INTERACTIONS OF SOIL MICROORGANISMS AND PLANTS WITH STABILIZING AMENDMENTS IN CONTAMINATED SOILS

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Thesis

This thesis is submitted in fulfillment of the requirements for the PhD degree at the Czech University of Life Sciences Prague, Faculty of Environmental Sciences.

Interactions of soil microorganisms and plants with stabilizing amendments in contaminated soils.

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Dedication

Dedicated to the memory of Guillermina Mendez and Lilia Salgado,
my life teachers.

Abstrakt

Hlavním cílem této práce bylo více porozumět procesům hrajícím roli při fytoremediaci, interakcím mezi půdou, mikroorganismy, rostlinami a činidly schopnými stabilizovat kovy a polokovy v kontaminovaných půdách. Výzkum byl zaměřen především na půdu kontaminovanou Pb, Zn, Cd a As a těžební a hutní oblast Příbram, Česká republika. První část projektu byla zaměřena na edafickou a biologickou charakterizaci lokality. Jako vzorkovací jednotka byla využita rhizosféra dominantních rostlinných druhů, včetně koncentrace a speciace kontaminantů v přilehlé půdě, arbuskulární mykorhizy (AM) asociované s kořeny a příjmu kontaminantů rostlinou, které byly vztaženy ke fluktuacím čtyř ročních období roku 2017. Poté byly některé cílové druhy testovány v kontrolovaných podmínkách za využití kompostu, biocharu, elementárního nanoželeza (nZVI), či kombinací těchto činidel. Přídavek kompostu snížil v rámci nádobového experimentu s *L. perenne* a *E. sativa* koncentrace kovů/polokovů v půdním roztoku a ve frakci extrahovatelné 0.01 M $CaCl_2$. Přídavek biocharu ke kompostu zlepšil růst rostlin a imobilizaci Zn, přičemž i urychlil kompostovací proces. Aplikace nZVI neprokázala žádný negativní účinek na vývoj *Agrostis capillaris* a *Festuca rubra* jak v hydroponických podmínkách, tak při testu klíčivosti, přičemž naopak zlepšila růst semenáčů a produkci biomasy. Pokud bylo však nZVI aplikováno v rámci nádobového experimentu s rostlinami inokulovanými AM, snížilo sice příjem kovů/polokovů rostlinou, zároveň však ale inhibovalo kolonizaci AM. Inokulace AM bez přítomnosti nZVI zlepšila růst rostlin a také snížila příjem kovů/polokovů. Z testovaných druhů se *A. capillaris* prokázal jako vhodný kandidát pro fytostabilizaci. Tento druh byl hlavním převažujícím druhem na lokalitě, prokázal vysokou odolnost ke kontaminaci a dobře se adaptoval na přítomnost nZVI vytvořením delších kořenů a větší biomasy. *A. capillaris* se však na lokalitě nachází obvykle v asociaci s jinými druhy trav, z nichž nejobvyklejší je *F. rubra*. Ta rovněž prokázala vysokou odolnost ke kontaminaci, žádnou negativní odezvu vůči aplikovaným činidlům a dobrou úroveň kolonizace AM. Naše výsledky ukazují, že všechna tři testovaná činidla mohou být s úspěchem aplikována za účelem stabilizace kontaminantů na cílové lokalitě a asociace *A. capillaris*/*F. rubra* je vhodná pro fytostabilizační účely. Další výzkum je však nezbytný pro porozumění dalším ekologickým a chemickým jevům provázejícím tuto asociaci, a to především v polních podmínkách a v delší časové škále.

