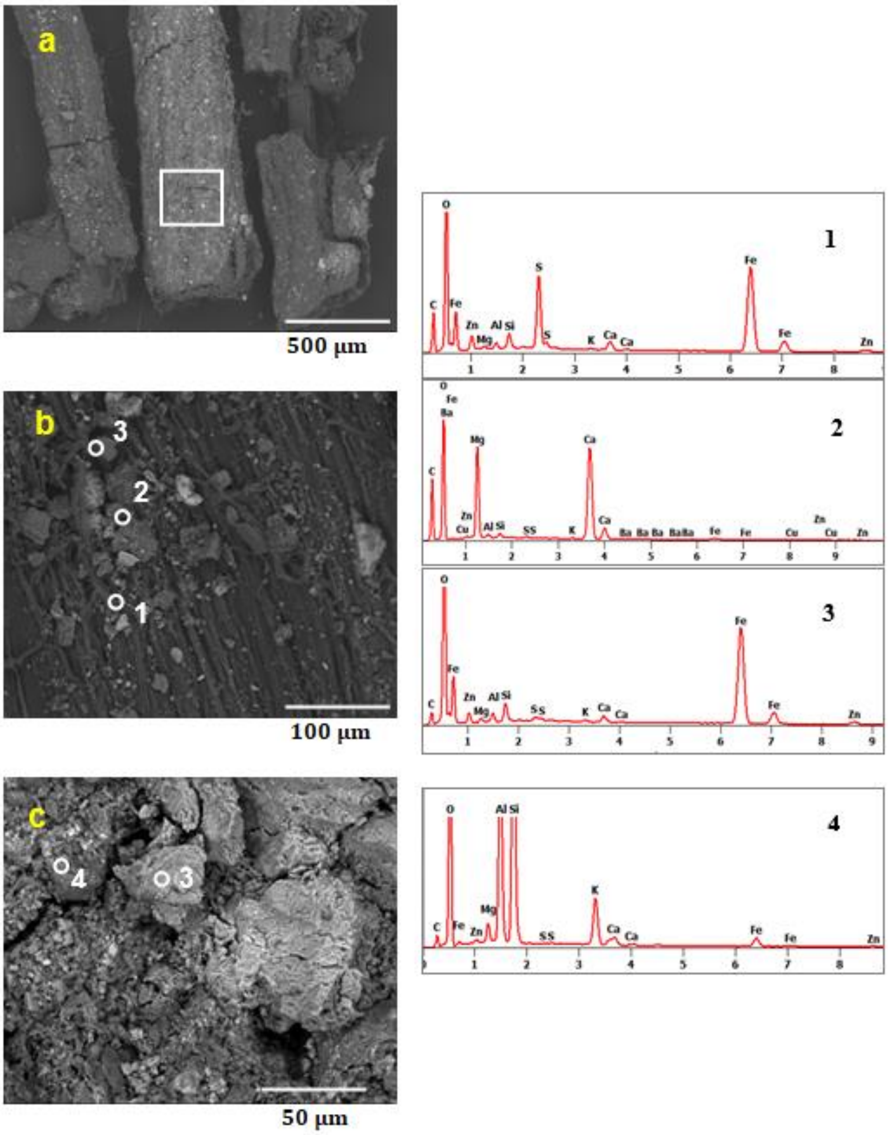


Figure 4.8. Spectroscopy-scanning Electron Microscopy (SEM) analysis of *Pinus halepensis* on root surface (**a**), minerals covering the root surface (**b**) and the presence of iron, aluminum, and silicon on the root surface (**c**); The indicated numbers (1–4) are the points where EDS spectra were acquired.

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, 4413.29, 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}) (Figure 4.9 and Table 4.5).

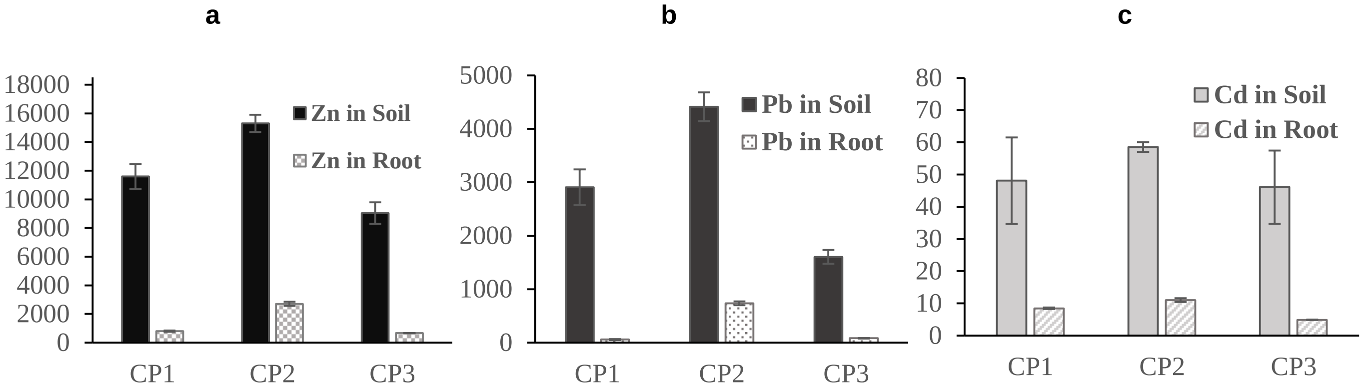


Figure 4.9. The mean concentration (mg kg^{-1}) of Zn (**a**), Pb (**b**); and Cd (**c**) **in** the collected *Pinus halepensis* roots and soils around the roots.

Table 4.5. Mean values of Zn, Pb, and Cd concentration in *Pinus halepensis* tissues samples; the data marked with * indicate the metal concentration of a single wood sample that had not adequate mass for performing a triplicate analysis; the number after ± is standard deviation.

Samples of Pinus halepensis				Mean value of total metal concentration (mg kg^{-1})		
				Zn	Pb	Cd
Roots	CP1			810.14±40.32	58.39±7.45	8.45±0.25
	CP2			2710.13±139.10	735.88±36.27	11.02±0.58
	CP3			664.65±12.04	81.86±2.90	4.86±0.06
Wood	CP1	1&2 Years		20.48*	5*	2.38*
		5 Years		31.73±7.21	3.08±0.82	2.28±0.04
		10 Years		21.7 ±1.49	5.09±0.51	1.31±0.12
	CP2	1&2 Years		9.27±1.03	2.80±0.70	0.55±0.09
		5 Years		10.9±2.55	5.70±2.40	0.70±0.14
		10 Years		10.7±.0.21	4.23±1.10	0.60±0.21
	CP3	1&2 Years		6.90±0.28	2.61±0.66	0.68±0.03
		5 Years		4.26±0.08	3.26±1.49	0.59±0.13
		10 Years		10.5±3.68	1.65±0.64	0.5±0.007
Barks	CP1	main stem		118.61±14.36	60.88±16.88	3.66±0.49
		1st branch		73.84±0.77	31.76±7.58	3.43±0.06
		2nd branch		105.72±1.81	25.01±6.53	3.66±0.06
	CP2	main stem		316.66±25.01	267.58±18.08	2.36±0.06
		1st branch		27.18±1.27	23.79±1.57	0.9±0.0003
		2nd branch		26.07±0.54	27.24±1.56	1.66±0.06
	CP3	main stem		583.84±3.05	582.41±1.01	3.13±0.06
		1st branch		54.69±1.39	81.79±3.01	1.26±0.06
		2nd branch		34.98±1.65	42.44±2.95	2.23±0.12
Needles	CP1	1st level	young	136.23 ±3.93	12.30 ±1.08	1.00 ±0.001
			old	219.14 ±6.36	31.82 ±6.54	1.66 ±0.06
		2nd level	young	156.38 ±1.81	8.69 ±0.90	1.30 ±0.10
			old	166.03 ±1.85	26.75 ±4.33	1.66 ±0.06
		3rd level	young	109.35 ±9.40	5.99 ±1.06	0.73 ±0.06
			old	150.46 ±2.32	18.78 ±0.80	0.97 ±0.06
	CP2	1st level	young	71.98±1.31	29.59 ±1.86	0.47 ±0.06
			old	83.15 ±2.60	25.75 ±0.45	0.57 ±0.06
		2nd level	young	78.76 ±5.27	6.69 ±0.43	0.37 ±0.06
			old	102.13 ±5.34	28.46 ±1.96	0.50 ±0.0003
		3rd level	young	65.01±3.96	4.75 ±0.57	0.40 ±0.0002
			old	99.78 ±3.48	26.70 ±2.37	0.57 ±0.06
	CP3	1st level	young	77.41 ±1.65	38.64 ±1.95	0.60 ±0.0001
			old	75.21 ±0.39	35.41 ±0.51	0.70 ±0.0003
		2nd level	young	51.23 ±1.68	9.75 ±0.25	0.50 ±0.0006
			old	89.15 ±3.89	46.84 ±1.37	0.67 ±0.06
		3rd level	young	43.45 ±2.19	6.92 ±0.20	0.37 ±0.06
			old	73.69 ±2.02	19.78 ±0.20	0.50 ±0.0005

Pinus halepensis bark samples

The XRD analysis of the bark sample in Figure 4.7 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 4.10b, point 4) and Al-silicates (Figure 4.10a, points 1 and 2) were mainly detected together with Zn and Fe in the external part of the bark sample (Figure 4.10b, 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 4.10c 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 4.10c). Furthermore, Figure 4.10a points 1 and 2 clearly show that silica is present in the external part of the barks, while Figure 4.10b (points 3 and 4) and Figure 4.10c (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.

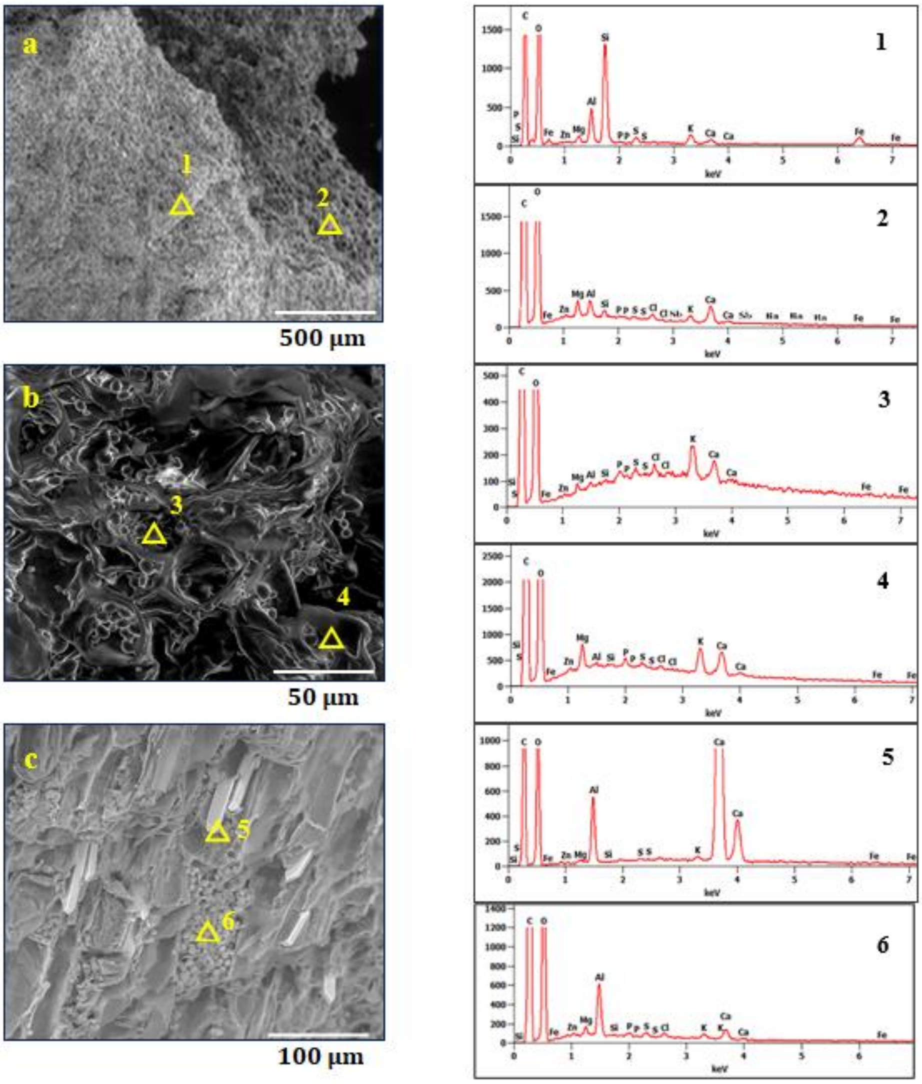


Figure 4.10. SEM-EDS analysis of selected *Pinus halepensis* bark samples**;** External part of bark **(a)**, inner part of fresh bark **(b)**, and the inner part of dried bark samples **(c)**. The indicated numbers from 1 to 6 are the points where EDS spectra were acquired.

Figure 4.11 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}).

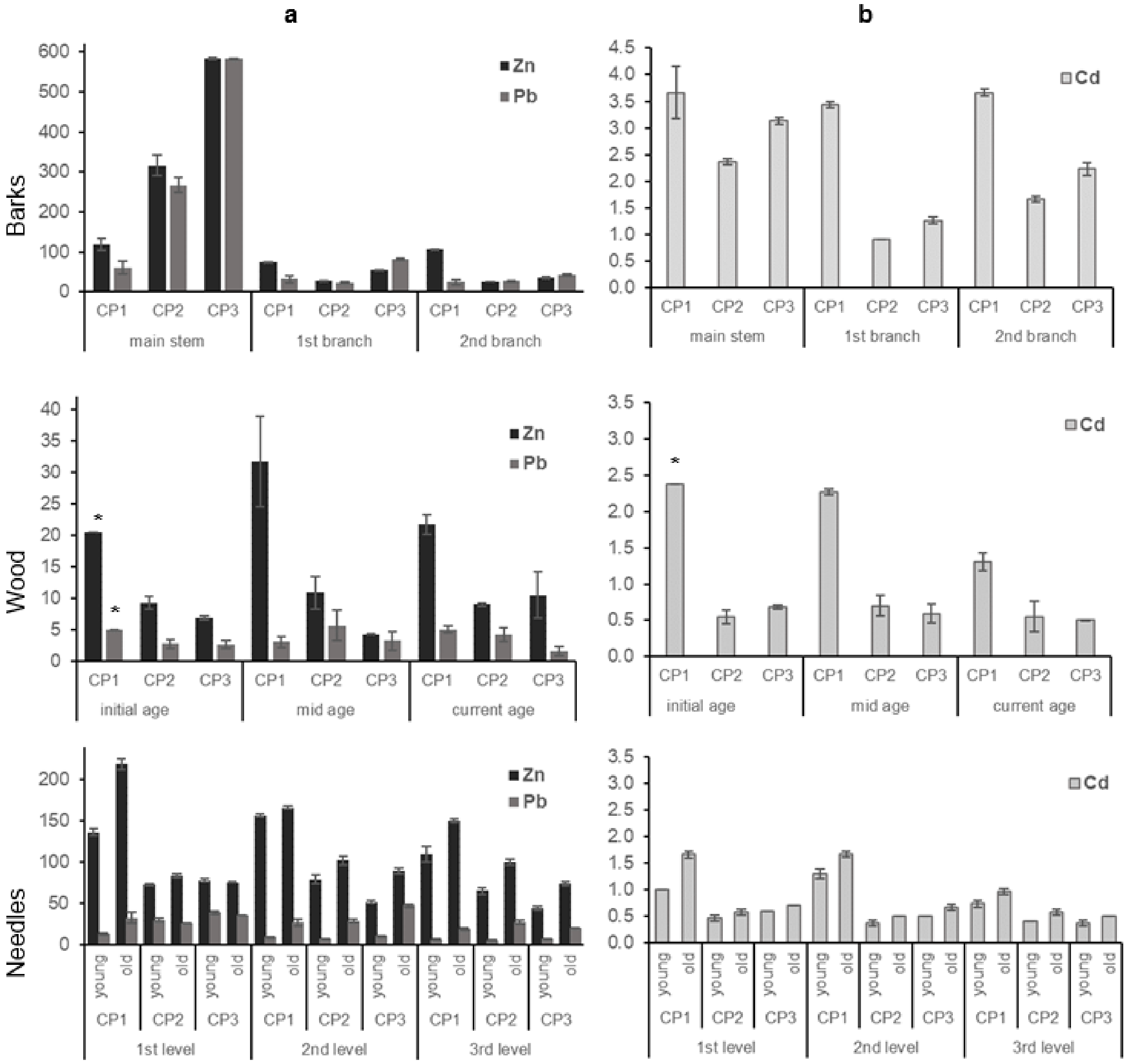


Figure 4.11. Mean concentration of Zn, Pb (**a**), and Cd (**b**) in *Pinus halepensis* tissues samples (barks, wood, and needles); n=3; the values marked with * indicate the single metal concentration value of the wood sample that had not adequate mass for performing a triplicate analysis.

Pinus halepensis wood samples

Figure 4.11 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 concentrations along the considered life spans.

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 4.2 and Figure 4.7). 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 Figure 4.12 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 (Figure 4.12 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 (Figure 4.12). 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).

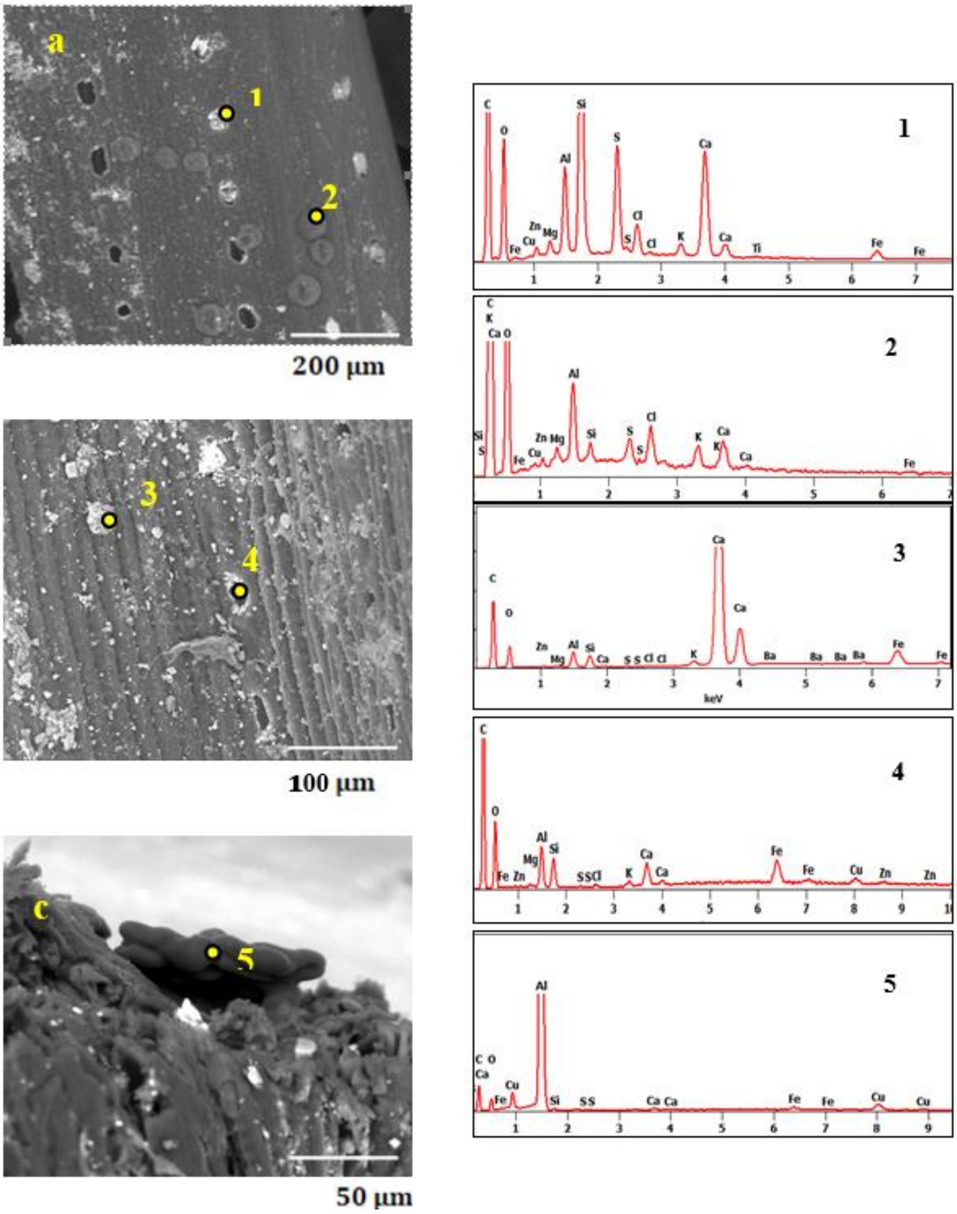


Figure 4.12. SEM-EDS analysis of the collected *Pinus halepensis* needles samples; external part of needles **(a)**, inner part of needles **(b)** and the details of the inner part of dried needles after samples were dried **(c)**; The indicated numbers from 1 to 5 are the points where EDS spectra were acquired.

4.3.2. Biological accumulation parameters

Figure 4.13 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 uptake 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).