Abstract

The main objective of this thesis was to understand part of the interactions involved in the process of phytoremediation between soil, microorganisms and plants with amendments that can potentially stabilize contaminants in soils with excess of metals and metalloids. The research was focused on a soil contaminated mainly by Pb, Zn, Cd and As, at the mining and smelting area of Přibram, Czech Republic. The first part of the project was focused on obtaining the edaphic and biological characterization of the site, using the rhizosphere of the dominant species as the sampling unit, including concentrations of contaminants in the soil and their speciation, arbuscular mycorrhiza fungi (AMF) associated to the roots, and plant uptake of contaminants, correlated to the fluctuation of the four seasons of one year (2017). Then, some of the targeted species were tested under controlled conditions using either compost, biochar, nano-scaled zerovalent iron (nZVI) or a combination of them. Addition of compost in a pot experiment with *L. perenne* and *E. sativa* reduced the concentrations of metal(loid)s in the 0.01 M $CaCl_2$ extractable and pore water fractions; the addition of biochar to the composting process improved plant growth and Zn immobilization, and hastened the composting process. Application of nZVI had no negative influence on development of *Agrostis capillaris* and *Festuca rubra* under hydroponic conditions and in a germination test, on the contrary, it improved seedling growth and biomass production. However, when nZVI was applied to a pot experiment using AMF - inoculated plants, it resulted in a decrease of plant metal(loid) uptake, but at the same time inhibited AMF colonization Inoculation by AMF without nZVI improved plant growth and also decreased metal(loid) uptake. Among the species tested, *A. capillaris* proved to be a suitable candidate for a phytostabilization process, being the main dominant species in the field, showing high resistance to contamination and adapting well to the presence of nZVI by developing longer roots and more biomass. However, *A. capillaris* is found in the field usually in association with other species, being *F. rubra* the most common one. *F. rubra* also showed high resistance to contamination, no negative response to the amendments, and good AMF colonization. Our results show that all the three amendments tested can be successfully applied as stabilizing agents in our target soil, and the association of *A. capillaris* and *F. rubra* is suitable for phytostabilization purposes. However, more research is necessary to understand other ecological or chemical elements of this association, particularly under field conditions and in the long term.

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CHAPTER

ONE

INTRODUCTION

The last decades have been characterized worldwide by notable growth in the population and development of industrial and urban activities. The production of wastes and residues as a consequence of this human activity affects negatively the biosphere, especially when it changes the chemical composition of soil, water and air (FAO 2015). This process is what we know as contamination, and it alters the normal functioning of ecosystems, living beings and human health. In the last years, the importance of this impacts has gained the general attention around the globe, and many actions are being taken in local, national and international contexts. The search for solutions requires a lot of specific information, especially in the social, environmental and technological areas (Austruy et al. 2013; Galan-Huertos and Romero-Baena 2008; Gucwa-Przepiora et al. 2007; Kucharski et al. 2005).

The impacts of contamination are wide and complex, and very site-depending, presenting different challenges according to the soil origin, weather, ecosystem composition and source of contamination (Galan-Huertos and Romero-Baena 2008). The research in the last decades has been oriented to find solutions that can properly reduce the risks instead of fixing or removing it. Research shows that the stabilisation of contaminants in the soil is the most realistic method, in therms of ecology, economy and pragmatism (**Table 2.1**). Thus, the use of stabilizing amendments with specific and well-known chemical characteristics, which can deal with toxic elements, preserve and/or improve plant growth and microorganisms, seems to be a promising way to deal with soil contamination (Mench et al. 2005).

The use of chemical amendments requires to consider the soil as a living system, and its complexity must be taken into account to propose reliable solutions. Therefore, the effect of the amendments over living organisms, specially plants, should be carefully studied. Research in the last years have shown that the use of well adapted plant - arbuscular mycorrhiza fungi (AMF) system is necessary for both, to reduce the risk caused by contamination, and to strengthen the adequate functioning of the ecosystem (Azcon et al. 2009; Krpata et al. 2008; Medina and Azcon 2010; Sudova et al. 2008; Wang et al. 2016b; Wu et al. 2014). Therefore, the system plant - AMF should be used and studied for its potential in the restoration of the ecosystem.

In order to contribute to the development of science and technology to face the challenge introduced here, the present thesis investigates different techniques and strategies oriented to the search for solutions, aiming particularly to the development of knowledge for a successful assisted phytostabilization of contaminated soils.

Particularly we started with an exploratory research of the native grassland established in a contaminated site in the Czech Republic (**Figure 3.2**) to select the best candidates for a phytostabilization process. Then we used the selected species in combination with different amendments to learn about their interactions with our selected grasses, and the relationships with arbuscular mycorrhiza fungi in the target soil. We expect that the knowledge gained here can contribute to the ecological restoration of the particular area, and in general, with the development of techniques and strategies for the reclamation of contaminated soils.

CHAPTER

TWO

STATE OF THE ART

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2.1 Inorganic contamination and its effect on ecosystems

Contamination includes the presence of materials that change the chemical composition of the soil, rising the concentration of some elements to levels that can impact negatively on living beings and change the natural functions of the ecosystem. The excess of inorganic materials in soils occur as a process related to geochemical anomalies connected to geological factors, such as ore deposits or rock materials. However, the main problem comes from anthropogenic sources, like urban waste disposal, industrial activities and agriculture, among others (Galan-Huertos and Romero-Baena 2008).

Mining and smelting activities are creating strong impacts and difficult challenges for the soils, due to the release of high amounts of inorganic contaminant such as Cd, Cr, Cu, Pb, Ni and Zn (Mudhoo et al. 2012), which are not easily degraded and highly toxic for living organisms. When these elements accumulate in high concentrations in soil and groundwater, are difficult to extract or treat, and can cause important problems to the environment (Science Communication Unit 2013). Numerous European base-metal mining districts have hundreds of years of mining and ore processing, during which contamination has been spatially and temporary superimposed over a particular area. Therefore, it is difficult to recognize the dispersion of the contaminants regarding the individual source (Zak et al. 2009). Mine tailings are another component of mine waste after metal extraction, and the major source of pollution from this industry. In 2000, metal contamination from mine tailings worldwide ranged from 10,000 to 600,000 metric tons of various metals (Mendez and Maier 2008). Nowadays the politics for environmental protection regulate the disposal of wastes for mine industry, however during historical periods, little attention was devoted to prevention of metal disposal. Particularly, in east European countries, wasteful practices were even escalated during the Communist period, and most of the problems inherited are caused by mining wastes, agriculture, outflows of unprocessed mine waters, accumulation of fine-grained waste from ore dressing, deposits of smelting slag and dust dispersion from ore mining, transport and crushing (Zak et al. 2009).

To date there is still not a clear understanding of the bioavailability or bioaccesibility of trace elements to plants (Kumpiene et al. 2017). What we know is that different fractions of metal(oid)s present in soil have different interactions with organisms. The soluble metals in the soil solution are directly available for plants, while other fractions are less available. Factors influencing the concentration and specialization of metals in the soil solution, such as pH, clay and hydrous oxide content, organic matter and redox conditions, will influence the bioavailability. The knowledge about the geochemical behaviour of potentially toxic elements in the soils is crucial to understand their interactions with the ecosystem (Rinklebe and Shaheen 2015). In particular, Mn and Zn compete for ion H^+, thus if pH decreases, Mn and Zn are more soluble and more available. Copper has been predominantly found to bound to organic matter fraction, also has been proved that Cu bound to organic matter increases as the pH decreases. Zinc is also affected by organic matter, but it does not compete for the same ion as Cu. Zinc has been found mainly as a free ion in soil solution, but its bioavailability decreases when organic matter increases. Clay and hydrous oxides (Al, Fe, Mn oxides) bind metals by specific adsorption, exchange, precipitation and coprecipitation, thus reducing their bioavailability (Reichman 2002).

The contamination by metal(oid)s affects the formation and development of the soil by triggering different chemical processes, specially when they impact directly to biologic organisms, affecting the growth and development of plants, animals and micro-organisms living in the rhizosphere (Wong 2003). Such processes depend on the chemical and physical form of the contaminant in the soil, as well as on the ability of the organisms to absorb it or exclude it. All plants need a variety of metal ions for basic growth and metabolic activity, however if the element is accumulated at high concentrations in an organism, it negatively alters certain biological functions (Galan-Huertos and Romero-Baena 2008), for instance As, Cr, Cd and Pb cannot be degraded, unlike organic compounds, and cleanup requires their immobilization or removal, and toxicity reduction (Mudhoo et al. 2012).

2.2 Role of plants

Plants are able to influence the solubility and speciation of metals in the rhizosphere by exuding chelators and manipulating rhizosphere pH. Most of our understanding of metal uptake comes from the study of Iron. Dicots and non-graminaceous monocotyledonous seem unable to directly absorb Fe(III) chelates or complexes, therefore reductants are excreted (organic acids) that can complex with Fe, and free Fe^{2+} is taken up by the plasma membrane. Graminaceous monocotyledonous excrete phytosiderophores that chelate with Fe and other metals, providing a ready supply for transport. Phytometallophores complex other metals as well as Fe, and so it has been assumed that the process for other metals is similar (Reichman 2002).

Accumulation of metals in the topsoil can be phytotoxic, thus reducing or inhibiting the establishment of vegetation. Metallic ions may also decrease available contents of soils mineral nutrients by inhibiting the mineralization processes and litter decomposition rate in ecosystems under pollution stress (Zaefarian et al. 2011). Perhaps the major environmental impact is found in temperate environments, where metal leaching and the formation of acid mine drainage can impact local streams and waterways by lowering pH and increasing toxic metal concentration (Mendez and Maier 2008).