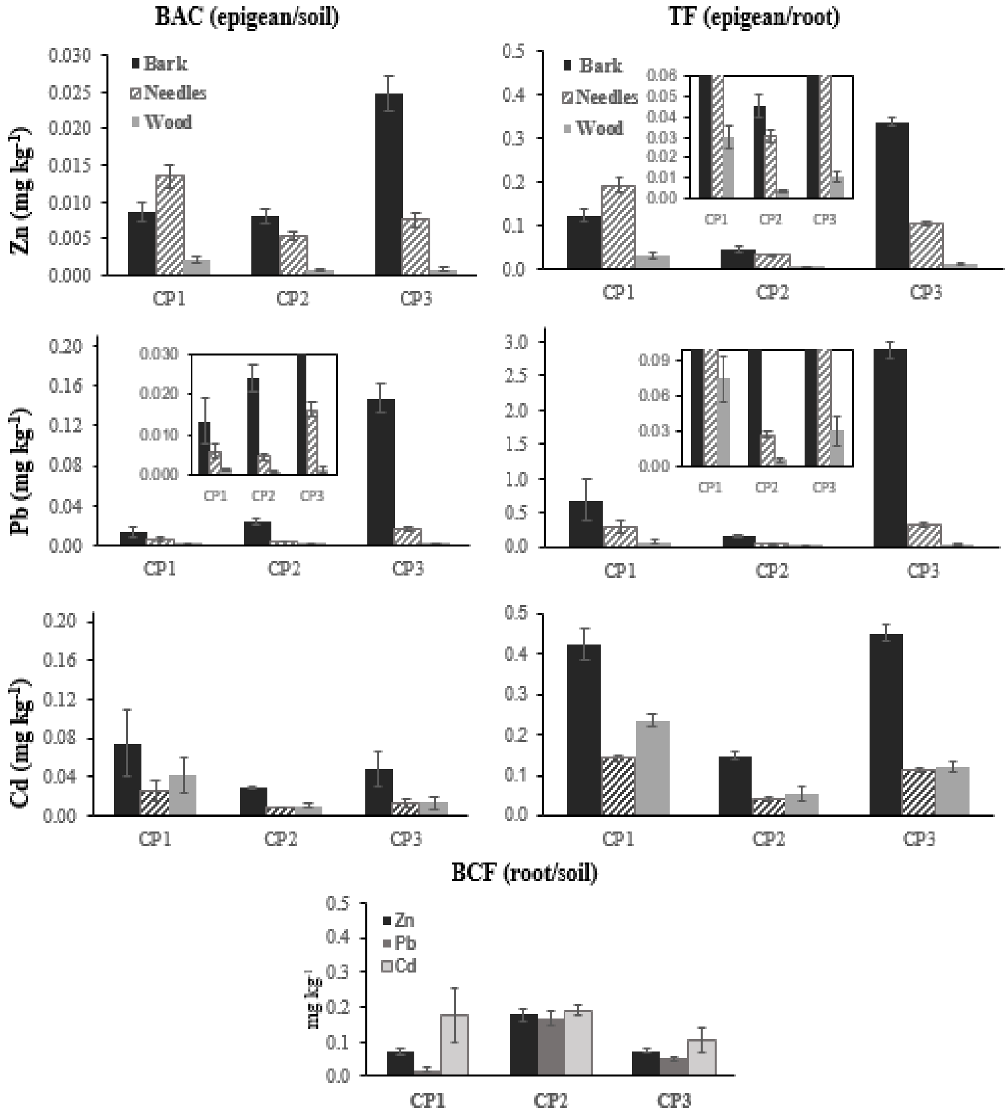


Figure 4.13. Biological accumulation coefficients (BAC, TF, and BCF) calculated from the data in table 2

4.4. Discussion

4.4.1 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 4.2) 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 4.11 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 sulfide (Figure 4.10a, 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 4.6). Among available literature, the findings of Disante et al. (2010) reveal 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 4.6). 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 4.6. 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 4.7. Compilation of the literature Zn and Pb data for some investigated plant species in the Campo Pisano mine area

	Total Zn concentration (mg kg^{-1})				Zn Biological parameters			Total Pb concentration (mg kg^{-1})				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.(2020a)
*Juncus acutus**	9700[e]		4700	480[c]	0.1	0.04	0.038								Medas et al. (2017 and 2023)
	20400[f]		10000	1140[e]	0.11	0.06	0.49								
	81400		3500	380[c]				6400		580	15[c]				
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

n.r. indicated not reported data
[a] CP experimental plot with Compost
[b] in plant leaves
[c] in plants shoots/ stems
[d] Average of data in the period May 2008–May 2010
[e] measure in Naracauli area
[f] measure in Sa masaarea
[g] measured in San Giorgio area
* plant species investigated in mine area of SW- Sardinia

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. (2020a) 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 4.7).

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. (2020a) 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.

4.4.2. The mineralogical investigation

The XRD data in table 4.2 confirms the mineralogical composition of the soil sample that indicates the primary mineral of tailing, metal sulfide, and carbonate (i.e., pyrite, calcite, and dolomite) with quartz. The secondary minerals (i.e., Sulfate 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 sulfate 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., 2019), *Euphorbia cupanii* Bertol. ex Moris and *Juncus acutus* L. (Medas et al., 2015 and 2019) 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 sulfate with mineral grains coating on root surface constituted of barite, Fe, Zn, and Al silicate. In roots samples of *P. halepensis*, where soluble Ca sulfate 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 4.10c, point 4.5).

4.5. 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 sulfate, iron oxide, and jarosite are present in the soil substrate around the roots as well as on the surface of plant roots. These sulfate 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.

CHAPTER 5

5. Assessing the geochemical forms of metals through BCR method

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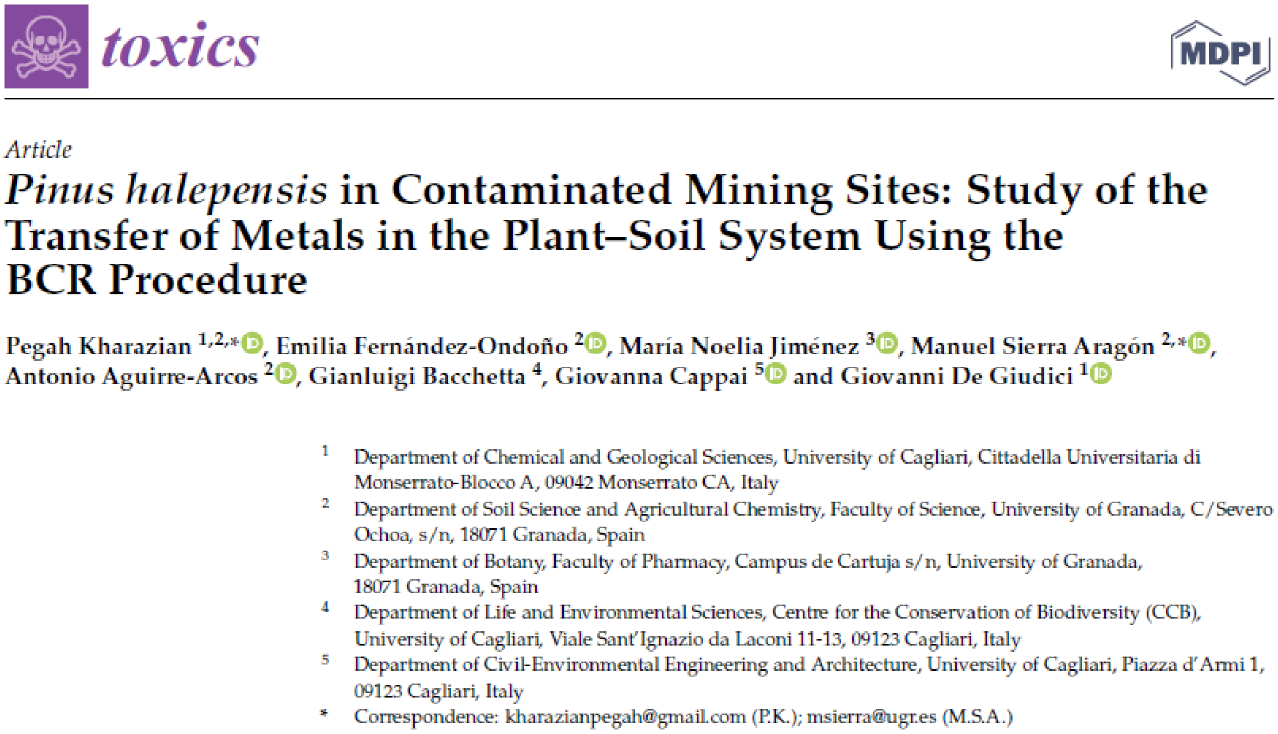

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Article

Pinus halepensis in Contaminated Mining Sites: Study of the Transfer of Metals in the Plant–Soil System Using the BCR Procedure

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Abstract:

The study aimed at evaluating the geochemical fractions of Zn, Pb, Cd and their bioavailability in soil in-depth and around the root of *Pinus halepensis* grown on heavily contaminated mine tailing in south-western Sardinia, Italy. The contaminated substrates were partly investigated in a previous study and are composed of pyrite, dolomite, calcite, quartz, gypsum, barite, iron-sulfate and iron-oxide. The geochemical fractions and bioavailability of Zn, Pb and Cd were measured through the BCR extractions method. Cadmium in the superficial contaminated substrates was mainly found in the exchangeable BCR fraction. Zinc and lead were often found in the residual BCR fraction. PCA confirmed that the uppermost alkaline-calcareous layers of mine waste were different with respect to the deeper acidic layers. We demonstrated that Pb and Zn were less present in the exchangeable form around the roots of *P. halepensis* and in soil depth. This can be due to uptake or other beneficial effect of rhizospheres interaction processes. Further studies will shed light to confirm if *P. halepensis* is a good candidate to apply phytostabilization in mine tailing.

Keywords: mine tailing; BCR sequential extraction; phytoremediation; phytostabilization; *Pinus halepensis;* geochemical characteristics

5.1. Introduction

Mine wastes are among the most hazardous sources of metal contamination for the surrounding area. Most often, minerals in the mine tailing sites undergo oxidation and dissolution due to exposure to atmospheric agents (Mendez and Maier, 2008). Indeed, the mine tailings are pollution sources often subjected to the mobilization and dispersion of metal contaminants by wind, seasonal heavy rainfalls, water run-off and water erosion [Concas et al., 2006; Barbafieri et al., 2011) and can seriously spread a high range of trace element contaminants in soils, ground-waters and plants (Cao et al., 2008; Bacchetta et al., 2015).

Some plant species that have adapted to grow in highly contaminated environments have been suggested as some of the most feasible and effective tools in phytostabilization through bio-mineralization processes (Jiménez et al., 2011 and 2021). This can eventually play an important role in the immobilization of elements around the root systems and the recovery of the polluted mine sites (DE Giudici et al., 2015; Lai et al., 2015; Conesa et al., 2019). In mine waste environments, soil elements can be associated with different geochemical forms, such as soluble exchangeable, carbonates, iron-manganese oxides, residues and organic materials. Indeed, the elements with the highest bioavailable potential are mainly those related to the water-soluble and exchangeable fraction in the soil–plant system, whereas the residual fraction is considered not to be bioavailable for plants (Rodríguez, et al., 2009). The sequential extraction method is generally used for assessing the potential of element mobility in different geochemical forms and analyzing their bioavailability (Tessier et al., 1979; Rauret et al., 1999; Gleyzes et al., 2002). The BCR three-step sequential extraction method (hereafter BCR) is an analytical tool that has been proposed by the European Community Bureau of Reference (Van Der Eijk, 1979; Rauret et al., 1999) and is widely used for performing element extraction analysis in mines. In this method, metals are extracted using chemical reagents in three steps and four fractions. In fact, the BCR method detects the different metals in the forms of (**i**) weak acid-soluble or exchangeable fraction bound to carbonates, (**ii**) reducible fraction related to Fe and Mn oxides, (**iii**) oxidizable fraction (organic matter and sulfides) and (**iv**) residual fraction bound to silicate and mineral structures that is a relatively resistant and stable fraction (Davidson et al., 1998; Fernández, et al., 2004; Bacon and Davidson, 2007).

The hypothesis of this work is that the geochemical fractions of Cd, Zn and Pb can be controlled by the physico-chemical properties and the mineralogical composition of soil as well as the plant root system. On one side, the physico-chemical properties of soil, such as pH, electrical conductivity, organic carbon, soil texture and the total metal concentration of elements in soil can affect the solubility of elements and the bioavailable fractions in soil (Cabała and Teper, 2007; Nowak-Winiarska et al., 2012; Fernández et al., 2017), waste and sediments (Ure et al., 1993; Gabarrón, et al., 2018). On the other hand, the mineralogical characteristics of soil (Conesa et al., 2019) and the plant root activity (Conesa et al., 2019) can rule trace element bioavailability. Hence, it is important to assess the mobility of elements and specify the stability of different forms of elements considering both the mineralogical and physico-chemical characteristics in the soil–plant system.

In this study, the soil and plants were from south-west Sardinia, an Italian mine area with a rich history of mine activities that have left large quantities of mine wastes in dumps and flotation tailings. The environmental risk of the area has increased due to inadequate actions for the mitigation of metal impact after the mines shut down (Bcchetta et al., 2012). Several pilot projects have been carried out focused on the rich plant diversity of the areas in order to identify the most suitable autochthonous plant species possessing the best phytoremediation capabilities to be exploited (Bcchetta et al., 2018; Medas et al., 2015 and 2019).

Pinus halepensis Mill. is a Mediterranean widespread tree species that has been frequently noted for its ability to tolerate high amounts of metals (Pulford and Watson, 2003; Conesa et al., 2019) and for the restoration of degraded soil in arid and semi-arid areas (Querejeta et al., 2008; Párraga-Aguado et al., 2013)*,* as well as the degraded and low fertile soils of mine wastes (Sardans et al., 2005). To our knowledge, little attention has been devoted to the study of the geochemical fractions of elements in the soil–root system of *P. halepensis* as well as in the different depth layers of soil where it grows spontaneously in the multiple heavy metal-contaminated mine tailing.

A preliminary study was carried out in the abandoned mine tailing site of Campo Pisano (SW-Sardinia) by our research group in 2020 to investigate the contaminated soils and different compartments (roots, barks, wood, and needles) of *Pinus halepensis* and to evaluate the metal content, plant accumulation, and translocation behavior as well as the main mineralogical characteristics of Zn, Pb and Cd (Kharazian et al., 2022a). The findings showed that despite the detection of a high concentration of metals in the soil the estimated Biological Accumulation Coefficient and Translocation Factor (BAC, BCF and TF<1) were very low. These findings indicated that *P. halepensis* has a qualified metal toleration capability and has limited metal accumulation and translocation factors in the aerial parts. Thus, this plant can be considered as an excluder plant and a candidate for phytostabilization projects (Kharazian et al., 2022a).

The aim of this study was to determine the geochemical fractions of Zn, Pb and Cd and the specific geochemical forms of elements present in the same sampling site through the three-step BCR extraction method in relation to the physico-chemical and mineralogical characteristics of the soil in depth and the soil-root system. In order to pursue this aim, the study exploited the pertinent data of mineralogical and chemical analysis of contaminated soils and roots samples available in Kharazian et al. (2022a).

5.2. Materials and Methods

5.2.1. Study area

The study area was Campo Pisano (CP) mine tailings dump that belongs to the Metalliferous Ring of the Sulcis-Iglesiente mining district, one of the most important mine regions of Europe since pre-Roman times, located in south-western Sardinia, Italy (Figure 5.1). The area has a Mediterranean pluviseasonal bioclimate with upper thermo-Mediterranean thermotypes and ombrotypes between the lower sub-humid and the upper dry (Bacchetta et al., 2009). The Campo Pisano ore area belongs to the Metalliferous Ring of Sulcis-Iglesiente mining district and geologically is characterized as a Paleozoic carbonate with the middle Cambrian limestone rocks and pre-Variscan sulfides (Zn and Pb) and non-sulfide deposits (Bechstädt and Boni, 1994). The main common minerals are pyrite

(FeS_2) cerussite ($PbCO_3$) and anglesite ($PbSO_4$), associated with nodules and residual galena (PbS), dolomite ($CaMg(CO_3)_2$), calcite ($CaCO_3$), quartz (SiO_2), barite ($BaSO_4$), and iron hydroxides (Aversa et al., 2002). The CP mine was extensively exploited before it became inactive in 1998 (Boni et al., 1999). The mine also exploited pyritic ores from Genna Luas, leading to tailings rich in pyrite. It should be noted that the surface layer of the Campo Pisano area is characterized by a carbonate lithology rich in alkaline waste materials coming from the Monteponi area (SW-Sardinia) (Bechstädt and Boni, 1994; Bacchetta et al., 2015). This alkaline cover was done to avoid the direct contact of air and water with the acid and pyrite-rich tailings. Moreover, the metal concentrations of CP mine wastes are highly heterogeneous due to the different extraction methods applied for mine exploitation activities (Boni et al., 1999; Bacchetta et al., 2018).

In this study, the term soil refers to the collected mine waste samples. In the previous research study, between the years 2008 and 2010, a successful phytoremediation study on some plant species was carried out in an experimental plot amended with compost produced from the organic fraction of Municipal Solid Waste (MSW) in a CP tailing site (Lai et al., 2015; Boi et al., 2019). The contaminated sampling points of this study are located in the same experimental site and its surrounding area.

5.2.2. Sampling

Six *Pinus halepensis* specimens with a nearly similar height (2 m) and age (10–12 years old) were selected from both non-contaminated and contaminated sites in the south-west of Sardinia (November 2020) where they grow spontaneously (Figure 5.1a and Figure 5.1b).

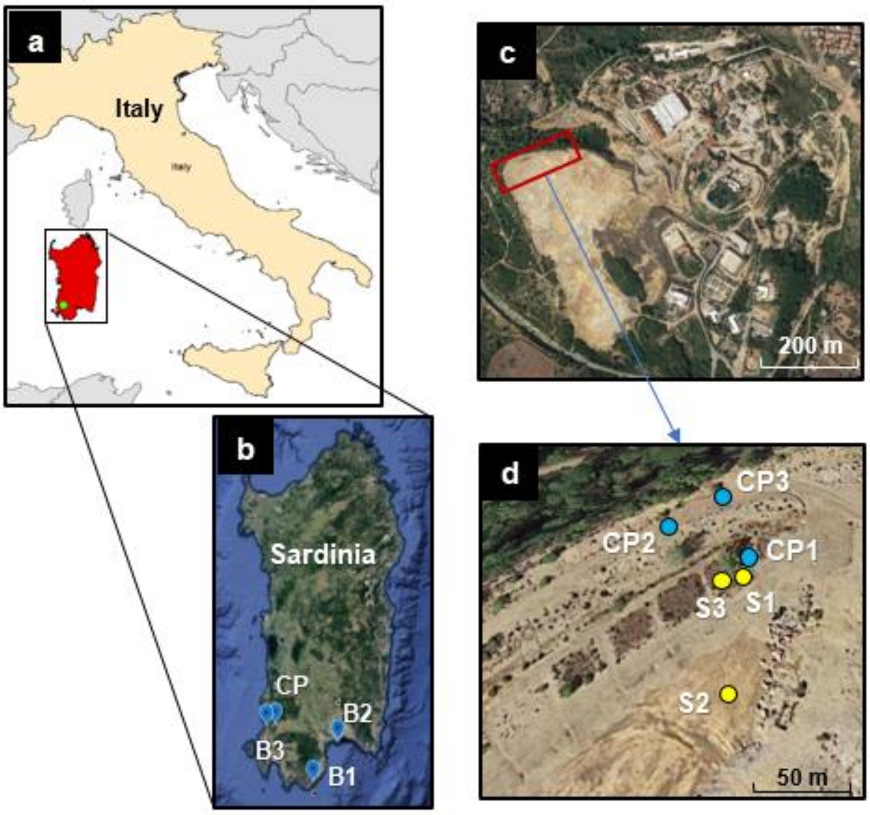


Figure 5.1. Location map of Sardinia (**a**); sampling sites of the collected soils around the root of *Pinus halepensis* in the contaminated Campo Pisano site (CP) and not-contaminated sites (Santa Margherita: B1; Calamosca: B2; and Fontanamare: B3) **(b)**; location map of the study area **(c)**; details of the sampling points and soil samples collected in the CP-contaminated site: the core-drilled soil samples are indicated by yellow spots (S1, S2 and S3); and the blue spots indicate the soil around the root in contaminated (CP1, CP2 and CP3) and not contaminated sites (B1, B2 and B3) **(d).**

Six bulk soil samples were collected from the soils around the root of *P. halepensis* together with its roots samples: (**i**) CP1: located inside the contaminated Campo Pisano (CP) site in the compost-amended plot aged 10 years old (Lai et al., 2015; Boi et al., 2019), (**ii**) CP2: outside of the CP experimental plot distancing 3–4 m from it aged 10 years old, and (**iii**) CP3: located outside of the CP experimental plot distanced 6–7 m from it and aged 12 years old (Figure 5.1c and Figure 5.1d); see more in (Kharazian et al., 2022a), (iv) B1: Blank sample in the non-contaminated site (Santa Margherita, Pula) at approximately 80 km far away from CP mine site, (v) B2: Blank sample in the not-contaminated site (Calamosca, Cagliari) at about 60 km far away from the CP mine site, (vi) B3: Blank sample from the less-contaminated site (Fontanamare, Gonnesa) at about 10 km distance from the CP mine site (Figure 5.1b). The contaminated soils around the root and root samples were exploited the previous study (Kharazian et al., 2022a).

Three soil in-depth core-drilled samples (approximately 70 cm) were also collected from the CP-contaminated mine sites using a core sampler (Atlas Copco's COBRA): **(i)** the first sample in the experimental plot at approximately 50 cm distance from the tree (S1), **(ii)** the second one in a bare site, out of the experimental plot, where there is no vegetation canopy (S2), **(iii)** the third sample in the amended plot at 1–2 m distance from the pine (S3) (Figure 5.1d); see more in Kharazian et al, (2022a). The three soil samples were selected from each core drilled sample, according to the visually recognizable color of the soil substrate and their mineralogical and geochemical characteristics that were investigated in our previous research study (Kharazian et al., 2022a). These layers were selected for BCR analysis, regardless of their soil depth horizon, to provide better interpretation of their metal availability. Samples were named as: uppermost soil [**1** (0–20 cm): S1-1, S2-1, S3-1)], interval depth [**2**: S1-2 (20–28 cm), S2-2 (37–41 cm), S3-2 (20–45 cm)], and subsamples [**3**: S1-3 (32–38 cm), S2-3 (47–50 cm), S3-3 (56–66 cm)]. The 20 cm uppermost soil of each collected core-drilled soil was considered the uppermost soil sample. Figure 5.1c and 5.1d provides the CP site location where the core-drilled soil samples were collected (see more about the collected samples in Supplementary Materials, Table 5.1 and Figure 5.2).

Table 5.1. *S*oil sampling sites, location and the number of collected samples; **SR**: soil around root, **SC**: soil core samples in-depth, **R**: root samples

Sampling points	Distance from *P. halepensis* in the plot	Code	Soil depth (cm)	nº of samples		
				SR	SC	R
In the CP amended plot	--	CP1		1	-	1
Out of CP amended plot	3–4 m	CP2		1	-	1
Out of CP amended plot	6–7 m	CP3		1	-	1
Not contaminated site (Santa Margherita)	20 cm	B1		1	-	-
Not contaminated site (Calamosca)	20 cm	B2		1	-	-
Less contaminated site (Fontanamare)	20 cm	B3		1	-	-
In the CP amended plot	50 cm	S1-1	0–20	-	1	-
		S1-2	20–28	-	1	-
		S1-3	32–38	-	1	-
Out of CP amended plot	4–5 m	S2-1	0–20	-	1	-
		S2-2	37–41	-	1	-
		S2-3	47–50	-	1	-
In the CP amended plot	1–2 m	S3-1	0–20	-	1	-
		S3-2	20–45	-	1	-
		S3-3	56–66	-	1	-

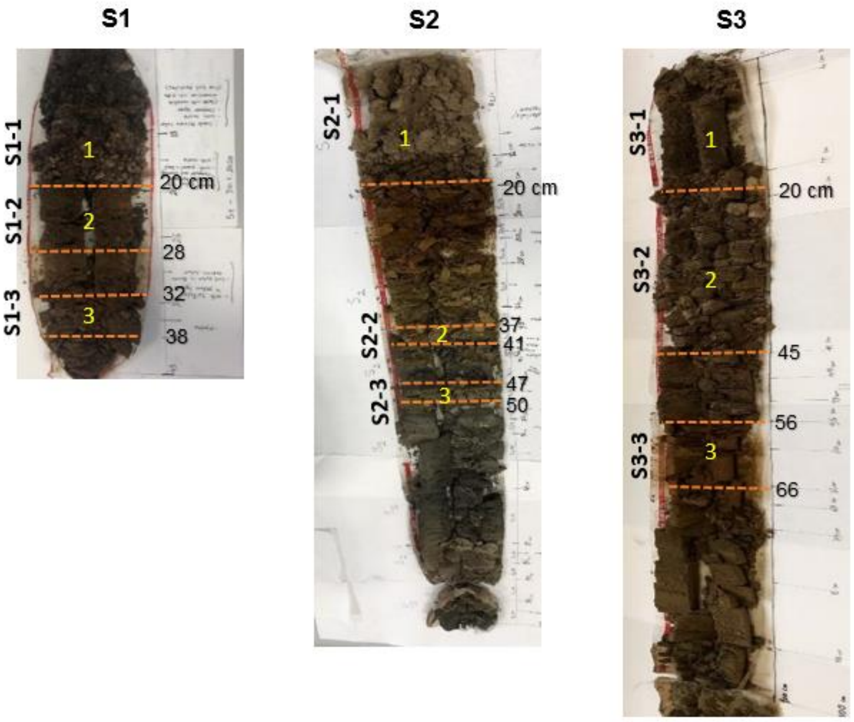


Figure 5.2. Soil samples selected from the different depth layers of core drilled samples in Campo Pisano mine tailing; numbers (1, 2 and 3) indicate the three selected soils for each core sample.

5.2.3. Physico-chemical and mineralogical characterizations of the soil and root

Samples, including bulk soils (rhizospheres solid materials), the soil grains particles around the plant roots (within 3–5 mm around the root) and the soil in deep layers as well as roots samples were all air-dried (for almost a week) at room temperature after gentle shaking, wiping and removal of the remaining particles. A detailed description of sample preparation and the chemical and mineralogical characteristics of the contaminated soils and root samples can be found in Kharazian et al. (2022a). Samples preparation and both SEM and XRD analyses were carried out at the Center for Research University Services (CESAR), Cagliari University, Italy and the remaining chemical and physical analyses, BCR sequential extractions, DTPA extractions and ICP-OES analyses were performed at the Department of Edaphology and Agricultural Chemistry, University of Granada, Spain.

Physical and chemical properties of the soil

Soil pH was measured in 25 mL distilled water with 10 g soil (ratio of 1:2.5) using a conductometer / pH-meter (914 Metrohm). Electrical Conductivity (EC) of the soil was obtained in distilled water (1:1) utilizing a conductivity meter (Eutech CON700). Calcium carbonate content (equivalent $CaCO_3$) was calculated following the method proposed by the Soil Conservation Service (1972). Total carbon and nitrogen as well as organic carbon (OC) content of the substrates were determined through an elemental analyzer LECO ® (TruSpec CN, St. Joseph, MI, USA) calibrated with the reference material (ore minerals). Organic carbon (OC) was analyzed after the soil samples were treated and acid-washed (HCl 1 mol L^{-1}) to remove carbonate content, according to Ussiri and Lal (2008). Amorphous forms of iron and aluminum (Fe and Al oxide) were extracted with 1 M ammonium oxalate and oxalic acid following the Schwertmann and Taylor (1977) procedure and measured by the Inductively Coupled Plasma Optical Emission in a Spectrometer ICP-OES (Perkin Elmer Avio ® 500, Waltham, MA, USA).

Mineralogical characterization of the soil and root

Soil and root samples were analyzed through XRD analysis using laboratory equipment (Pan analytical X'Pert Pro, X'Celerator detector) and software X'Pert High Score Plus (PAN-analytical B.V., Almelo, The Netherlands) to clarify the mineral phases**.** The microscopic characteristics, as well as the element distribution of samples, were clarified under low-pressure conditions through Energy Dispersive Spectroscopy (Thermo Ultra Dry EDS Detector, Pathfinder, Waltham) and Scanning Electron Microscopy (SEM) imaging (ESEM QUANTA 200, FEI) (Medas et al., 2015). The detailed description and the outcome of the mineralogical characteristics (XRD and SEM) of the CP-contaminated soil and root samples can be found in Kharazian et al (2022a).

5.2.4. Total metal concentration of the soil and root

The chemical characteristics of soil samples were analyzed following the official Italian analytical methods (D.M. 13-09-1999) (Lindsay and Norvell, 1978; Barbafieri et al., 1996) and the total metal concentrations in soil and root samples were assessed using the Environmental Protection Agency (EPA) method 3052 (Bacchetta et al., 2012). Total metal concentration in the soil was assessed using acid digestion (9 ml concentrated HNO_3 65% and 3ml of HF 48%) through the laboratory microwave system (CEM Mars® XP1500 Plus, Mathews, CN, USA). Zn, Pb and Cd concentrations of both root and soil samples were determined by ICP-OES, Perkin Elmer Avio® 500, MA, USA, Waltham. The detailed description of the method performed was reported in Kharazian et al (2022a). We also calculated the Biological Concentration Factor (BCF) as the ratio between the metal content in the roots and in soil samples that are available in Kharazian et al (2022a).

5.2.5. Bioavailable concentration of elements (DTPA)

The bioavailable contents of Zn, Pb and Cd were evaluated for all contaminated and non-contaminated soils around the roots through a single extract method performed by using 0.005 M DTPA solution (1.96 g of DTPA, 14.92 g triethanolamine and 1.47 g of $CaCl_2.2H_2O$ dissolved in the final solution of 1L by distilled water adjusted to pH~7.3) added to the substrate samples (Barbafieri, et al., 1996; Guan et al., 2010). The concentration of the extracted metal was measured through the ICP-OES technique (the Agilent 725-ES method). DTPA extraction is considered as an efficient procedure that prevents the carbonate's dissolution and consequently, the release of the bounded metals (Lindsay and Norvell, 1978; Feng et al., 2005). The metal bioavailability in soil was calculated in terms of the percentage values according to equation (1).

$$\text{DTPA bioavailability (\%)} = (M_{\text{Metal bioavailability in soil}}) / (M_{\text{Total metal concentration in soil}}) \times 100 \qquad (1)$$

5.2.6. Sequential extraction procedure (BCR)

The three-step BCR sequential extraction method of the European Community Bureau of Reference was applied to differentiate metal fractions in all collected soils (Rauret et al., 1999; Mossop and Davidson, 2003). Accordingly, the four extracted fractions were defined as exchangeable (**F1**); reducible (**F2**); oxidizable (**F3**) and residual (**F4**) fractions performed through the application of modified reagents in 1 g soil. The mixture of each fraction was centrifuged for separating and measuring the extracted metals through the ICP-OES technique. The remaining materials at the end of each BCR fraction were washed with 20 ml distilled water, shaken for half an hour, and centrifuged to be prepared for the next fraction step (Figure 5.3).

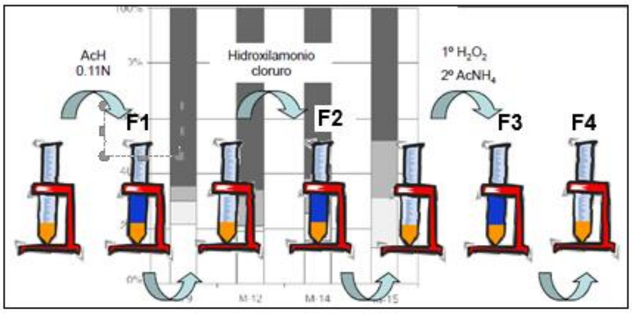


Figure 5.3. the four extracted fractions of BCR procedure

The exchangeable fraction (F1) was performed by extracting metals during 16 h shaking and using 40 mL of 0.11 mol/L acetic acid. In the reducible fraction (F2) 40 mL of 0.5 mol/L hydroxylamine hydrochloride at pH 2.0 was added to the residue of the first step and shaken 16 hr. In the oxidizable fraction (F3) 50 mL of 1 M ammonium acetate (adjusted at pH=2 with HNO_3) was applied following the oxidation process with 10 mL of H_2O_2 (acid-adjusted pH 2) for an hour at room temperature and an hour-heated to 85°C with occasional agitation (PerkinElmer-SPB 50-48S). The same process was repeated until the liquid volume was <1 mL. The residual remained material in the fourth extraction (F4) was oven-dried at 45°C for a day and acid digested with the same digestion procedure as the total metal concentrations. The Zn, Pb and Cd concentration extracted in each BCR fraction was determined through Inductively Coupled Plasma Optical Emission in a Spectrometer (ICP-OES, Perkin Elmer Avio® 500, MA, USA, Waltham).

The precision of the chemical test was evaluated by performing a triplicate sample. Blank solutions and different reference materials were applied to ensure the reliability of the analytical methods in the analysis of the total metal content of the soil samples. The analytical quality control and the accuracy of the analyzed data were verified by analyzing a certified reference material for soil (CRM052-050, Sigma-Aldrich, USA, St. Louis, MO). All standards and blanks were matrix-matched with the samples and reagents.

The ratios between the metal concentrations (mg kg^{-1}) of each BCR fraction (F1, F2, F3, and F4) and the total metal concentration in all BCR fractions were calculated (in percent) for all samples. Moreover, to confirm the reliability of the BCR outcomes, the ratios between the sum of BCR fractions (F1, F2, F3, and F4) and the total metal concentration for each element were measured in all soil samples. The sum of the first three extractions represents the available fraction and correlates with soil properties, such as elements adsorbed onto mineral surfaces, reactivity and solubility of minerals (Gabarrón et al., 2018). The values generally corresponded to the recovery (in percent) of the BCR extraction method and varied from 70.1% to 126.9 %. The variable and poor recovery calculated for some elements could be described by the high heterogeneity characteristics of mine waste samples

(Van Her-rewegh et al., 2003; Kabala et al., 2011) and the process of washing samples with distilled water at the end of each BCR fraction step (Cappuyns et al., 2007).

5.2.7. Data analysis

Statistical analyses were performed using IBM SPSS Statistics 23.0. The result values are expressed as the mean ± standard deviation and the significant level of statistical analysis in all cases considered <0.05. The correlation among metal extracted in BCR fractions with total metal contents in soils, metal concentrations in roots, soil properties and the concentration of DTPA extracted metals were explored using Pearson correlations coefficients. The matrix of Principal Component Analysis (PCA) was carried out to examine the correlations between measured parameters of elements in BCR fractions, all physico-chemical soil properties and DTPA extracted metals in the contaminated and non-contaminated soil samples using the statistical software CANOCO 5 following the recommendations proposed by ter Braak and Smilauer (2002) and Lepš and Šmilauer (2003).

5.3. Results

5.3.1. Physical and chemical soil properties

Table 5.2 summarizes the physico-chemical properties of all the examined soil samples. The data show that the pH values of CP soil samples around the *Pinus halepensis* root varied from 7.4 (CP3) and 7.7 (CP2), whereas the in-depth soil samples were generally more acidic than the upper ones. Indeed, the pH showed higher value in the uppermost soil layer of the amended plot samples S1-1 (6.8) and S3-1 (6.9) than in the soil depth S1-3 (2.3) and S3-3 (2.5). The carbonate content of soil samples was high in all CP soils around the roots (49.7% in CP1, 45.4% in CP2 and 41.03% in CP3). Moreover, the highest value was detected in the uppermost soil samples: S2 (59.4%) > S3 (53.6%) > S1 (52.07%), while it decreased in the deep layer of the amended plot with the lowest (0.19%) in the amended deep layer S1-3 (Table 5.2). The values of total carbon (TC) and nitrogen (TN) content of the CP soil around the *P. halepensis* root ranged from 5.65% to 9.97% and from 0.27% to 0.70% respectively. In the deeper soil layers, the TC and TN contents of the soils were decreased in the amended plots S1 (0.36% TC, 0.03% TN) and S3 (0.38% TC, 0.02% TN).

The highest organic carbon (OC) content was measured in the uppermost soil layer of the compost-amended plot sample S1-1 (1.9%), whereas the lowest was in the deeper soil layer of S1-3 (0.02%) where Electrical Conductivity (EC) reported its highest value (29.3 dS m^{-1}). Table 5.2 shows that Fe and Al oxide had different concentrations in the deep layers of the soil samples. The lower soil layer of the amended plot S3-3 showed the highest amount of Fe-oxide (up to 42948.9 mg kg^{-1}). Moreover, Al-oxide content was high in the CP soil around the roots and in the upper most soil samples.

Table 5.2. The physic-chemical properties of the contaminated soil samples in the different soil depths (S1, S2 and S3); the soil around the roots of *Pinus halepensis* (CP1, CP2 and CP3); and the not contaminated soil around the *P. halepensis* roots (B1, B2 and B3); Number after ± is standard deviation.

Soil	Soil depth (cm)	Soil properties						Mean value (mg kg^{-1})	
		pH	EC (dS m^{-1})	$CaCO_3$ (%)	Total Carbon (%)	Total Nitrogen (%)	Organic Carbon (%)	Fe-oxide	Al-oxide
Bulk soil in-depth									
S1-1	0–20	6.8	12.05	52.07±0.7	8.9±0.1	0.17±0.01	1.9±0.1	10565.3±301.3	160.4±6.2
S1-2	20– 28	4.5	15.3	4.05±0.1	0.71±0.04	0.010±0.001	0.22±0.01	29658.3±3026.05	17.2±2.9
S1-3	32–38	2.3	29.3	0.1±0.08	0.36±0.001	0.03±0.01	0.020±0.001	14920.9±751.9	3.4±1.7
S2-1	0–20	7.3	1.9	59.4±0.4	8.3±0.1	0.04±0.02	0.35±0.02	19160.8±537.9	189.5±10.3
S2-2	37–41	6.7	14.2	37.5±.0.5	5.00±0.05	0.020±0.0003	0.020±0.0004	5909.6±128.6	101.5±18.3
S2-3	47–50	7.6	11.3	47.6±0.7	6.59±0.02	0.002±0.001	0.180±0.003	11498.5±326.8	37.2±4.9
S3-1	0–20	6.9	10.7	53.6±1.5	8.6±0.2	0.15±0.02	1.5±0.2	12334.9±99.6	112.9±6.7
S3-2	20–45	7.1	11.2	55.5±.2	7.62±0.08	0.002±0.001	0.140±0.005	17411.7±2030.7	61.2±6.3
S3-3	56–66	2.5	19.1	1.40±0.02	0.38±0.005	0.02±0.01	0.190±0.002	42948.9±5084.9	14.3±3.9
Bulk soil around the roots of *P. halepensis*									
CP1		7.6	8.3	49.7±1.7	9.97±0.05	0.70±0.08	0.35±0.03	9194.4±129.9	211.95±7.08
CP2		7.7	2.2	45.4±1.2	5.7±0.1	0.27±0.02	0.060±0.002	18265.6±159.4	112.8±9.8
CP3		7.4	8.8	41.03±1.03	8.9±0.2	0.51±0.03	0.33±0.04	10936.2±269.4	245.6±2.8
B1		6.7	0.3	0.32±0.02	1.2±0.2	0.06±0.01	0.77±0.07	726.8±116.9	215.2±14.3
B2		7.9	3.9	4.07±0.30	10.07±0.04	0.66±0.01	7.61±0.04	992.2±26.8	2323.9±37.9
B3		8.8	1.1	19.03±0.50	3.83±0.08	0.080±0.005	0.78±0.06	467.6±62.5	195.2±8.4

5.3.2. Mineral composition in the soil and plant roots

The XRD analysis performed on the collected bulk soil samples shows that carbonate (dolomite: $CaMg(CO_3)_2$), silica, quartz (SiO_2), and gypsum ($CaSO_4$) were the predominating minerals in all soil samples; see more in Kharazian et al (2022a). It shows that iron sulfide (pyrite) was found in all bulk soil layers, except in the layers of the amended plots S1-2 and S3-3. Moreover, SEM analysis detected iron oxide (goethite) and iron sulfate (jarosite) in all the soil around the root samples (Figure 5.4a, b and c) together with zinc carbonate (smithsonite) (Figure 5.4c). Muscovite ($KAl_2[(AlSi_3O_{10})(OH)_2]$) was present in the uppermost soil samples of amended plot S1 and S3 (0–20 cm) and the soil around the roots of CP3 (Figure 5.4c).

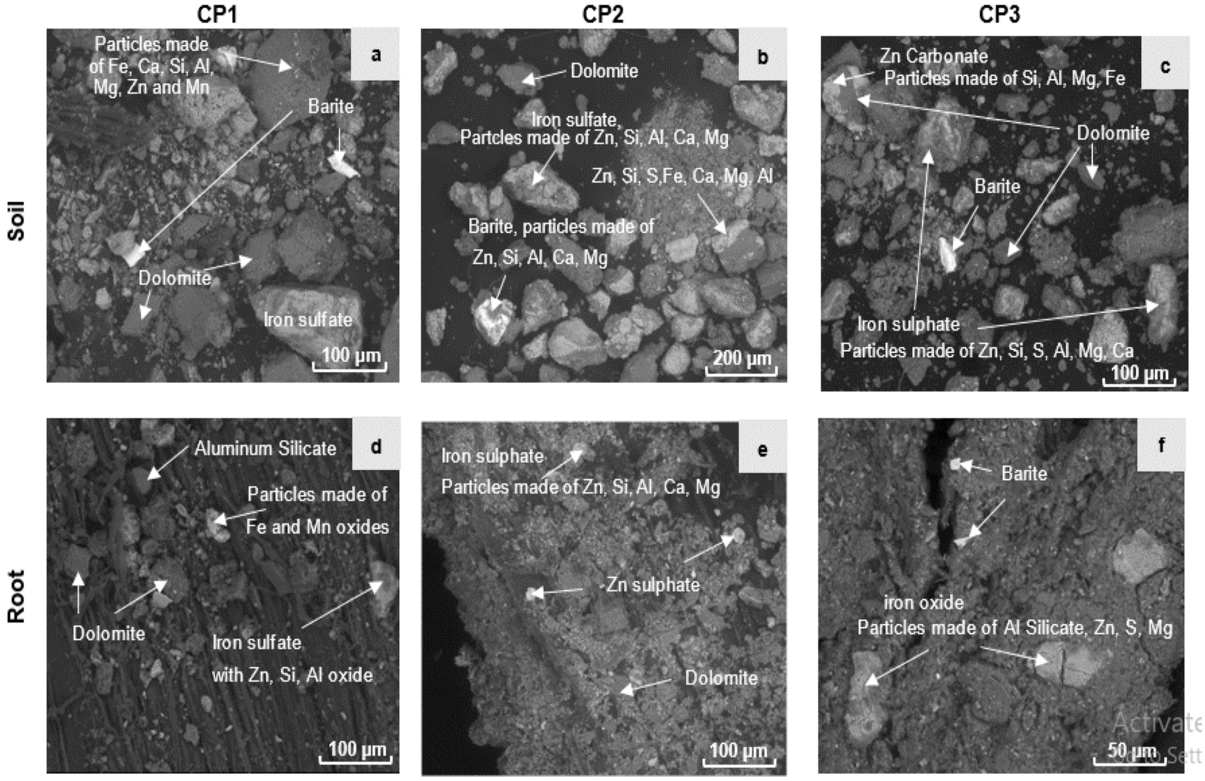


Figure 5.4. Spectroscopy-scanning Electron Microscopy (SEM) analysis on minerals of the root and the soil around the root of *Pinus halepensis* samples (CP1, CP2 and CP3); in the soil samples (**a, b** and **c**), and the root surface (**d, e** and **f**)

Moreover, illite [(K, H_3O) (Al, Mg, Fe)$_2$ (Si, Al)$_4$ O_{10} (OH)$_2$, (H_2O)] was found in the bulk soil samples S2-2, S3-2 and CP1 and the main minerals detected through XRD analysis on the root samples were dolomite, whewellite (Ca (C_2O_4)·2(H_2O), silica and barite (Figure 5.5). The SEM analysis performed on soils and particle materials shows that these mineral particles were embedded and adhered to the external part of *Pinus halepensis* root samples and mostly detected iron-sulfate (Figure 5.4d and e) and iron oxide phases (Figure 5.4f), as well as a mixture of other elements, mainly Al, Si, Zn and Fe, embedded on the external part of the root samples (Figure 5.4d, e and f) (see more in Figure 5.6).