Plants response to contamination

When the toxic elements in plants operate as stress factors, they can cause physiological reaction changes, reducing vigor or inhibiting plant growth. Some of the most described effects of toxicity by metals are the stunned growth, leaf epinasty and chlorosis (Reichman 2002; J. E. Yang et al. 2008). However, the effects vary according to the element and plant specie. For example, Zn is an essential element to plants: it is a constituent or a co-factor for several enzymes and plays an important role in regulating the nitrogen metabolism, cell multiplication, photosynthesis and auxin synthesis. It is assimilated early by plants, reason why its main problem at high concentrations is growth inhibition. Other general symptoms of Zn toxicity are stunning of shoot, curling and rolling of

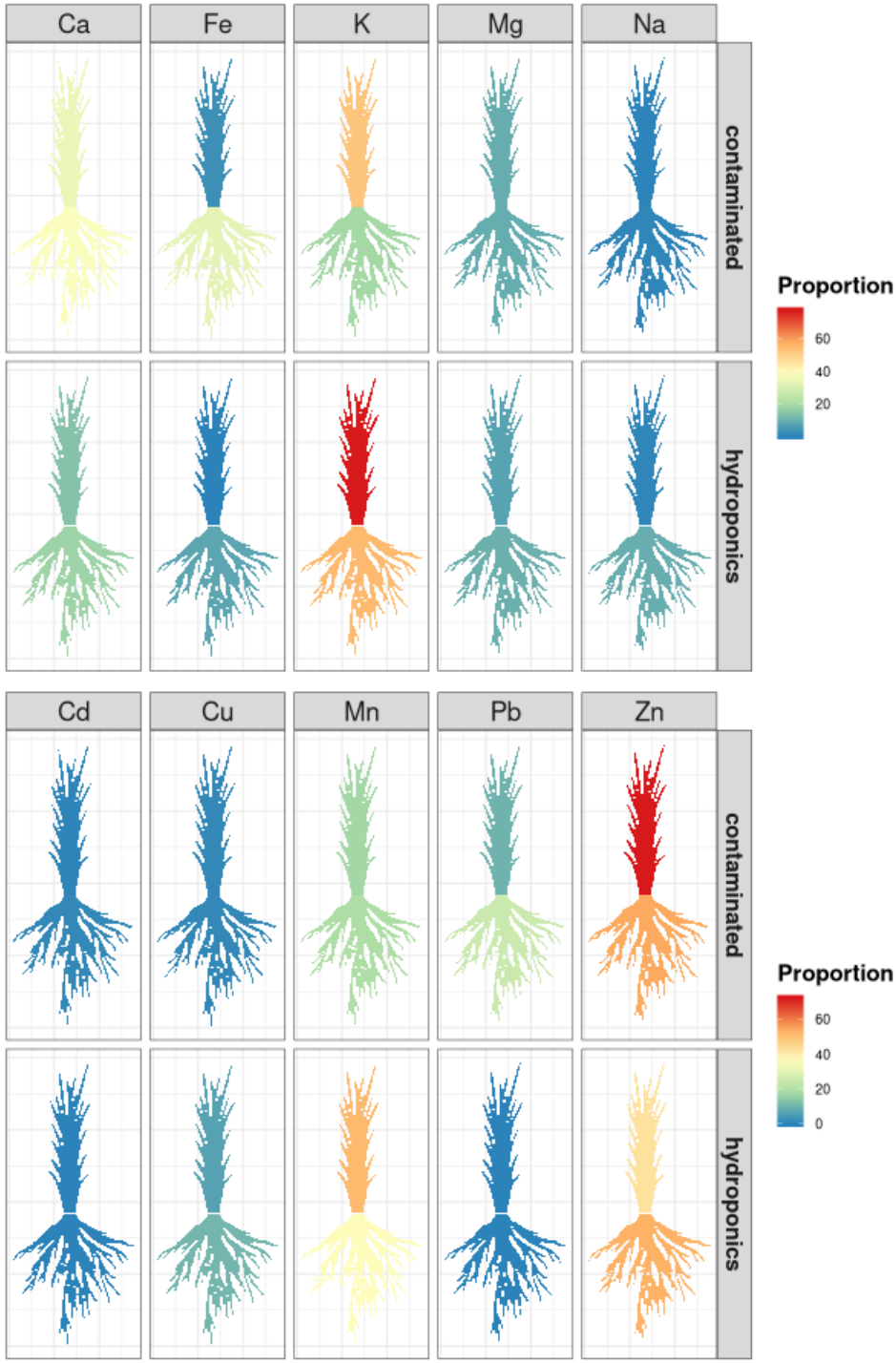

Figure 2.1: Proportion of main nutrients and contaminants (relative to the elements on each row, per plant) of *A. capillaris* plants growing under contaminated and ideal (hydroponics with hoagland solution) conditions.

young leaves, death of leaf tips and chlorosis (Premananda 2003). Arsenic, one of the most toxic elements, can be taken up to the plant system due to its function as phosphate analogue, specially As(V), when is transported across the plasma membrane via phosphate transport systems. Once in the cytoplasm, As reacts with sulfhydryl groups (-SH) of enzymes and tissue proteins, inhibiting cellular function and causing death (Garg and Singla 2011). Lead is considered a general protoplasmic poison, which is cumulative, slow acting and subtle. It is easily taken up by plants, and accumulated in different organs, specially roots. In small amounts, it produces a wide range of adverse effects on key physiological processes, while at high concentrations usually causes cell death. Among the main problems caused by Pb toxicity are inhibition of enzyme activities, disturbed mineral nutrition, water imbalance, change in hormonal status and alteration in membrane permeability (Sharma and R. S. Dubey 2005).

Metal uptake by plants is regulated by the electrochemical potential gradient for each metal ion that exist across the plasma membrane of root cells. On the other hand, metal ions in the cytoplasm are maintained at low activity to prevent harmful redox reactions. However, such reactions can result from the presence of free ionic forms of these reactive metals. Therefore, there is a little need for the plant to use thermodynamically active processes for the uptake of metal ions. The exact mechanism which controls influx across the plasma membrane is not yet known (Reichman 2002). Availability of bivalent cations for plant roots increases at acidic conditions: when the rhizosphere is exposed to such conditions, plant growth is reduced. In order to counteract this effect, plants can increase the pH of the rhizosphere, but this effect is dependent on certain conditions such as organic matter content, forms of nitrogen present and the availability of phosphorus and iron (Ovecka and Takac 2014).