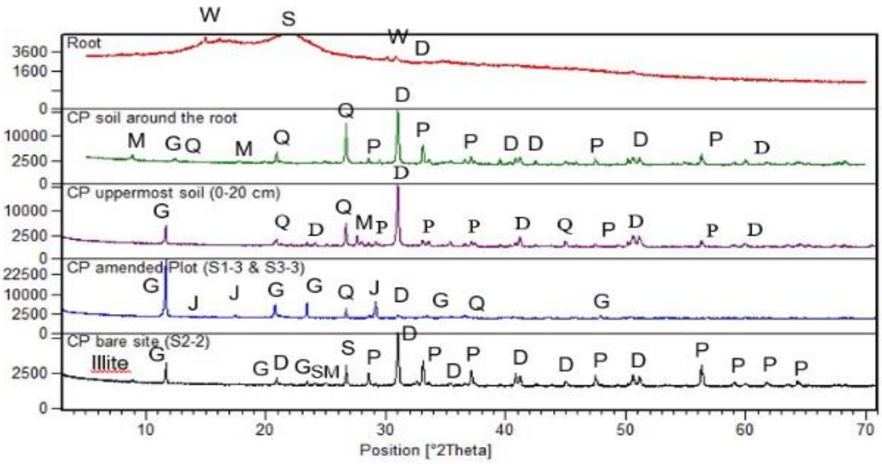
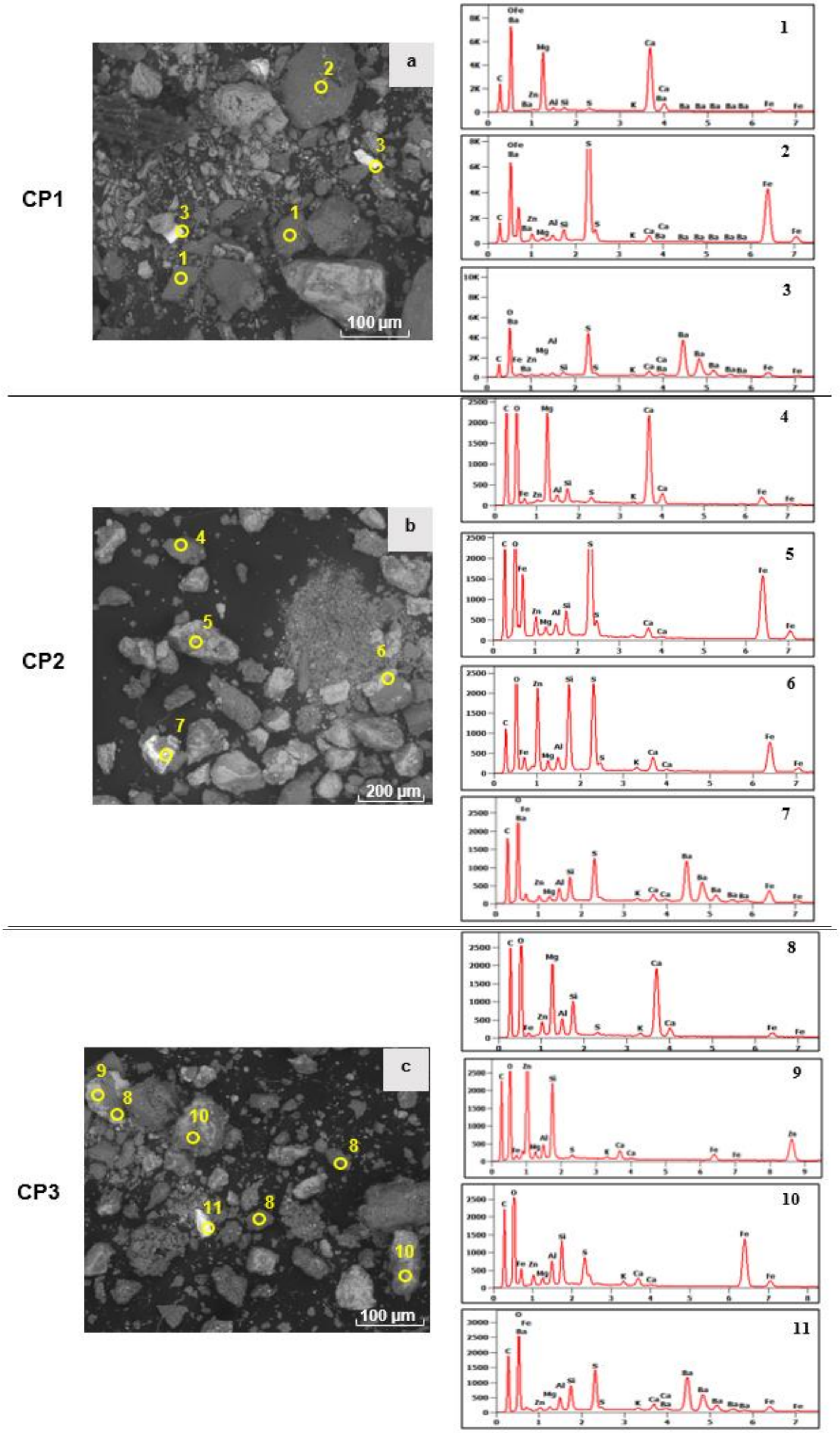


Figure 5.5. XRD patterns of *Pinus halepensis* root and CP polluted soil samples; Mineral legend: Gypsum (G), Quartz (Q), Dolomite (D), Pyrite (P), Muscovite (M), Silica (S), Whewellite (W), Smithsonite (Sm)

Soil


CP1
a
100 µm
CP2
b
200 µm
CP3
c
100 µm
1
2
3
4
5
6
7
8
9
10
11
C
O
Fe
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Al
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S
K
Ca

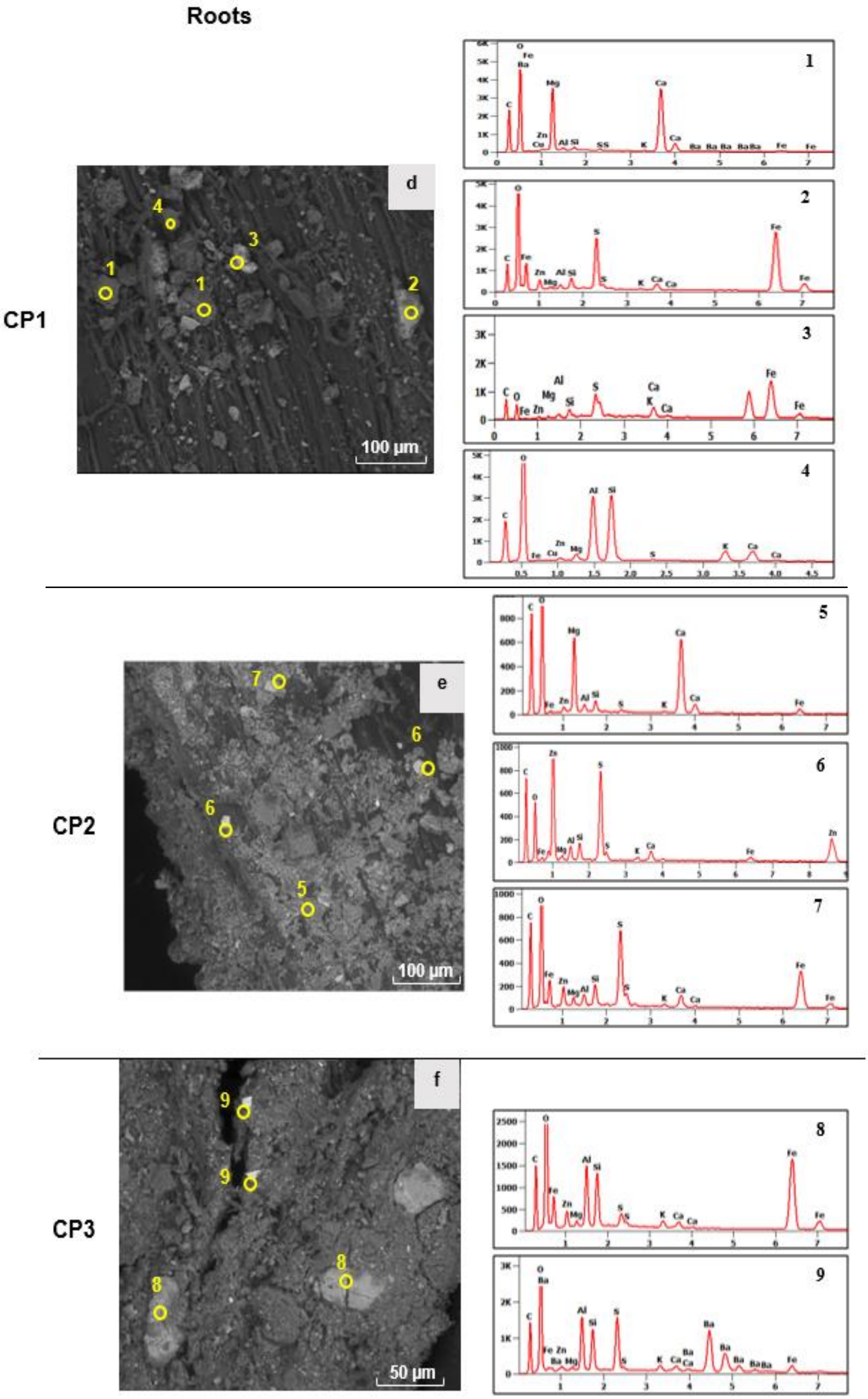

Figure 5.6. Spectroscopy-scanning Electron Microscopy (SEM) analysis on the soil around the roots (a, b and c) and on the root surface (d, e and f) of *Pinus halepensis* grows in Campo Pisano contaminated mine site (CP1, CP2 and CP3); the indicated numbers (1–11 for soil and 1–9 for roots samples) are the points where EDS spectra were acquired.

5.3.3. Total Zn, Pb and Cd concentrations

The chemical data of Zn, Pb and Cd concentration in all contaminated soil samples showed that Zn was the most abundant metal followed by Pb (Figure 5.7); see more in Kharazian et al. (2022a). In the non-contaminated soil samples, total metal contents ranged from 10.2 to1305.7 mg kg^{-1} for Zn, 27.6 to 450.7 mg kg^{-1} for Pb and 0.88 to 8.9 mg kg^{-1} for Cd. In the contaminated soil samples, CP2 had the highest total Zn, Pb and Cd content in the soil around the root samples. Moreover, Zn and Cd concentrations decreased in the lower soil layers of the amended plot S1-3 (3421.5 mg kg^{-1} for Zn and 5.9 mg kg^{-1} for Cd) and S3-3 (4224.8 mg kg^{-1} for Zn and 11.4 mg kg^{-1} for Cd). However, in the same samples, the total content of Pb was higher and ranged from 3430.04 in S3-3 to 4537.6 mg kg^{-1} in S1-3 (Figure 5.7). The BCF values and the data for the root samples were taken from the previous study available in Kharazian et al. (2022a); (see more in Figure 5.8 and Figure 5.9).

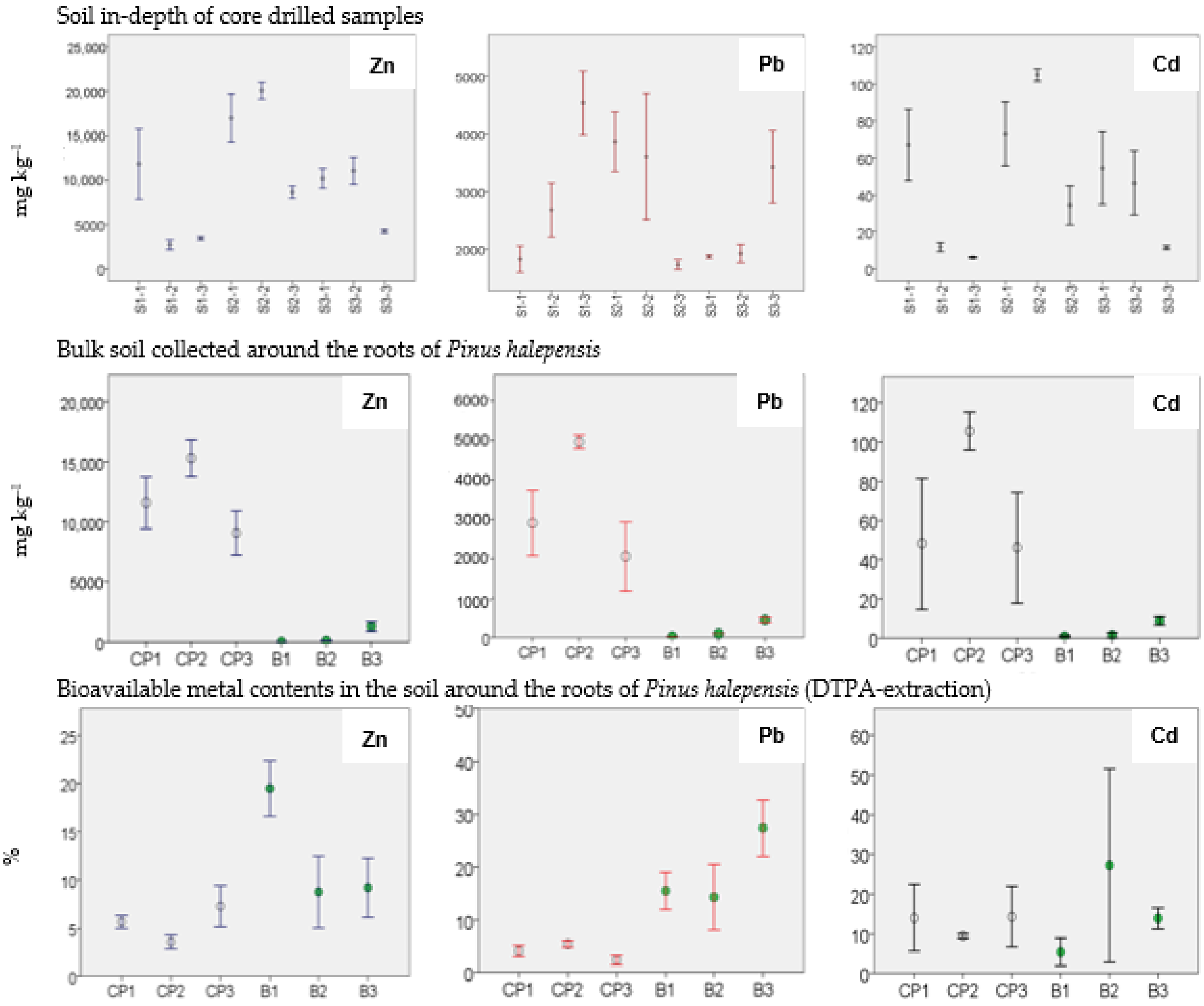

Figure 5.7. Total Zn, Pb and Cd content (mean values and standard deviation) in core drilled samples at different depths. Total metal content and bioavailable fraction (DTPA extraction) in soil around the roots of *Pinus halepensis* at Campo Pisano (CP1, CP2 and CP3) and in not contaminated sites (B1, B2 and B3); non-contaminated soils are indicated with green circles

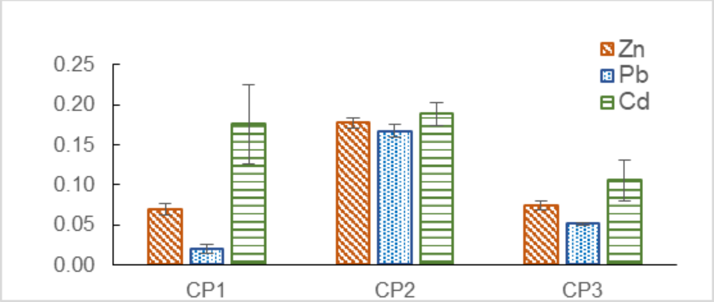

Figure 5.8. Biological concentration factor (BCF) calculated for all substrates collected around the roots of *Pinus halepensis*

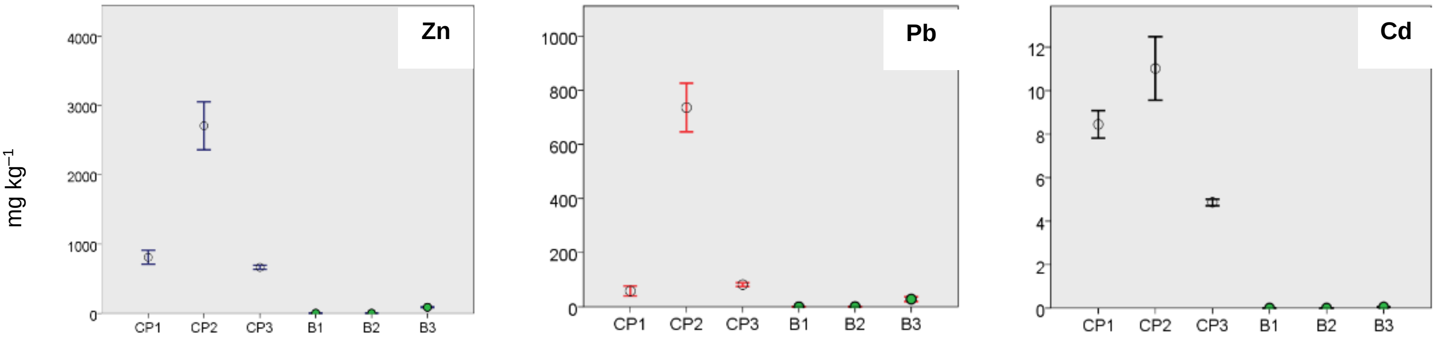

Figure 5.9. Metal concentration in the roots of *P. halepensis* samples exploited from Kharazian et al (2022a)

5.3.4. Bioavailable content of Zn, Pb and Cd (DTPA)

Figure 5.7 reports the bioavailable metal content (percentage) of the soil collected around the roots in CP contaminated (CP1, CP2 and CP3) and in non-contaminated sites (B1, B2 and B3). The data of contaminated soil around the root sample CP3 showed the highest Cd and Zn bioavailable fraction (13.8% Cd > 7.3% Zn), and the lowest bioavailability of Pb (2.4%); see more in Kharazian et al. (2022a). The values show that the Cd bioavailable fraction was remarkably high in all contaminated soils around the roots from 9.5% in CP2 to 13.8 % in CP3 and 13.5% in CP1. The data show a variable bioavailable fraction in non-contaminated soils with the highest Pb (27.3%) in B3, Cd (25.2%) in B2 and Zn (19.5%) in B1. Thus, this test confirmed that contaminated samples had a significant bioavailable fraction, even below 20% of the total amount. As the bioavailable fraction was much lower than the bulk concentration, these data are relevant for a risk analysis of the Campo Pisano area.

5.3.5. The BCR sequential extraction

Figure 5.10 shows the geochemical fractions (percentage) of Zn, Pb and Cd in all soil samples through the BCR sequential extraction method. The data show that in CP soils around the roots and in the uppermost soil layers, Zn and Pb were often present in the residual fraction (F4), whereas the highest fraction of Cd was in the exchangeable fraction (F1). The data show that the highest Pb was present in F4 in the deep layers of the amended plot samples S1-3 (96.2%) and S3-3 (94.1%) where it was less bound to the exchangeable fraction (F1) (Figure 5.10). Moreover, in all CP soil samples, some part of Zn was mainly present in exchangeable fraction F1 (ranging from 10.8% to 51.8%) more than in the oxidizable fraction F3 (7.9%–22.1%) > F2 (0.1–11.5%). The highest Zn in F1 was reported in S2-2 (51.8%) where it showed the highest total metal content and there was no vegetation. Unlike CP-contaminated soils, the non-contaminated and less contaminated soils showed variable extracted elements in BCR fractions. For instance, in the less contaminated soil sample (B3), Zn and Pb were more present in F2 (66.8% Pb > 57.9% Zn) and F1 (32.5% Zn > 21.4% Pb) than F4 (7.6% Pb > 4.6% Zn) and F3 (5.1% Zn > 4.1% Pb) (Table 5.3).

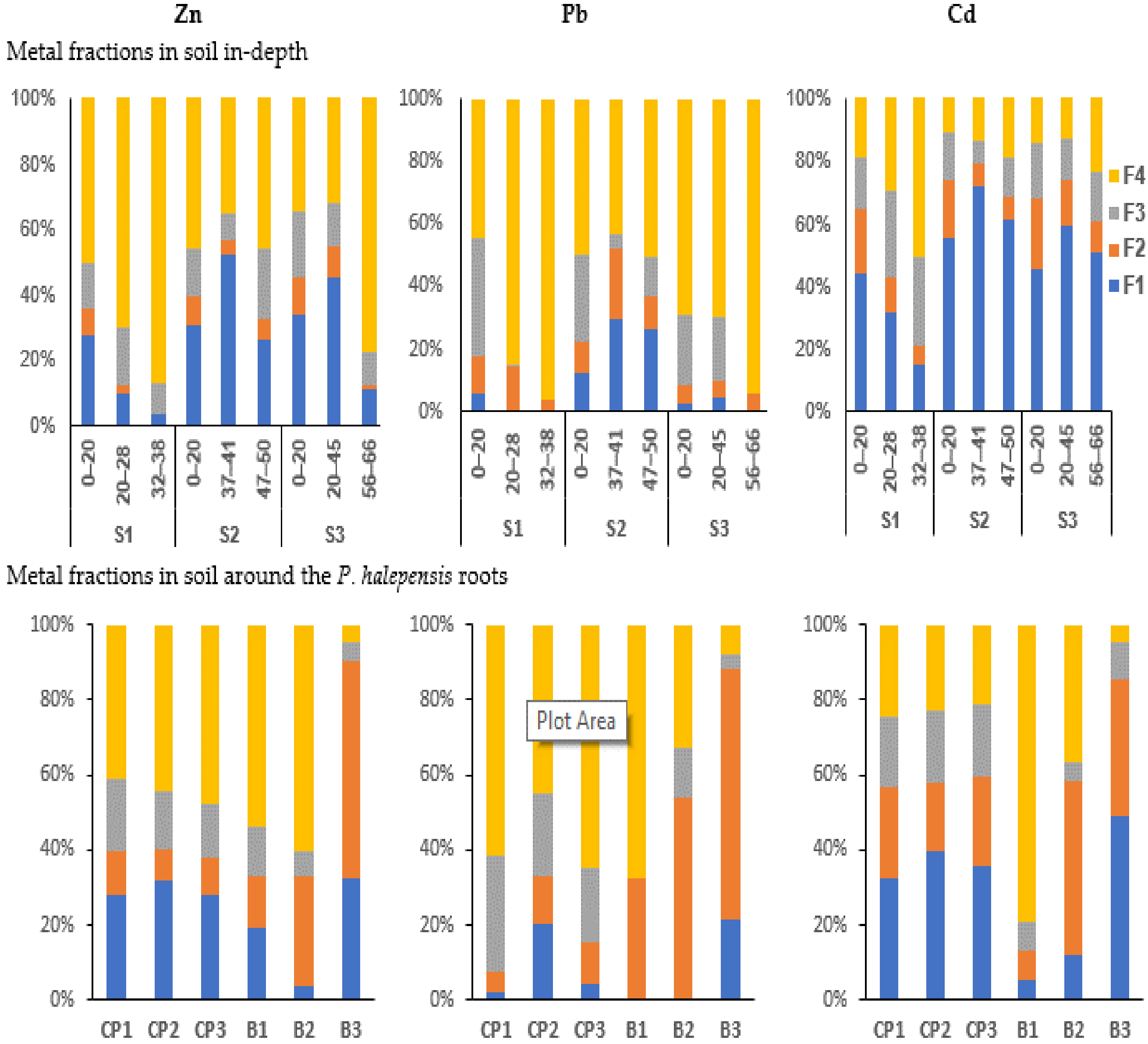

Figure 5.10. Geochemical fractions of Zn, Pb and Cd obtained through BCR sequential extraction: acid-extractable (F1), reducible (F2), oxidizable (F3) and residual fraction (F4) in the soil collected from depth layers of the core samples (S1, S2, and S3) and around the collected *Pinus halepensis* root samples in Campo Pisano mine tailings (CP1, CP2, and CP3) and in the non-contaminated areas (B1, B2 and B3).

Table 5.3. The ratio of metal concentrations extracted in the BCR fractions (F1, F2, F3 and residue F4) in all collected soil samples

Soil depth (cm)		Zn (%)				Pb (%)				Cd (%)			
		F1	F2	F3	F4	F1	F2	F3	F4	F1	F2	F3	F4
Soil in-depth samples													
S1-1	0–20	27.2	8.2	13.9	50.3	5.8	12.2	37.3	44.7	44.05	20.8	16	19.2
S1-2	20– 28	9.8	2.6	17.7	69.9	0.8	13.8	0.2	85.2	31.7	10.9	28	29.3
S1-3	32–38	3.3	0.1	9.3	87.2	0.1	3.5	0.2	96.2	14.7	6	28.5	50.8
S2-1	0–20	30.3	8.9	14.9	45.8	12.3	10.1	27.5	50.1	55.2	18.7	15	11.1
S2-2	37–41	51.8	4.9	7.9	35.3	29.8	22.3	4.8	43.1	72	7.3	6.8	13.9
S2-3	47–50	26.1	6.1	22.1	45.7	26.1	10.9	12.7	50.3	60.9	7.3	12.9	18.9
S3-1	0–20	34	11	20.1	34.8	2.7	5.6	22.8	68.9	45.4	22.3	17.9	14.3
S3-2	20–45	45.3	9.5	13.2	31.9	4.3	5.2	20.8	69.6	59.1	14.8	13.3	12.8
S3-3	56–66	10.8	1.2	10.2	77.8	0.5	5.1	0.3	94.1	50.4	9.8	16.3	23.4
Soil around the roots of *P. halepensis*													
CP1		28.2	11.5	19.2	41.2	2.3	5.5	30.5	61.7	32.6	24.3	18.6	24.5
CP2		31.7	8.3	15.4	44.6	20.1	13	22.5	44.7	39.5	18.5	19	23
CP3		28.1	9.9	14.6	47.4	4.3	10.9	20.2	64.7	35.9	23.7	19.2	21.2
B1		19.01	13.8	13.3	53.9	0.17	32	0.29	67.5	5.7	7.5	7.5	79.3
B2		3.5	29.5	6.5	60.4	0	53.8	13.3	32.9	11.8	46.8	5	36.4
B3		32.5	57.9	5.1	4.6	21.4	66.8	4.1	7.6	48.9	36.4	10.1	4.6

5.3.6. Correlation between BCR fractions and metal content in the soil and in the plant–root system

The Pearson correlation between Zn, Pb and Cd extracted in the BCR fractions with the total metal content in the soil and in the *Pinus halepensis* root samples, the concentrations of DTPA-extracted metals, the sum of all BCR fractions and the soil properties are shown in Table 5.4. The soil core-drilled samples and the soil around the roots were considered separately in order to indicate the details of the data correlations. In the soils around the roots, all metals (Zn, Pb and Cd) bound in F1 appeared to be positively correlated with their concentrations in soil and *P. halepensis* root samples. Moreover, the concentrations of Zn and Cd extracted in all BCR fractions were highly positively correlated with their concentration of DTPA-exchangeable availability in the same soil samples. The matrix shows that the total Zn concentration in the soil and Cd concentration in roots samples were significantly positively correlated with Zn extraction in all BCR fractions. The Fe-oxide content in the soil around the roots samples was significantly positively correlated with all elements extracted in the BCR fractions, except for Cd in the residual fraction (F4). In the same soil sample, all metals in F4 appeared to be positively correlated with the $CaCO_3$ content; and only Zn and Pb showed a positive correlation with Fe-oxide in the same fraction. In the core soil samples, $CaCO_3$ presented a positive correlation with Zn and Cd concentration extracted in F2 and F3. The concentration of Cd found in F2 and F3 correlated positively with Al-oxide in the soil core-drilled samples, while there was no reported positive correlation in the soil around the roots for Al-oxide in the BCR steps.

Table 5.4. The correlation matrix between BCR fractions and the total metal content in soil (T-Zn; T-Pb; T-Cd), the total metal content in *Pinus halepensis* root (Zn-R; Pb-R; Cd-R), the bioavailable content of Zn, Pb and Cd (DTPA), the sum of BCR fractions (SF-Zn; SF-Pb; SF-Cd), and the soil characteristics; Correlation (r>0.8) is significant at the 0.05 level (2-tailed).

Samples	**F1**			**F2**			**F3**			**F4**		
	Zn	**Pb**	**Cd**	**Zn**	**Pb**	**Cd**	**Zn**	**Pb**	**Cd**	**Zn**	**Pb**	**Cd**
Bulk soil around the roots of *P. halepensis*												
T- Zn	0.96	0.86	0.90	0.81	-	-	0.89	0.92	0.93	0.88	-	0.80
T- Pb	0.98	0.92	0.94	0.76	0.85	-	0.86	0.95	0.97	0.89	0.83	-
T- Cd	0.92	0.95	0.96	-	0.92	-	-	0.96	0.94	0.87	0.87	-
Zn-R	0.96	0.98	.99	-	0.95	-	-	0.98	0.99	0.87	0.90	-
Pb-R	0.94	0.99	0.99	-	0.97	-	-	0.97	0.97	0.86	0.93	-
Cd-R	0.93	-	0.82	0.9	-	-	0.98	0.84	0.88	0.80	-	-
DTPA-Zn	0.83	-	0.88	0.80	-	0.97	0.88	-	0.92	0.85	0.82	0.89
DTPA-Pb	0.82	0.83	-	0.85	0.87	-	-	0.81	-	-	-	-
DTPA-Cd	0.85	-	0.89	0.80	-	0.98	0.89	-	0.93	0.87	0.84	0.90
SF-Zn	0.99	-	0.98	0.86	-	0.95	0.98	0.97	0.95	0.96	0.96	0.98
SF-Pb	0.96	0.91	0.95	0.73	0.92	0.82	0.87	0.99	0.92	0.94	0.98	0.89
SF-Cd	0.98	0.92	0.99	0.85	-	0.98	0.97	0.94	0.99	0.98	0.96	0.98
pH	0.86	-	-	-	-	-	-	0.84	0.82	0.89	-	0.94
EC	-	-	-	-	-	-	-	-	-	-	-	-
$CaCO_3$	0.88	-	0.80	0.92	-	0.98	0.92	0.80	0.94	0.88	0.83	0.94
Total C	-	-	0.92	-	-	-	-	-	-	-	-	-
Total N	-	-	-	-	-	-	-	-	-	-	-	-
OC	-	-	-	-	-	-	-	-	-	-	-	-
Fe-oxide	0.97	0.80	0.98	0.74	0.82	0.90	0.93	0.98	0.98	0.99	0.94	-
Al-oxide	-	-	-	-	-	-	-	-	-	-	-	-
Bulk soil in-depth												
T- Zn	0.85	-	0.96	-	-	-	-	-	-	0.89	-	0.90
T- Pb	-	-	-	-	-	-	-	-	-	-	-	-
T- Cd	0.86	-	0.93	-	-	-	-	-	-	0.89	-	0.94
SF-Zn	0.97	0.97	.95	0.81	-	-	-	-	-	0.89	-	0.91
SF-Pb	-	-	-	-	-	-	-	-	-	-	0.80	-
SF-Cd	0.90	-	0.97	0.80	-	-	-	-	-	0.88	-	0.93
pH	-	-	-	0.85	-	-	0.89	-	-	-	-	-
EC	-	-	-	-	-	-	-	-	-	-	-	-
$CaCO_3$	-	-	-	0.92	-	0.85	0.89	0.83	0.89	-	-	-
Total C	-	-	-	0.91	-	0.88	0.87	0.85	0.90	-	-	-
Total N	-	-	-	-	-	-	-	-	-	-	-	-
OC	-	-	-	-	-	-	-	-	-	-	-	-
Fe-oxide	-	-	-	-	-	-	-	-	-	-	-	-
Al-oxide	-	-	-	-	-	0.94	-	0.92	0.92	-	-	-

The PCA was performed to assess the distribution and similarity among the collected soil samples and the correlation between Zn, Pb and Cd extracted in all standardized BCR fractions with soil properties and total metal concentration in the soil and roots samples. The parameters were spatially ordinated within a diagram according to the collected soil in deep layers (S1, S2 and S3) (Figure 5.11) and the soils around the root of the *P. halepensis* samples on the non-contaminated soil (B1, B2 and B3) and contaminated CP soils (CP1, CP2 and CP3) (see more in Figure 5.12).

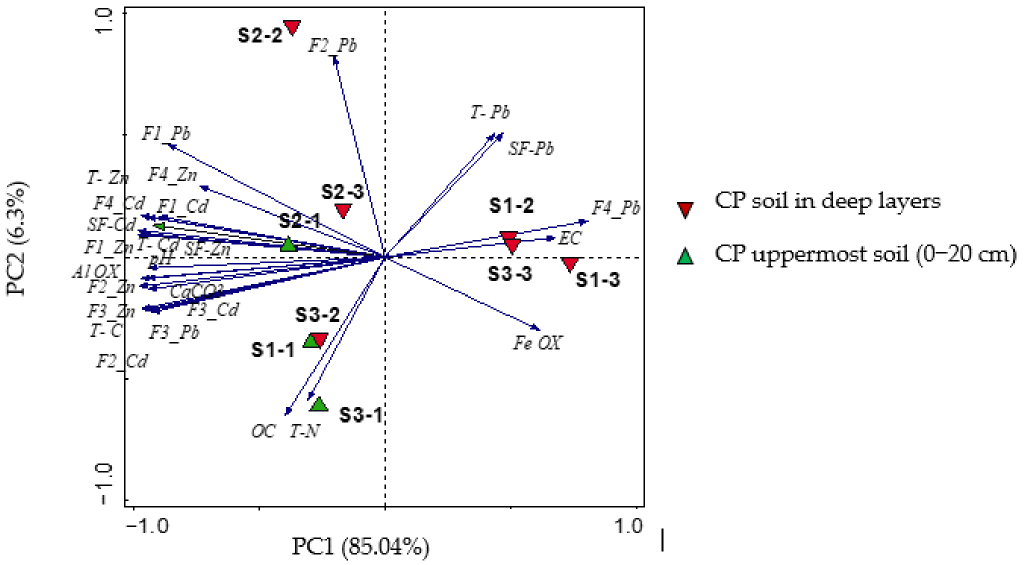

Figure 5.11. PCA for the collected soils in deep layers. Elements extracted in F1, F2, F3 and F4 BCR Fractions; the total metal content in soil (T-Zn; T-Pb; T-Cd); the sum of the BCR fractions (SF-Zn; SF-Pb; SF-Cd); electrical conductivity (EC); organic carbon (OC); total nitrogen (T-N); total carbon (T-C) in the uppermost soil samples (S1-1, S2-1 and S3-1) and the deep layers (S1-2, S1-3, S2-2, S2-3, S3-2, S3-3).

Figure 5.11 shows that the first component of the diagram (PC1) had 85.04% of the total variation. Different parameters, such as Fe, EC, residual Pb (F4-Pb), total Pb (T-Pb) and the sum of BCR for Pb (FS-Pb) were all presented in PC1 with a positive correlation. The other parameters, such as $CaCO_3$, pH, Al-oxide as well as Zn and Cd extracted in F2 and F3 showed strongly negative correlation in the inverse section of the first principal component. The second principal component (PC2) explained 6.3 % of the total variation with a highly positive correlation for Pb extracted in the second fraction (F2-Pb) and a negative correlation for organic carbon (OC) and total nitrogen (T-N) on PC2. The deep layers of the soil-amended plot samples S1-2, S1-3 and S3-3 are distributed on the positive side of the first axis and the main variables that had a positive correlation with them were Fe-oxide, EC, the total content of Pb (T-Pb), residual of Pb (F4-Pb) and the sum of BCR fractions for Pb (SF-Pb). Moreover, the uppermost soil samples S1-1, S2-1 and S3-1 are distributed on the negative side of the first component axis and exhibited high levels of $CaCO_3$, pH, Zn and Cd extracted in all BCR fractions, and the sum of BCR fractions with low levels of total Pb (T-Pb) and the sum of BCR fraction for Pb (SF-Pb). Further, the availability of Zn and Cd extracted through the BCR fractions decreased in the deep soil layers of the amended plot S1-3 and S3-3, which had high EC, Fe-oxide and residual Pb (Figure 5.11).

Figure 5.12 shows 91.8% of the total variation in the first principal component (PC1) for finding the correlation between the same parameters in the soil samples around the roots. Most of the parameters are mainly presented in the negative side of axis PC1, while only Organic Carbon (OC) and Al-oxide are presented in PC1 with positive correlation. The parameters, such as pH, $CaCO_3$, Fe-oxide and EC as well as Zn, Pb and Cd extracted in all BCR fractions show strongly negative correlation in the inverse section of CP1. The second principal component (PC2) explained 3.6% of the total variation with a high positive correlation for pH and negative correlation for total nitrogen (T-N). The non-contaminated soils (B1 and B2) are distributed in the positive side of the first axis show a positive correlation with OC and Al. Moreover, the soil around the root samples (CP1, CP2 and CP3) are distributed on the negative side of PC1 axis and exhibited high levels of $CaCO_3$, pH, Zn, Pb and Cd extracted in all BCR fractions and the total BCR fractions. Indeed, the availability of Zn, Pb and Cd extracted through BCR fractions are decreased in CP soil around the roots which have high Al and organic carbon.

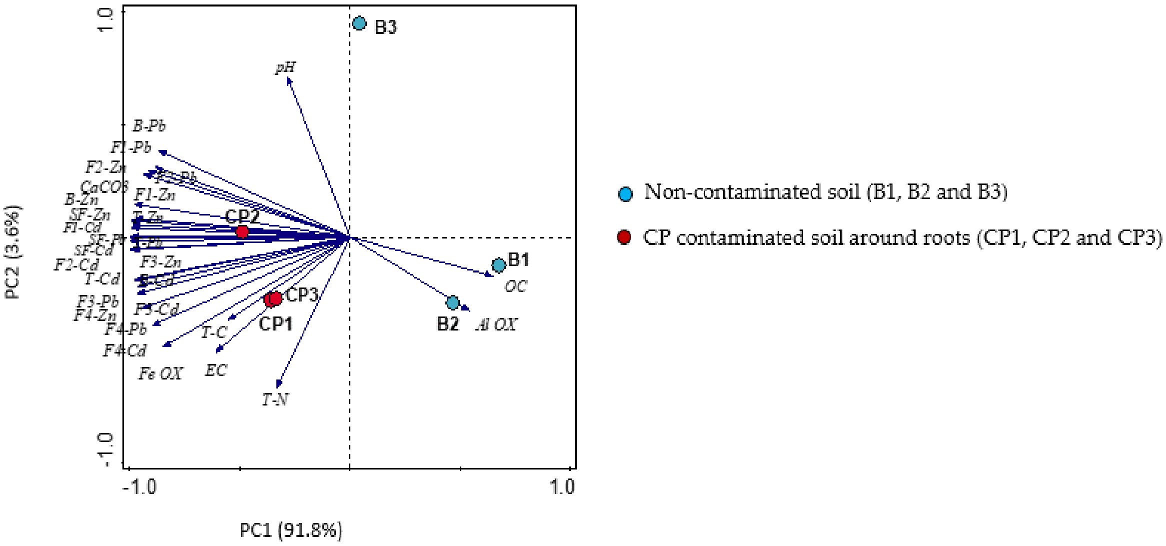

Figure 5.12. PCA for the collected soil around the *Pinus halepensis* root samples. Elements extracted in F1, F2, F3, F4, BCR fractions; the total metal content in soil (T-Zn; T-Pb; T-Cd); the sum of BCR fractions (SF-Zn; SF-Pb; SF-Cd); electrical conductivity (EC); organic carbon (OC); total nitrogen (T-N); total carbon (T-C), Aluminum oxide (Al OX), iron oxide (Fe OX), bioavailable fractions of metals (B-Zn, B-Pb, B-Cd); in Campo Pisano contaminated and non-contaminated soil around the *P. halepensis* root samples.

5.4. Discussion

The CP soil samples had a high content of Zn, Pb and Cd due to the high concentration of heavy metals originated from the minerals present in the extremely polluted mine site (Cidu et al., 2002). On the other hand, the heterogeneous mineral composition and the different physico-chemical properties of CP soils could affect the mobility of elements in soil and root samples. Thus, the BCR fractions were evaluated in relation with the soil properties and physico-chemical and mineralogical characteristics of soil in depth and around the root samples.