Once inside the plant, each metal will behave differently depending on its function and conditions. An abstraction of this can be observed in **Figure 2.1**, which represents the balance of different elements in biomass of *A. capillaris* growing under ideal (hydroponics) and contaminated conditions. Arsenic is usually taken by the roots as As(V), possibly via the phosphate transport mechanism, and a small fraction is exported to the shoot, however As(V) will not normally have high enough cytoplasmic concentrations to exert toxicity because As(V) is rapidly reduced to As(III) in plant tissue. Thus the majority of As

remains in roots, immobilizing it below ground (Garg and Singla 2011). Lead retention in roots is based on binding of Pb to ion exchangeable sites on the cell wall and extra-cellular precipitation, mainly in the form of Pb carbonate deposited in the cell wall. Lead binds strongly to the carboxyl groups of the carbohydrates galacturonic and glucuronic acid in the cell wall, which restricts its transportation via apoplast. Lead moves predominantly into the root apoplast and thereby in a radial manner across the cortex, and accumulates near the endodermis, which acts as a barrier to the movement of Pb to the shoot. It appears that casparian strips of the endodermis are the major limiting factor restricting Pb transport across endodermis into the central cylinder tissue (Sharma and R. S. Dubey 2005). Copper in xylem sap is bond to amino acids. Even under toxic conditions, plants are able to regulate complexation of Cu within the sap. Zinc is predominantly complexed to citric and malic acid. At excess, small amounts of soluble Zn-phosphate were found in the sap. Under conditions of elevated metal supply, the majority of metals are restricted to roots. Redox potential, pH, ionic strength and organic constituents will determine loading, transport and unloading of metals. However, phloem cells are alive, therefore its sap is more responsive to changes in the internal plant environment: pH value is above 8, is more reducing and has a higher solute concentration and ionic strength. It is unknown whether plants are able to maintain stable pH and redox state, and the information on the speciation of Cu, Mn and Zn within the phloem is scarce (Reichman 2002).