5.4.1. The mineralogical investigation related to the soil properties

The mineralogy of the investigated soils comprised primary minerals, i.e. metal sulfide (pyrite), carbonate (calcite and dolomite) and quartz as well as the secondary mineral, i.e. iron sulfate (jarosite) and iron hydroxides, in agreement with previous studies carried out by De Giudici et al (2015) and Lai et al (2015). In fact, the formation of secondary minerals compounds is generally due to the weathering minerals process or induced by biogeochemical processes occurring in the soil-root system of the plant. Sulfate minerals, such as jarosite (iron sulfate) and gypsum (Calcium sulfate) are formed during the pyrite (iron sulfide) oxidative-dissolution process (Jambor et al., 2000; Jerz and Rimstidt, 2003). This can increase the dissolved ions and eventually elevate the electrical conductivity (EC) in S1-3 and S3-3. PCA showed that the surface layer was different with respect to the deep layer. Surface layers showed alkaline pH values consistent with alkaline and carbonate lithology of the Monteponi and Campo Pisano mines (Bechstädt and Boni, 1994; Bacchetta et al., 2015). The deep layers showed acidic pH values due to the pyritic tailings rich in pyrite and with low carbonate content (Aversa et al., 2002). Indeed, the oxidation of pyrite generates H_2SO_4 and involves a sequence of reactions beginning with the release of Fe^{2+}, which is converted into Fe^{3+} under oxidizing conditions. In pH > 4.5, Fe^{3+} precipitates as a hydroxide generating more acidity. On the contrary, in pH < 4.5, the Fe^{3+} can act as an oxidant of the pyrite to generate much greater acidity (Martín et al., 2008). In this regard, the oxidation of sulfur generates sulfate anions that form soluble salts (Pastor-Jáuregui et al., 2020), and release large amounts of iron (Simón et al., 2001). Unlike the soil in deep layers, the soil around the root samples was mainly characterized by a pH close to neutral and more total carbon and carbonate contents (Table 5.1). The neutral pH (6.8–7.2) and higher organic carbon content in the CP uppermost soil can decrease the availability of metals (Zn, Pb and Cd) and enhance plant growth to facilitate the phytostabilization process (Wong, 2003; Young et al., 2015; Luo et al., 2018). Moreover, other studies have also suggested that soil organic amendments can decrease metal availability by the formation of organic metallic complexes. According to García-Carmona et al (2019a) and García et al (2009), pH appears to be the main property controlling Zn and Cd availability, increasing its solubility in acidic soil conditions. However, Jacquat et al (2008) reported that in neutral soils, organic matter, clay, minerals, and carbonates become more influential than pH in Zn availability. Moreover, Sierra-Aragón et al (2019) reported that Cd availability is strongly reduced by the increase in OC and the rise in pH that causes a decrease in the soluble forms of Cd and the fraction of Cd (Cambier,et al., 2019; Swed et al., 2022).

5.4.2. The BCR fractions related to the soil properties and mineralogy

The soil around the roots with high total metal content (i.e., CP2) (Figure 5.7) showed a higher ratio of metals extracted in F1 (Figure 5.10). The Pearson analysis showed highly significant correlations between the total Zn, Pb and Cd content and all metals were found in the F1 fraction (Table 5.4) which indicates that the metal availability increased with the total metal content. This is in agreement with the previous studies carried out by Rodríguez et al (2009), Fernández-Ondoño et al (2017) and Swed et al (2022).

Metals in the exchangeable fraction (F1)

The results showed that Cd was mainly associated with the exchangeable fraction (F1) more than Zn and Pb in all contaminated soil samples except S1-3 (Figure 5.10). Similar results were obtained by Favas et al (2011) in the Ervedosa mine area of northeastern Portugal, and Swed et al (2022) in the Silesia-Cracow region in southern Poland. This may be due to: (i) the presence of carbonates and alkaline waste materials originated from the Monteponi area (SW-Sardinia) that have been loaded on the surface materials of CP mine waste; (ii) in the soil around the root samples, the presence of sulfide and sulfate (Figure 5.4) and the sulfuric acid production caused by sulfide weathering enhanced the high content of Cd that was found in F1 fraction. (iii) On the other hand, the findings of Jerzykowska et al (2014) and Sutley et al (1999) explained that Cd is mainly associated with smithsonite ($ZnCO_3$) in calamine mine areas. This could describe the highest exchangeable fraction (F1) of Cd (72%) and also Zn (51.8%) measured in the deep soil layer of the S2-2 sample (Figure 5.10), where smithsonite and Zn-bearing dolomite were detected as the most important host minerals of Cd. It should be noted that the highest total Cd and Zn contents was measured in the same soil sample.

The results showed that Zn and Cd contents in all BCR fractions were highly positively correlated with their concentration in DTPA-exchangeable availability in the soil around the root samples (Table 5.4). This suggests that these metals may be controlled by the rhizosphere processes. Further, the results of the bioavailable Zn and Cd contents in the CP soil around the root samples indicated that the extraction in DTPA was weaker than the dilute acetic acid applied in the exchangeable fraction of BCR (F1). Indeed, Zn and Cd were more bioavailable during the first BCR fraction (up to 31.7% for Zn and 39.5% for Cd) (Figure 5.10), compared to DTPA-exchangeable fraction (up to 7.2% for Zn and 13.8% for Cd) (Figure 5.7). This is in accordance with the findings of Luo et al (2019) that investigated the phytostabilization of Zn and Cd in smelting slag site in northwestern Guizhou, China (Luo et al., 2018).

Moreover, the F1 fraction showed the lowest Zn and Pb which were found in the deep soil samples of amended plot S1-3 and S3-3. This can be explained by the low total metal and/or carbonate contents that contributed to precipitation of these metals in mineral forms associated with carbonate (F1) **(**Monterroso et al., 2014). Soil carbonates may affect metal solubility in water through their surface interactions, providing specific adsorption or precipitation reactions (Simón et al., 2010; García-Carmona et al., 2019). However, soil carbonates have a limited capacity of controlling metal water solubilization and severe metal soil pollution can exceed the thresholds of toxicological concern even in carbonated soils (Jacquat et al., 2008). The significant lower content of Pb that was presented in F1 revealed that Pb was less soluble and less mobilized in all the CP-amended soil samples. This can reduce its toxic impact on the environment (Rozek et al., 2015).

Metals in reducible fraction (F2)

The results showed a low content of Zn < Cd in the reducible fraction (F2) for CP soil samples. This mainly refers to the presence of Fe-oxides (Figure 5.4). Moreover, the highest reducible fraction (F2) for Zn (11.5%) and Cd (24.3%) occurred in the soil around the root sample CP1. The Zn-bearing iron oxide phase can be considered to be the main source of Zn minerals mainly detected in the CP uppermost soils and the soil around the roots samples (Figure 5.4). In non-contaminated soils, the elevated concentration of Pb found in F2 that was bound to Fe and Mn oxides (up to 66.8% in B3) could be caused by the adsorption and accumulation of these elements in the oxide form, favored by alkaline conditions (pH=8.7) that are favorable for the formation of Fe and Mn oxides.

Metals in oxidizable fraction (F3)

In CP soil samples, some part of Zn was presented in the oxidizable fraction F3 > F2 (Figure 5.10). This could be connected to organic material and sulfides which were oxidized to sulfate. Thus, sulfide could partially dissolve and increase the metal content in the soil with high content of sulfide minerals (i.e. pyrite, sphalerite and galena) (Swed et al., 2021, 2022). In addition, organic matter is a natural sink of Zn in soils where it is easily absorbed (Kumpiene et al., 2008; García-Carmona et al., 2018). Moreover, in the soil around the roots as well as the uppermost soil samples, Pb was partly found in the oxidizable fraction (F3) (Figure 5.10). According to the findings of Cappuyns et al (2007) some of the jarosite group minerals could release Pb in the solution after the second step of BCR extraction (Cappuyns et al., 2007). This could describe the presence of Pb in oxidizable fraction (F3). On the other hand, Romero-Freire et al (2015) and Coppola et al (2010) pointed out that soil organic matter is one of the main soil properties controlling lead availability, and it can be attributed to the formation of organic complexes (Coppola et al., 2010; Romero-Freire et al., 2015). Different authors have observed that high levels of organic matter mainly found in the surface layers of soils are an important sink for lead (Kabata-Pendias, 2011; Aragón et al., 2019).

Metals in residual fraction (F4)

In the CP soil samples, Zn and Pb were mainly associated with the residual fraction (F4). The highest Zn (up to 87.2%) and Pb (up to 96.2%) were measured in the deep soil layer of compost-amended plot S1-3 (Figure 5.10). This could be linked to the more acidic soils that indicate metal sulfides and specifically pyrite (Table 5.2) and the presence of Pb and Zn in minerals with low solubility, such as hemimorphite, smithsonite, anglesite or cerussite even if these minerals could be formally attributed to the first three fractions. The study carried out by Cappuyns et al (2007) indicated that Zn-sulfide minerals and pyrite may not completely be dissolved in the F3 fraction by H_2O_2 and its dissolution can be completed in the last step of BCR fraction. This can also explain the high Zn content in the residual fraction (F4) (Figure 5.10).

5.5. Conclusions

The BCR results revealed that the uppermost soil and the deep soil layers of mine waste tailing affected by the rich carbonates lithology and mineralogy were significantly different in relation to the soil properties. Moreover, the bioavailability of Zn, Pb and Cd in the first fraction of BCR was positively correlated with the total metal contents in the soil around the root samples. Cadmium showed the highest bioavailability in the contaminated soil samples as it was found through the first step of BCR fractions (F1).

Zinc was present in F1 as smithsonite ($ZnCO_3$) > in F3 as sphalerite (ZnS) > in F2 as Fe-oxide in the soil around the root samples. However, the results showed that Pb and Zn were often found in high percentages in the residual fraction (F4), with the highest percentage measured in deep layers of the compost-amendment soil in mine tailing. That was mainly due to the presence of Zn and Pb ore minerals that, having a lower solubility, can resist leaching in F1, F2 and F3 extraction. The different metal fractions indicated that Pb and Zn were less bioavailable for *P. halepensis* roots. It is not clear if the geochemical fractions of metals and immobilization processes were influenced directly by the pyrite dissolution and weathering process or were induced by the rhizospheres and root activities. Further investigations may provide a better insight of *Pinus halepensis* phytostabilization and its physiological adaptation to better trace the mineralization process on similar contaminated mine tailing sites.

CHAPTER 6

6. *Ex-situ* investigation under controlled conditions of greenhouse

Ex-situ investigation on phytoremediation capability of *Pinus halepensis* Mill. a pioneer tree in abandoned mine tailing

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Abstract

Tailings and mine dumps are often a pollutant source and pose a serious environmental threat to the surrounding areas. The use of pioneer vascular plant species to extract or stabilize metals is considered among the more effective reclamation technique in mine tailings. The study aimed at evaluating the phytoremediation potential of *Pinus halepensis* in abandoned mine tailing (Campo Pisano-SW Sardinia, Italy). The plant ability to tolerate high Zn, Pb and Cd concentration and their accumulation in plant tissues (roots and aerial parts) were assessed at greenhouse conditions. Experiments were carried out on 45 healthy seedlings from the plant nursery planted in three different substrates (mine tailings, mine tailings amended with compost and reference substrate) as well as on 15 seedlings spontaneously grown in the contaminated mine site, that were investigated with their own substrates. The phytostabilization potential of plant was evaluated through the biological accumulation and translocation indexes as well as plant survival and biometric parameters. The outcomes showed the adaptability of *P. halepensis* to grow and survive in contaminated substrates. Compost addition did not improve/ did worsen plant survival and growth, however, it enhanced total carbon and nitrogen contents of soil, restricted metal bioavailability and metal accumulation in the aerial parts of P. halepensis. These findings confirm that *P. halepensis* is an appropriate candidate for phytostabilization with great potential to limit the toxicity of Zn, Pb and Cd in plant tissues by applying compost amendment in metal-contaminated mine sites.

Keywords: metal tolerance; phytoremediation; phytostabilization; *Pinaceae*; translocation indexes; biometric parameters

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Novelty statement

The novelty of this study is the selection of *Pinus halepensis* Mill. as a proper tree species for long-term phytoremediation of multi-heavy metal mine tailing sites. This plant species not only had adaptation to the Mediterranean climate and could tolerate high temperatures and high metal concentrations, but also showed high survival and growth percentage of its roots and epigean organs in the highly contaminated mine tailing. *P. halepensis* could limit metal accumulation and toxicity in the aerial part of the plant with the addition of compost amendment. This study demonstrates that compost amendment can enhance the soil properties and modify the metal bioavailability. The outcomes from this study can also be beneficial for the phytostabilization project and restoration of similar Mediterranean mine sites.

6.1. Introduction

Tailings are the most important sources of toxic heavy metals in mine sites. The exposure of fine-grained minerals to atmospheric agents makes them subjected to oxidative dissolution processes (Jerz and Rimstidt 2003; Mendez and Maier 2008). Metals can be mobilized through aeolian dispersion and surface runoff and mostly spread a high range of contaminants in the soils and ground waters of the surrounding areas (Concas et al. 2006; Cao et al. 2008; Barbafieri et al. 2011; Bacchetta et al. 2015). For this reason, mine areas are often affected by low vegetation survival and growth, which in turn may result in the loss of biodiversity due to the high content of heavy metals and the low nutrients and organic carbon in soil (Ahirwal and Pandey 2021; Baker et al. 2010). These unfavorable conditions are common environmental challenges in most of the heavily contaminated mine areas and require effective sustainable remediation.

The pioneer plant species growing spontaneously in these highly polluted environments are sufficiently resilient, well-adjusted to the local climate and can tolerate the high concentration of metals (Cao et al. 2009; Concas et al. 2015; Lai et al. 2015; Bacchetta et al. 2018). Indeed, some of these autochthonous plant species can be effective and beneficial for *in-situ* reclamation of mine tailing sites utilizing phytostabilization processes (Mendez and Maier 2008; Conesa et al. 2019; Lai et al. 2015; De Giudici et al. 2015; Medas et al. 2017). In general, phytostabilization is considered an appropriate technique because through its bio-mineralization processes it helps the low accumulation of metals in the aerial parts of plants and the long-term establishment of plant canopy in mine wastes (Conesa et al. 2019; Pandey et al. 2015; Monaci et al. 2020). This can be improved by appropriate soil amendments capable of contributing to the recovery of organic matter and essential nutrient elements (N, P, K, Cr, Cu and Zn) in soil, enhancing water retention, improving root penetration and plant growth (Barbafieri et al. 2011). Among the investigated soil amendments, compost seems to play an important role in optimizing the phytoremediation process by lowering the metal bioavailability and its accumulation in plant tissues (Bernal et al. 2007; Fagnano et al. 2011; Bacchetta et al. 2015; Conesa and Párraga-Aguado 2019).

Southwest Sardinia (Italy) has a rich history of mine activities dated back to the pre-Roman time. The mine activities together with the related inadequate actions performed to alleviate the metal impacts after the mine closure have left a high content of metal pollutants in the mine wastes estimated to be at about 70 million m^3 (Jiménez et al. 2014; Bacchetta et al. 2018).

Several pilot project studies have been carried out in Sardinian abandoned mining areas availing of its rich biodiversity in order to identify the appropriate autochthonous plant species performing the best phytoremediation capabilities (e.g., Concas et al. 2006; Cao et al. 2008; Barbafieri et al. 2011; Bacchetta et al. 2012). Moreover, the metal tolerance adaptability has been investigated as well as the survival capability of several Sardinian grasses, half-shrubs and shrubs growing spontaneously on the highly contaminated mine waste, such as *Pistacia lentiscus* L., *Scrophularia canina* L. subsp. *bicolor* (Sm.) Greuter, *Dittrichia viscosa* L. Greuter, *Cistus salviifolius* L., *Euphorbia cupanii* Bertol. ex Moris, *Helichrysum microphyllum* Cambess. subsp. *tyrrhenicum* Bacch., Brullo & Giusso, *Phragmites australis* (Cav.) Trin. ex Steud, *Euphorbia cupanii* Bertol. ex Moris and *Juncus acutus* L. (Jiménez et al. 2005, 2011, 2021; Bacchetta et al. 2012, 2015, 2018; De Giudici et al. 2015, 2019; Medas et al. 2015, 2017; Lai et al. 2015; Boi et al. 2019, 2020a, 2020b and 2021). However, few available findings investigated the use of woody tree species despite their potential for the long-term stabilization of mining sites (Domínguez et al. 2009; Disante et al. 2010; Conesa and Pàrraga-Aguado 2019).

Pinus halepensis (Aleppo pine), is a circum-Mediterranean tree species (Tutin et al. 1993; Fady et al. 2003; Farjon 2017; Pesaresi et al. 2017; Pignatti 2017–2019) that can grow spontaneously in different substrates often in marls, limestone (Barbéro et al. 1998; Mauri et al. 2016; Farjon 2017) and low fertile soil at the altitudinal range from sea level up to 1700 m a.s.l. in Morocco (Farjon 2017). It is a thermophilous and xerophilous plant that grows in areas with annual average rainfall between 350 and 700 mm (Barbéro et al. 1998; Mauri et al. 2016) and tolerates low temperatures between 2 and 10°C (Querejeta et al. 2008; Pérez-Piqueres et al. 2018). In the Mediterranean Basin, the forests dominated by this species cover more than 3.5 million ha but despite the species name comes from the town of Aleppo in Syria concentrated mainly in the western part, they are more scattered in the eastern side (Pesaresi et al. 2017). In Italy, *P. halepensis* is considered a native species in many regions, as well as the islands of Sicily and Sardinia (Bartolucci et al. 2018; Bartolucci et al. 2020). The usefulness of *P. halepensis* in the restoration of degraded soil in arid and semi-arid soils is documented in the literature (Querejeta et al. 2008; Pàrraga-Aguado et al. 2013), because of its high metal tolerance and eligibility to survive in low nutrient conditions (Sardans et al. 2005). These findings make this plant species a suitable candidate for performing phytoremediation experiments. In this study, in order to better assess the process of phytoremediation of *P. halepensis*, one of the widespread evergreen tree species growing on the Mediterranean mine substrates, the experiment was carried out under the greenhouse-controlled condition.

The aims of this study were to assess the capability of *P. halepensis* to tolerate high concentrations of Zn, Pb, and Cd, in terms of: (**i**) plant survival and plant growth (elongation and biomass (dry weight) production of roots and epigean organs); (**ii**) metals bioavailability and its accumulation in the roots and the aerial parts of the plant through the evaluation of biological parameters and (**iii**) mitigation of heavy metals stress in the plant by assessing the effectiveness of compost organic amendment.

6.2. Materials and Methods

6.2.1. Study area

The study area is Campo Pisano mine tailing (CP) which is located in the Iglesiente biogeographic subsector (Fenu et al. 2014), South-Western Sardinia, Italy (Figure 6.1). The area is characterized by a Mediterranean pluviseasonal bio-climate ranging between the upper thermo- and the lower meso-Mediterranean (Bacchetta et al. 2009), within 600 mm annual mean precipitation, 17°C temperature, around 57% evapotranspiration and 24% runoff (Cidu et al. 2001; Lai et al. 2015). The surface layer of CP mine waste is generally classified as neutral or slightly alkaline (pH 7.3) and sandy-loam (70% sand, 13% silt and 17% clay) based on the USDA classification (USDA 1998). Moreover, the deep layers of Campo Pisano characterized differently and are more acidic due to the metal sulfides and pyritic-rich materials (Aversa et al., 2002; Kharazian et al., 2022b). The application of different mine extraction methods during the exploitation activities caused the intensive heterogeneity of metal concentrations (e.g. Zn, Pb and Cd) and poor nutrient contents in CP mine wastes (Boni et al. 1999). In this study, the mine waste samples were collected in the Campo Pisano mine site (Figure 6.1) at a maximum depth of 30 cm, transported to the laboratory and mixed and homogenized several times with a shovel.

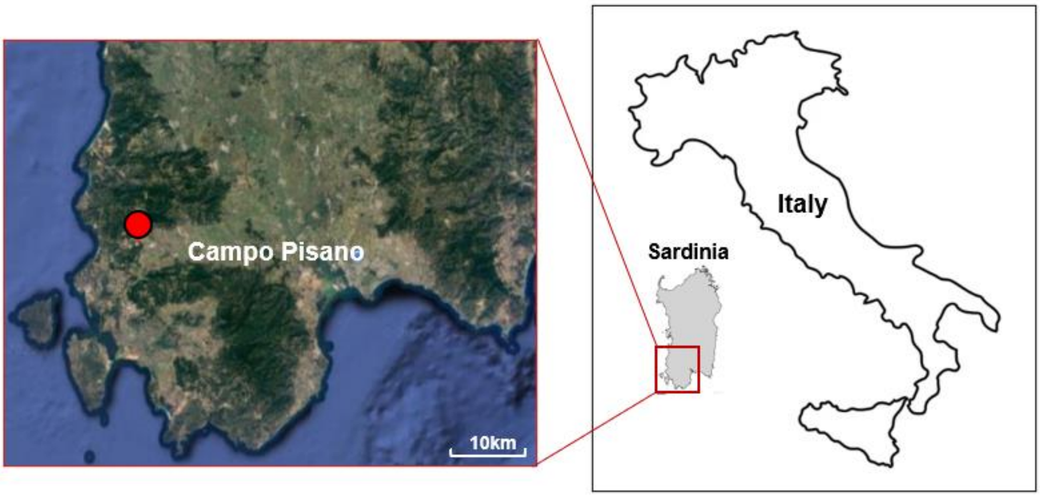

Figure 6.1. Location maps of the study area in south-west of Sardinia, Italy; the red sign indicates the sampling site of Campo Pisano mine tailing dumps

6.2.2. Experimental design

The selection of *P. halepensis* was based on the preliminary study on this plant species made by Kharazian et al. (2022a and 2022b), its adaptability to the local geochemical and mineralogical characteristics, climate and its option to grow spontaneously in this highly polluted environment.

Forty-five healthy seedlings of *P. halepensis* (roughly 4–6 months old) provided by the Bagantinus plant nursery (PN) of the “Forestas Agency” of “Regione Autonoma della Sardegna” (Decimomannu, South Sardinia) have been selected (out of approximately 100 similar specimens) and placed for some days into the greenhouse to be adapted to the new environment. Thereafter, these seedlings were transplanted randomly and individually in three distinct sets of 15 pots (1.7 L-polyethylene pots; 16×13×10 cm). The pots of each set were filled with 1.5 L of a specific homogenized substrate: (**i**) not-contaminated reference treatment (NOC) consisting of 40% peat, 45% commercial soil and 15% sand; (**ii**) Campo Pisano contaminated mine waste (CP), and (**iii**) Campo Pisano mine waste amended with 30% w/w of compost from Municipal Solid Waste (MSW) organic fraction (CPC) (Figure 6.2), provided by "Tecnocasic S.p.A." (Cagliari, Italy) and characterized by 24% Carbon and 1.9% Nitrogen content, pH~ 6.6 with the total content of 500 mg kg^{-1} Zn, 140 mg kg^{-1} Pb and 1.5 mg kg^{-1} Cd. 30%w/w of Compost was recommended to examin its effectiveness in enhancing soil properties and plant growth considering the specific characteristics of CP substrate and the previous phytoremediation experiences in the same site. Moreover, fifteen *P. halepensis* seedlings (about 3–6 months old) spontaneously grown in Campo Pisano contaminated site (PS), were also collected randomly (out of approximately 50 similar specimens), transferred into similar pots and filled with about 1.5 L of their own CP-contaminated substrate (S).

All the pots were placed into the greenhouse for being investigated under controlled conditions (constant temperature at 20°C, humidity at 60–65%, and 12 hours of light/ 12 hours of darkness as photoperiods) at the greenhouse laboratory of Sardinian Germplasm Bank (BG-SAR) (Porceddu et al. 2017), University of Cagliari, Italy for six months (Figure 6.2). Plants were watered twice a week with 30 mL of distilled water for the first two months and continued 3 times a week with 50 mL until the end of the experiment. The amount of water used was based on the plant needs and according to the findings of the previous phytoremediation lab-scale experiments carried out on the Mediterranean plant species (Bacchetta et al. 2015; Boi et al. 2021). Before greenhouse trials began (time T0), homogenized substrates and plants were characterized. The total Zn, Pb, and Cd concentration and their bioavailable fraction were assessed in the four selected substrates (NOC, CP, CPC, S). As for plant specimens, five seedlings of pines (out of the overal polulation of 100 similar specimens) from the nursery (PN) and five seedlings of pine (out of 50 similar specimens) grown naturally in the Campo Pisano site (PS) were randomly selected at the beginning of experiment (T0), at the same time of selecting plants and planting in pots, washed several times with distilled water and dried at 45°C. Each specimen was split into roots and epigean organs and characterized in terms of length and biomass dry weight (Figure 6.2). The accumulation of Zn, Pb and Cd was then measured in a single composite sample (out of five samples) for root as well as epigean organs separately at the beginning of the experiment (T0). All these parameters were evaluated again at the end of six months experiment (T6), by removing plants from the pots and carefully washing them before analyses.

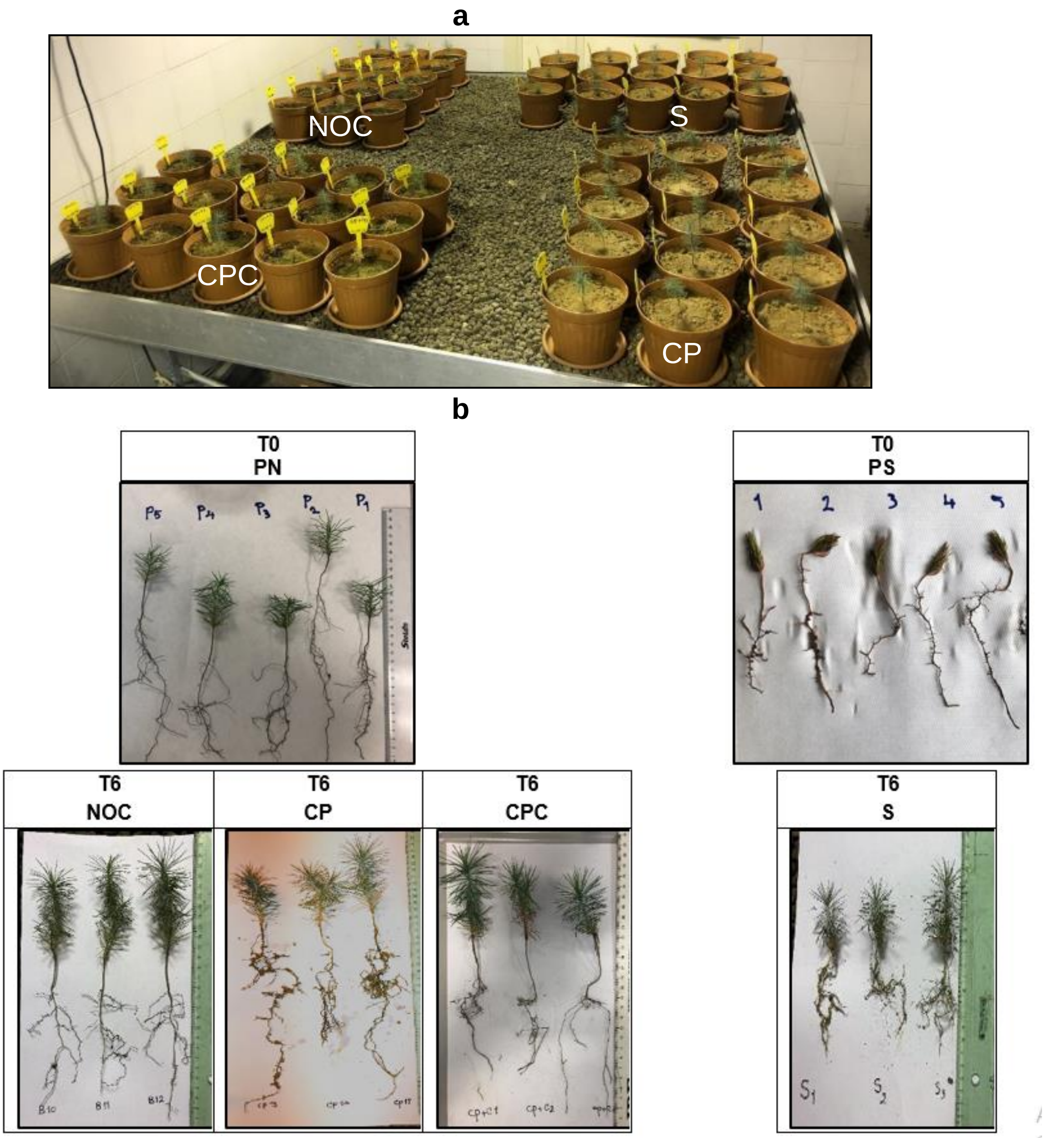

Figure 6.2. The lab scale experiments on *Pinus halepensis* samples in different substrates. (**a**) Pots of the considered treatments: seedlings from nursery planted in not-contaminated reference (NOC); seedlings from nursery planted in Campo Pisano substrate (CP); healthy seedlings from nursery planted in CP substrate amended with compost (CPC); contaminated substrates with its plant samples collected from the CP mine site (S). (**b**) Seedlings from the nursery (PN); seedlings from the contaminated site (PS), before planting (T0) and after six months (T6) are shown as representative of collected plant samples.

6.2.3. Substrate analysis

Physico-chemical characteristics of substrates

The substrate of each treatment (15 pots) was mixed and homogenized in a single composite sample, sieved (<2 mm), ground in fine powder in an agate mortar and oven-dried at 45 °C (Binder GmbH, Tuttlingen, Germany) in order to be prepared for the analysis. The chemical characteristics of substrates were analyzed following the Italian society protocol of the soil science D.M.13.09.1999 (Barbafieri et al. 1996; Lindsay and Norvell 1978). Soil pH was measured in distilled water (1:2.5) using a pH-meter (ORION model 230A). The Electrical Conductivity (EC) of substrates was calculated in 50 mL distilled water (1:1) utilizing a conductivity meter (HANNA-DiST4). Total carbon and total nitrogen as well as organic carbon (OC) content of the substrates were determined through Micro-analyzer (CHNS/O 2400 SERIE II PERKIN ELMER) calibrated with the reference material (ore minerals). Organic carbon (OC) was analyzed after soil samples were treated and acid-washed (HCl 1 mol L^{-1}) to remove carbonate content, according to Ussiri and Lal (2008).

Total metal content and bioavailable concentration of metals in substrates

The total metal concentrations in substrates were assessed using the Environmental Protection Agency (EPA) method 3052 (see for more details at Bacchetta et al. 2012). It was performed using duplicate samples in microwave-assisted acid digestion (Start D, Milestone, Sorisole, Italy) following the procedure according to Bacchetta et al. (2018). Samples were processed with certified reference materials, GBW-07403-GSS-3 for substrates as well as the blank solution (the EnviroMAT-pure water high reference solution, EP-H-1150). All reference materials and blank samples were matrix-matched with reagents and samples.

The bioavailable content of Zn, Pb, and Cd was evaluated for all substrates through a single extraction method on duplicate samples performed by using DTPA solution (diethylenetriaminepentacetic acid), calcium chloride ($CaCl_2$) and triethanolammine (TEA) adjusted to pH 7.3 following the procedure made by Lindsay and Norvell. (1978), Barbafieri et al. (1996) and Feng et al. (2005). This extraction procedure is recognized to be an efficient method to prevent the dissolution of carbonate as well as the release of bounded metals (Feng et al. 2005). The bioavailable fractions of metals (bf) were measured (in percent) considering the mean values of bioavailable fractions of metal (M_{bf}) and the total metal content (M_{total}) in the substrates.

$$\text{bf} (\%) = (M_{bf}) / (M_{total}) \times 100 \qquad (1)$$

The total metals content (Zn, Pb and Cd) as well as the bioavailable fractions of the substrate, were both determined by Inductively Coupled Plasma Emission Spectrometry (ICP-OES, Perkin Elmer Optima DV 7000, Waltham) after cooling, filtering and diluting the prepared solutions using distilled water (Guan et al. 2011; Barbafieri et al. 1996).

6.2.4. Plant analysis

Metal accumulation in plant tissues

Before metal analyses, the roots and the epigean organ samples were separated and ground into powder using an electric grinder (Ultra Centrifugal Mill ZM 200, Retsch GmbH). Metal accumulation (Zn, Pb and Cd) in plant tissues were measured following the same procedure described in Kharazian et al. (2022). Data quality was also assessed by processing certified reference materials, GBW-07603-GSV-2 (bush twigs, and leaves) and the concentration of metals was analyzed by ICP-OES using the same wavelengths performed for the substrates. Due to the low biomass production of plants at the end of six months (T6), for each treatment, a single composite root sample was produced (out of 15 specimens) as well as three composite samples of aerial parts (out of each 5 samples).

Assessment of the biometric parameters: plant growth and phytoremediation potential

The variation of biometric parameters (BP) was calculated in each treatment at the end of the phytoremediation experiment (T6) in comparison with the control sample (T0).

$$\text{BP Variation (\%)} = (BP_{T6}–BP_{T0}) \times 100 / BP_{T0} \quad (2)$$

The phytoremediation potential of *P. halepensis* to extract metals from the substrate and translocate them to the epigean organs of the plant was assessed using the biological indexes, namely Biological Accumulation Coefficient (BAC), Translocation (TF) and Biological Concentration Factors (BCF).

The BAC was measured to estimate metal transferring from the soil (M_S) to the epigean organs (M_E) (Marchiol et al. 2013) as $BAC = (M_E) / (M_S)$ (3)

The metal translocation from roots (M_R) into the epigean organs (M_E) (Brunetti et al. 2009) was measured as $TF= (M_E) / (M_R)$ (4)

The BCF reported the ratio between the metal content in roots (M_R) and soil (M_S) according to Fellet et al. (2007): $BCF = (M_R) / (M_S)$ (5)

6.2.5. Data analysis

One-way ANOVA was used to evaluate statistical differences between plant growth in different substrates and to assess variations of the growth rate from T0 to T6 in terms of elongation and the biomass production of root and the epigean organ of *P. halepensis*. Moreover, the significant differences highlighted by ANOVA were afterward analyzed by the post-hoc pairwised comparisons T-test (Tukey test). The result values are expressed as the mean ± standard deviation and the significant level of statistical analysis in all cases considered $p<0.05$, using Sigmaplot 11.0 software. The matrix of Principal Component Analysis (PCA) was performed to examine the correlations between the total metal contents in soil and the metal accumulation in plants with the biological parameters and DTPA-extracted metals in the contaminated and not-contaminated soil samples using the statistical software CANOCO 5 proposed by ter Braak and Smilauer (2002).

6.3. Results

6.3.1. Substrates analysis

Table 6.1 shows the main physico-chemical properties of soil samples as well as the total Zn, Pb and Cd concentration and the bioavailable fractions of metals in all substrates. The data shows that the substrates of CP and S were slightly alkaline (pH 7.2–7.8) and presented low EC (3.4–6.8 dSm^{-1}), whereas the compost-amended substrate (CPC) showed acidic (pH 6.1–6.7) and high EC (20.2–21.2 dSm^{-1}). Both CP and S substrates showed low content of organic carbon (up to 0.7%) and nitrogen contents (up to 0.1%). The highest total carbon (up to 13.5%) and organic carbon (up to 6.3%) were reported for CPC substrate.

Table 6.1. Total metal content (Zn, Pb and Cd) and physico-chemical characteristics of substrates (means ± SD, n=2); NOC: not contaminated substrate; CP: Campo Pisano substrate with plants from nursery; CPC: Campo Pisano substrate with compost; S: Campo Pisano substrate with plant grows naturally in contaminated site; bf: bioavailable fraction; T0: before planting; T6: after six months study

	Parameters		NOC	CP	CPC	S
Zn	Zn-total (mg kg^{-1})	T0	59.2±0.9	28041.7±3153.5	20193.9±769	34842.3±2899.3
		T6	50.9±4.6	30038.6±2367.03	21334.9±1257.5	30444.08±4525.3
	Zn-bf (%)	T0	17.4	8.8	0.9	7.3
		T6	16.5	7.9	0.2	8.0
Pb	Pb-total (mg kg^{-1})	T0	18.9±2.9	17270.5±2404.4	13324.5±48.6	24338.01±2242.6
		T6	18.3±2.3	15258.5±2708.5	14859.04±42.5	19644.3±4420.9
	Pb-bf (%)	T0	31.8	6.5	0.2	3.6
		T6	27.7	8.2	0.4	6.3
Cd	Cd-total (mg kg^{-1})	T0	0.92±0.5	121.9±11.6	108.4±0.5	141.4±6.4
		T6	0.37+0.06	106.1±5.1	113.8±0.05	124.9±7.4
	Cd-bf (%)	T0	14.8	10.6	2.1	11.6
		T6	29.4	13.05	1.1	11.5
pH		T0	7.8	7.2	6.1	7.5
		T6	7.8	7.3	6.7	7.8
EC (dSm^{-1})		T0	4.2	4.7	21.2	3.4
		T6	3.1	6.8	20.2	3.5
Total C (%)		T0	6.9±0.9	7.8±0.06	13.5±0.2	6.6±0.2
		T6	6.5±0.4	7.6±0.1	13.2+0.2	6.3±0.1
Total N (%)		T0	0.4±0.05	0.1±0.01	0.3±0.03	0.1±0.02
		T6	0.4±0.06	0.1±0.01	0.3±0.02	0.1±0.01
Organic C (%)		T0	6.5±0.2	0.3±0.02	6.3±0.5	0.7±0.06
		T6	6.3±0.2	0.3±0.01	6.1±0.2	0.5±0.01

The chemical data of metal concentration in substrates shows that the most abundant metal was Zn, followed by Pb and Cd in each treatment. Indeed, the total metal content was high in S and CP substrates with the highest values reported for Zn (up to 34842.3 mg kg^{-1}), Pb (up to 24338.01 mg kg^{-1}) and Cd (up to 141.4 mg kg^{-1}) in S substrate. All metals concentration remained almost constant in each substrate at the end of the trial in T6. The total metal content (mainly Zn and Pb) measured in CPC was lower than in CP and S, indicating that the compost addition has a diluting effect on mine waste (Table 6.1).

The bioavailable metal content shows that the bioavailability of all investigated metals in not contaminated (NOC) substrate was higher than CP, S and compost-amended (CPC) substrates (Table 1). Cadmium was the most bioavailable metal in all substrates through the phytoremediation test (T6), except compost-amended substrate (CPC). The lowest bioavailable fraction for Cd was reported in CPC up to 2.1% in T0 which decreased to 1.1% in T6. Zinc (0.9%) and lead (0.4%) were also the lowest bioavailable metals in the same substrate. The bioavailable values for Cd were reported 13.05% in CP and 11.6% in S substrates.

6.3.2. Plant analysis

The preliminary metal analysis assessed in plant tissues (T0) showed that root and epigean organs of *P. halepensis* accumulated Zn > Pb > Cd which is followed also through the phytoremediation test (T6) in each treatment (Table 6.2). The Zn, Pb and Cd concentrations measured at T6 were generally higher in roots than in epigean parts of the plant in all substrates. The highest accumulation of Zn, Pb and Cd in plant tissues was detected in S followed by CP and CPC. In the compost-amended substrate (CPC), Zn and Pb were accumulated mainly in the root samples of *P. halepensis* (534.7

mg kg^{-1} Zn and 707.3 mg kg^{-1} Pb) and their accumulation in the epigean organs (24.2 mg kg^{-1} Zn and 21.9 mg kg^{-1} Pb) was lower than the contaminated substrates (S: up to 505.6 mg kg^{-1} Zn and 260.06 mg kg^{-1} Pb). Due to the low biomass production of plant roots, the assessment of total metal content in roots was performed on a single composite root sample (one replication) for each treatment. Then after, three replication of one composite root sample were checked for the quality of analysis. Indeed, three values were checked for analytical quality according to the aim of the research.

Table 6.2. Zn, Pb and Cd concentration (mg kg^{-1}) in *P. halepensis* tissues before planting (T0) and after 6 months of experiment (T6); R: roots; Epi: Epigean parts; <dL: lower than the detection limit; for epigean parts mean ± SD, n=3

Metals	Parameters		**NOC**	**CP**	**CPC**	**S**
Zn	Zn-R (mg kg^{-1})	T0	52.7	52.7	52.7	7459.5
		T6	45.6	6254.7	534.7	10768.5
	Zn-Epi (mg kg^{-1})	T0	0.6±0.1	0.6±0.1	0.6±0.1	701.8
		T6	0.9±0.4	263.1±56.5	24.2±4.2	505.6±27.7
Pb	Pb-R (mg kg^{-1})	T0	5.7	5.7	5.7	6885.7
		T6	12.06	5342.4	707.3	11427.2
	Pb-Epi (mg kg^{-1})	T0	<dL	<dL	<dL	535.2
		T6	<dL	173.1±53.5	21.9±3.4	260.06±21.2
Cd	Cd-R (mg kg^{-1})	T0	<dL	<dL	<dL	95.05
		T6	<dL	44.7	4.6	97.7
	Cd-Epi (mg kg^{-1})	T0	<dL	<dL	<dL	7.3
		T6	<dL	1.6±0.4	<dL	6.4±0.3

6.3.3. Plant biometric parameters: survival, elongation and biomass growth

Figure 6.3 and Figure 6.4 show the assessment of biometric parameters measured for *P. halepensis* in terms of root and epigean elongation and biomass production at the beginning (T0) and at the end (T6) of the phytoremediation experiment as well as plant survival (at T6) for each treatment. The highest plant survival percentage was reported in not contaminated substrate (NOC) and in S (up to 100 %) followed by CP (93.3%) at T6. Further, the plants grown in the compost-amended substrate (CPC) showed 60% of survival rate (Figure 6.3, row 1). ANOVA shows no statistically significant differences (p<0.05) among treatments (Figure 6.3a, row 2) in terms of root and epigean elongation, except for the plants that grew on the S substrate which were significantly lower compared to the other treatments. Significant differences (p<0.05) were assessed in the epigean elongation during the time (Figure 6.3b, row 2) in all the treatments, whilst root elongation varied significantly over time only in CP and S substrates. The epigean biomass production in substrate S was significantly lower than that observed in the other treatments, whereas the root biomass production was similar in NOC and CPC and significantly higher in CP (Figure 6.3a, row 3). During the time, the epigean biomass significantly grew in all the treatments, whilst roots biomass showed no significant changes (p<0.05) after 6 months of experiment in CPC and NOC (Figure 6.3b, row 3). The plant survival (at T6) is presented in figure 6.4.

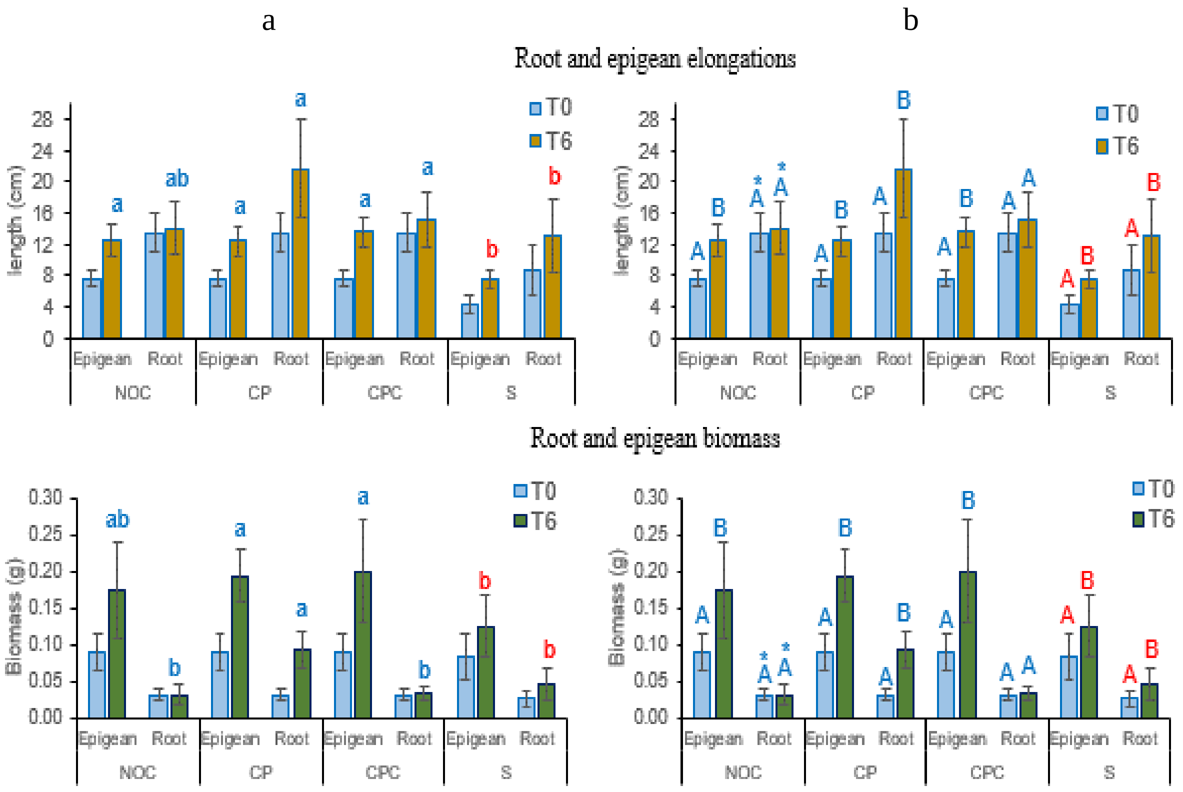

Figure 6.3. Biometric parameters of *P. halepensis* during the trial (mean values± SD; n=15) and statistical analysis: (**a**) statistical analysis of biometric parameters among treatments at a fixed time (T6); (**b**) statistical analysis of biometric parameters in each treatment over time (from T0 to T6); different letters indicate statistical significant differences at $p<0.05$; different colors of letters indicate plant references (blue color for plant obtained from nursery and red for the plant collected from CP site)

Figure 6.4 summarized the variation of biometric parameters calculated in T6 for all studied treatments in comparison with the control sample (T0). It shows that plant survival was very high in all the substrates, with the exception of the compost-amended substrate (60%). The root length and biomass increased over time, especially in CP and S, the most contaminated substrates, whilst this increase was reduced in CPC and even more in the reference treatment. The Epigean length and biomass show similar increases among treatment substrates.

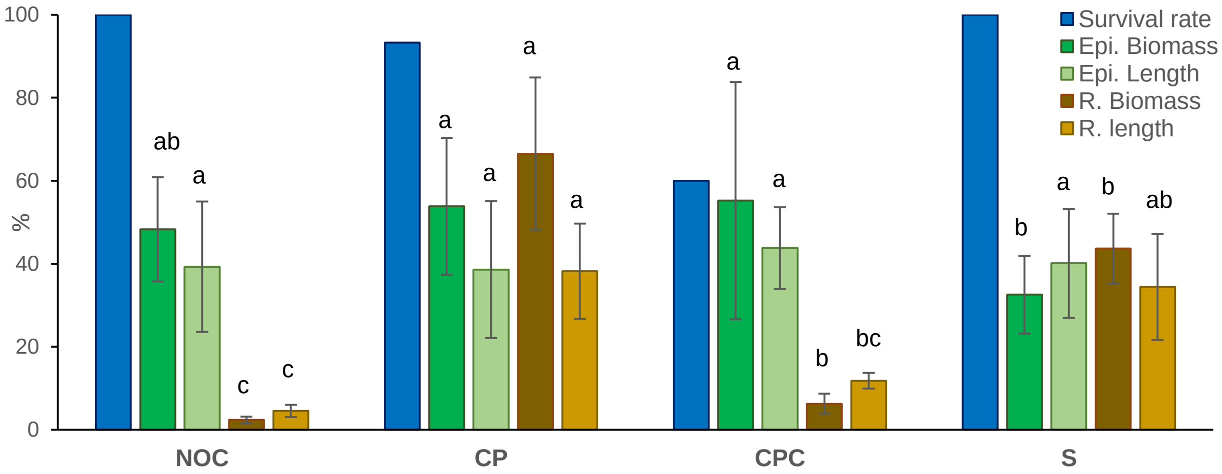

Figure 6.4. Variation of biometric parameters calculated in all treatments at the end of phytoremediation experiment (six months); R: root; Epi: epigean; different letters indicate statistical significant differences at $p<0.05$ for each parameter among treatments

6.3.4. Biological accumulation parameters and phytoremediation potential

The biological accumulation parameters (BCF, BAC and TF) were calculated for root and epigean samples through the phytoremediation test in order to evaluate the phytoremediation potential of *P. halepensis* (Figure 6.5). In all substrates, the measured values of investigated metals are consistently very low (<1). In detail, the BCF values of CPC for the uptake of all metals from soil to roots are significantly very lower than those of CP and S. In not contaminated substrate (NOC), the BCF values for Zn and Pb were higher (0.8 Zn and 0.6 Pb) than those of CP and CPC samples. The BAC values for Zn and Pb were generally very similar for all substrates (0.01–0.03), except in CPC that shows the lowest BAC for all metals. The BAC values related to the uptake of Cd in S (0.05) and CP (0.02) substrates were higher than in not-contaminated and compost-amended ones.

The translocation of Zn, Pb and Cd to the plant epigean organs was generally low (TF<1) in all substrates. Higher TF values for Cd have been measured in S (0.06) and CP (0.04). However, the TF of Zn and Pb were the same in all measured substrates. The lowest BAC and BCF value for all metals were measured in CPC samples (Figure 6.5).

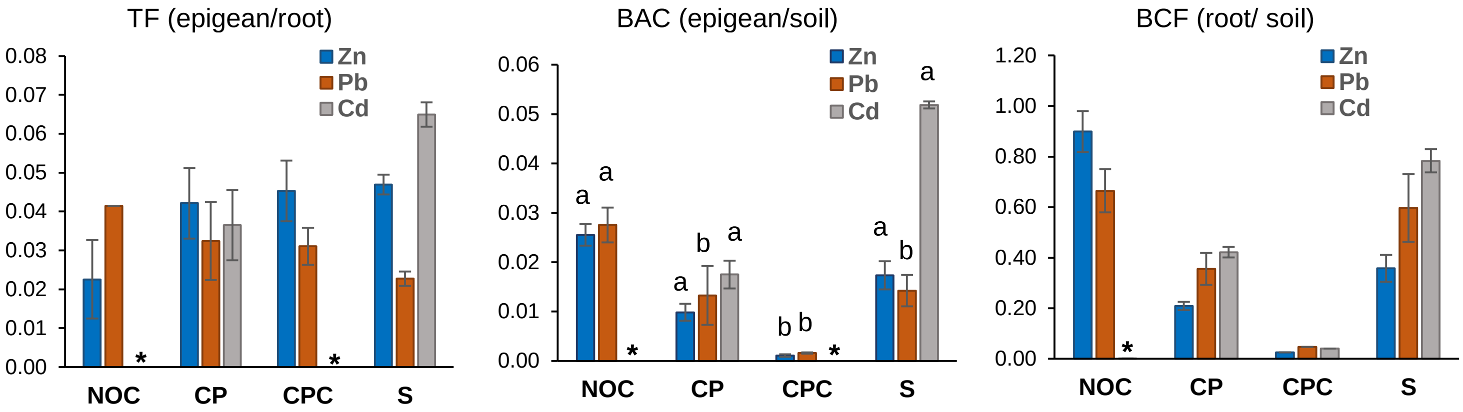

Figure 6.5. Biological accumulation indexes (BAC, TF, and BCF) through the phytoremediation test (T6) and calculated from the data in table 1 and Table 2; * indicated that the calculated values for Cd were lower than detection limit in some substrates (<dL); different letters indicate statistical significant differences at p<0.05 performed only for epigean organs, but not for root samples (one replication) among treatments.

Figure 6.6 shows the PCA analysis that was performed to assess the distribution and similarity among contaminated substrates and the correlation between total Zn, Pb and Cd concentration in their substrate, roots and epigean organs of *P. halepensis*. These parameters are spatially ordinated within a diagram according to the substrates (NOC, CP, CPC and S). The first component of the diagram (PC1) shows 96.9% of the total variation. Moreover, parameters, such as the biological parameters for Pb (Pb-TF, Pb-BAC and Pb-BCF), Zn-BAC and Zn-BCF having positive correlation were all presented in PC1. The other parameters, such as Cd-TF, Cd-BAC, Cd-BCF, total metal content in the substrate (Zn-tot, Pb-tot and Cd-tot), metals accumulation in roots (Zn-R, Pb-R and Cd-R) and in epigean organ (Zn-E, Pb-E and Cd-E) shows strongly negative correlation in the inverse section of the first principal component. The second principal component (PC2) explained only 2.6% of the total variation with a high positive correlation for all metals bioavailable fraction (Zn-bf, Pb-bf and Cd-bf), Pb-BCF and Zn-BAC. The not contaminated substrate (NOC) was distributed on the positive side of the first axis and the main variables that had a positive correlation with it were biological parameters for Pb (Pb-BAC and Pb-TF) and Zn (Zn-BCF). Both CP and S were distributed on the negative side of the first component axis and exhibited correlation with the high levels of Zn, Pb and Cd concentration in their substrates as well as roots and epigean organs of *P. halepensis* (Figure 6.6).

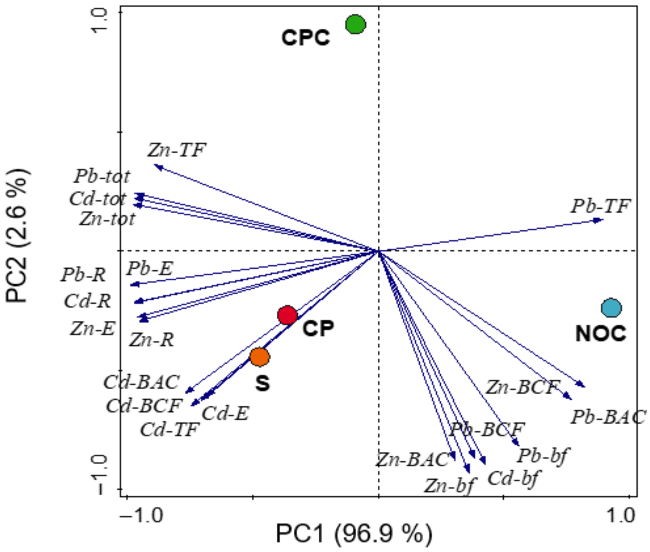

Figure 6.6. PCA for roots and epigean organs of *P. halepensis* grows in different substrates: NOC: not contaminated; CPC: Campo Pisano compost-amended; CP: contaminated Campo Pisano; S: plant and substrate from Campo Pisano site; tot: total metal in the substrate; R: root; E: epigean; TF: the translocation factor; BAC: biological accumulation coefficient, BCF: biological concentration factor; bf: the bioavailable fraction of metals

6.4. Discussion

The results showed that Zn, Pb and Cd concentrations measured in the contaminated (CP and S) and the compost-amended (CPC) substrates were above the maximum threshold contamination levels estimated by Italian law for the industrial purpose (1500 mg kg^{-1} Zn, 1000 mg kg^{-1} Pb and 15 mg kg^{-1} Cd; Decreto Legislativo n.152. 2006, Guri 2006) with the exception of Cd in CPC substrate that was below the threshold limit.

It should be considered that Zn is an essential element for plant growth, whereas Pb and Cd are highly toxic for plants (Nagajyoti et al. 2010; Kabata-Pendias 2011). Further, all metals concentration measured in root and epigean organs of *P. halepensis* grown in CP and S samples were above the reported phytotoxic levels for plants (20–100 mg kg^{-1} Zn, 10–20 mg kg^{-1} Pb and 5–10 mg kg^{-1} Cd; Rathore et al. 2019) at the end of the trial, except for Cd in epigean organs. This indicates the *P. halepensis* tolerance and adaptability to grow, and its capacity to develop the mechanisms to survive in the contaminated mine waste. It should be considered that all metal concentrations measured in roots were higher than in epigean organs of *P. halepensis* samples, indicating a metal tolerance and exclusion mechanisms of *P. halepensis* for these metals. These outcomes are in agreement with the previous studies carried out by Disante et al. (2010) reporting high Zn accumulation in roots than in the aerial parts of *P. halepensis*. The effect of organic amendments to reduce the metal uptake in roots are reported for some plant species growing in similar polluted mine sites (Bacchetta et al. 2012, 2015; Conesa and Párraga-Aguado 2019; Boi et al. 2021). Likewise, the finding of this study also reveals that the metal content (total metal and bioavailable fractions) and the metal uptake into the plant tissues were generally lower in the presence of compost (30% w/w) in CPC substrate than in CP and S (see Table 6.1). Indeed, all metals accumulation in epigean organs and Cd in roots of *P. halepensis* grown in CPC were below the phytotoxic levels for the plant at the end of the 6 months study (T6). Thus, the addition of compost can have a positive effect on the reduction of Zn, Pb and Cd bioavailability in soil and their concentration in plant tissues over time (see Table 6.2).

The calculated variation of biometric parameters in T6 shows that plant survival was higher in the contaminated substrates. This finding shows the higher adaptability of *P. halepensis* to highly contaminated mine waste than other native plants species (i.e., *H. microphyllum* subsp. t*yrrhenicum*, *P. lentiscus* and *P. australis*) that were investigated in different phytoremediation experiments in the same mine tailing (Bacchetta et al. 2015; Boi et al. 2021). This confirms the great potential of *P. halepensis* to tolerate polluted substrates and matrix toxicity, even though, the visual symptoms of metal toxicity, such as chlorosis were distinguished in the needles of seedlings grown in CP substrate at the end of the trial. Moreover, the exposure of young seedling to the stress conditions after the addition of 30 w/w% compost could have detrimental effects and induced negative effect on plant survival in compost amended substrates (CPC).

Moreover, the significant increase of plant growth (roots elongation and biomass) in both CP and S substrates and similar growth of epigean parts in all substrates reveal that compost addition seems not to influence the survival rate nor the elongation and biomass production of root and epigean organ in *P. halepensis*. However, compost could improve the physico-chemical properties of the contaminated substrate by slightly increasing organic carbon, total nitrogen and electrical conductivity and decreasing pH value to slightly acidic conditions (see Table 6.1). This is in agreement with the findings of Concas et al. (2006), which reveal that organic amendments could be able to improve the physical properties of mine tailings in terms of water retention and bulk density. Other studies have also suggested that soil organic amendments can decrease metal availability by the formation of organic metallic complexes (Clemente et al. 2006; Rodríguez et al. 2016). Moreover, the CP and S substrates showed lower organic carbon contents than the compost-amended sample (CPC), indicating the presence of inorganic carbon in the form of carbonate (i.e., calcite and dolomite) which is consistent with the carbonates lithology rich in alkaline waste materials in Campo Pisano mine area (Bechstädt et al. 1994; Bacchetta et al. 2015).

Further, the addition of 30%w/w compost, especially at the first stage of plant growth, and the exposure of young seedlings to stress conditions on planting in the contaminated matrices induced increasing plant mortality in a short time (Disante et al. 2010). This may explain the low elongation and biomass production in root samples in the compost-amended substrate (CPC). Thus, further investigation on the long-term application of amendment is advisable to better understand the effectiveness of compost on the growth of *P. halepensis* root samples over a long time.

The analysis of PCA confirmed that both CP and S substrates are similar and statistically correlated with Zn, Pb and Cd concentration in their substrates as well as in the *P. halepensis* tissues, considering their differences with the compost-amended substrate. Moreover, the biological accumulation parameters were generally very low (BAC, TF <1) in all substrates, indicating the phytostabilization/revegetation potential of *P. halepensis* that can restrict the translocation of Zn, Pb and Cd from soil to roots and more specifically to the aerial parts of the plant. Thus, due to the low capability to accumulate the Zn, Pb and Cd into the epigean organs, and according to what has already been reported in the *in-situ* investigation carried out by Kharazian et al. (2022a), *P. halepensis* with low translocation factor (TF< 1) can be considered as a pioneer species suitable for revegetation in highly polluted mine waste materials.

6.5. Conclusion

This *ex-situ* study was carried out to evaluate the phytoremediation potential of *Pinus halepensis* growing on the highly contaminated mine waste to provide information on revegetation and recovery of the mine tailing. The results reveal that *P. halepensis* can possess suitable characteristics for phytostabilization projects: (**i**) the high concentrations of Zn, Pb, and Cd in the contaminated substrates and in roots samples as well as the overall plant survival and the growth data in the short-term highlighted the tolerance and adaptability of *P. halepensis* to survive and grow in the contaminated mine substrates; (**ii**) the assessed metals bioavailability and the low BAC, BCF, and TF values (<1) emphasize the phytostabilization potential of *P. halepensis*;(**iii**) the addition of compost-amendment had a negative influence on the plant survival and growth, however it improved the organic carbon and nitrogen contents in the substrate and mitigated the heavy metals toxicity in the plant through decreasing the total metal content in the soil, the low bioavailable fraction and metal accumulation in roots and epigean organs of *P. halepensis*. Thus, compost can be advisable in phytostabilization projects considering further *in-situ* investigations applying the optimal compost quality to provide better insight on *P. halepensis* physiological adaptation in the mine tailing site.

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CHAPTER 7

7. Summary and general conclusion

The PhD thesis focused on the geochemical, mineralogical and physico-chemical properties of the soil-plant system considering the capability of Sardinian native species *Pinus halepensis* Mill., growing spontaneously in highly contaminated abandoned mine tailing (Campo Pisano, SW-Sardinia, Italy). The idea was to devise a sustainable, easy-to-replicate and cost-effective technique for the reclamation of highly contaminated mine sites.

To start the experiment, *P. halepensis* was selected as a pioneer phytoremediation tree species growing spontaneously on the Campo Pisano mine tailing site (CP) with multiple heavy metal contamination. Plant materials (roots, barks, wood and needles) and soils (in-depth and around the roots) were collected aimed at evaluating the main mineralogical characteristics, metal content, plant accumulation and translocation behaviour. The outcomes showed that the polluted substrates were composed of pyrite, dolomite, calcite, quartz, gypsum, and barite with iron sulfate, and iron oxide. Zn ore minerals (smithsonite) and muscovite were detected mostly in the deeper soil layers. Iron sulfate was present in the soil substrate around the roots as well as on the surface of the plant roots. These sulfate and iron phases indicated the presence of some minerals that were discharged in the Campo Pisano mine waste, notable pyrite that undergo dissolution reaction. However, it was not clear if the pyrite dissolution reaction is fastened by rhizosphere processes. Zn was the most abundant metal in the substrate as well as plant tissues. Roots accumulated high metal concentration (up to 2710 mg kg^{-1} Zn, 735.88 mg kg^{-1} Pb, and 11.02 mg kg^{-1} Cd) reflecting high metal contamination in soil. The biological accumulation and translocation values were reported below one (BCF, TF, BAC <1) for all plant tissues which indicated 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 potentially eligible for long-term phytostabilization projects in abandoned mine tailing sites.

Further investigation was carried out at assessing the geochemical forms of elements through the BCR three-step sequential extraction method in relation to the physico-chemical and mineralogical characteristics of the soil in depth and the soil-root system. The results showed that Cd in all contaminated substrates was mainly present in the exchangeable BCR fraction, while Zn and Pb were often found in the residual BCR fraction and were less bioavailable for *P. halepensis* roots. That was mainly due to the presence of Zn and Pb ore minerals having a lower solubility that can resist leaching in BCR extraction or can be due to other beneficial effects of rhizosphere interaction processes. Moreover, the uppermost alkaline-calcareous layers of mine waste, were affected by the rich carbonate lithology and mineralogy of CP and were different with respect to the deeper acidic layers. As far as the soil around the plant root is concerned, the bioavailability of all metals in the first fraction of BCR was positively correlated with the total metal contents. Pb and Zn were less bioavailable for roots and often found in high percentages in the residual fraction, mainly in deep layers of the compost-amended soil.

Further investigation was beneficial for acquiring a better assessment of *P. halepensis* phytostabilization capability, its adaptation, growth (elongation and biomass) and survival rate under the greenhouse-controlled condition. Thus, for these aims, a six-month ex-situ experiment was carried out under the controlled condition of the greenhouse laboratory of Sardinian Germplasm Bank (BG-SAR), Hortus Botanicus Karalitanus (HBK), Cagliari. Young pine seedlings were planted in pots aimed at evaluating plant ability to tolerate Zn, Pb and Cd concentrations in different substrates (not-contaminated substrate, CP mine tailings, CP mine tailings amended with compost, CP mine tailing with their own pine and substrates) and to assess metals bioavailability and accumulation in plant tissues. The phytostabilization potential was evaluated through the assessment of plant survival, elongation and biomass production. The results indicated the adaptability of *P. halepensis* to tolerate high Zn, Pb and Cd concentrations, to grow and survive in highly contaminated substrates and to limit their accumulation and translocation to the aerial parts. It showed that compost addition did not improve the plant survival and growth, however, it enhanced the soil organic carbon and nitrogen contents and restricted the metal bioavailability and metal accumulation in the aerial parts of *P. halepensis*.

All these *In-situ* and e*x-situ* findings confirm that *P. halepensis* is an appropriate candidate for long-term phytostabilization and revegetation processes with great potential to limit the toxicity of Zn, Pb and Cd in plant tissues by applying compost amendment in metal-contaminated mine sites. However, it was not clear if the geochemical fractions of metals and immobilization processes were influenced directly by the pyrite dissolution and weathering process or were induced by the rhizospheres and root activities. Further investigations may provide a better insight of *Pinus halepensis* phytostabilization and its physiological adaptation to better trace the mineralization process and profit from its phytostabilization capabilities in similar Mediterranean mine tailing sites.

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