The metals are transported via xylem and phloem. Therefore the cell wall represents an important barrier against the symplastic entry of metal ions (Ovecka and Takac 2014). Metal ions movement is essentially driven by mass upward flow of water created by the transpiration stream. Composition, pH and redox potential of the xylem sap would effect the types and amount and therefore, movement of metal species in the xylem sap. Plants appear to be able to maintain xylem pH and redox potential at fairly constant levels of deficiency and sufficiency; there is not enough research to understand the effects of excess, but the little that exists suggests that plants are able to maintain pH, redox potential and ionic strength (Reichman 2002). Apoplastic metal ion transport can be reduced by the adaptive development and accelerated maturation of the exodermis, endodermis and other extracellular barriers (Ovecka

and Takac 2014).

Plant-microorganism system

Soil is a complex system that provides nutrients and water for a variety of organisms, the diversity and abundance of living beings in soil is greater than in any other ecosystem (Azcon et al. 2009; Baba et al. 2016). The interactions of these organisms among themselves and its environment is what makes the system function, making possible the decomposition of organic matter, fixation of nitrogen and transformation of nutrients, changing soil aggregation and porosity, thus allowing water and oxygen fluxes. Soil organisms decompose different kinds of organic compounds, preventing them from entering the water and become pollutants (Castillo et al. 2011). However, when one of this components, processes or functions change in a bigger scale, it generates a negative impact known as degradation.

Contamination in soil affects whole ecosystem that gives life to the rhizosphere. In this context, microorganisms are of special importance due to their function in supporting the development and growth of plants (**Figure 2.3**). Some fungus develop symbiotic associations with the plant roots, supplying them with carbohydrates, and in turn, the extensive mycelial network enables the plant to acquire nutrients and water from a volume of soil otherwise inaccessible to plant roots (Zaefarian et al. 2011). The roots of most of vascular plant species are colonized by some kind of mycorrhizal fungi, specially Arbuscular Mycorrhizal Fungi (AMF) which colonizes most of the herbaceous species (Doubkova and Sudova 2016). Many rhizosphere colonizing bacteria typically produce metabolites, such as siderophores, biosurfactants or organic acids that stimulate plant growth (Azcon et al. 2009). Contamination by metals and metalloids alters the activity and development of bacteria and fungi in the soil, changing the structure and function of the local communities, reducing the content of organic matter and important nutrients such as C, N and P (Bouasria et al. 2012; Donkova et al. 2009; Lacatasu et al. 2001).

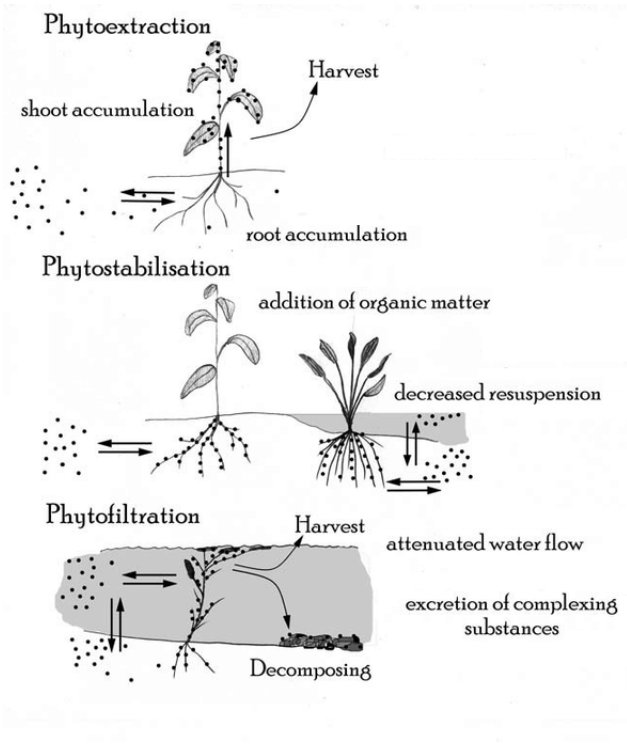

Figure 2.2: Main plant mechanisms to cope with metal(oid) contaminants. Adapated from Fritioff and Greger 2003.

2.3 Phytotechonologies for soil restoration

In contrast with organic contaminants, which can undergo biodegradation and mineralization, metals and metalloids remain in the environment and their speciation and bioavailability change over time. In such cases one of the strategies is to completely remove the contaminant from the soil, or to reliably immobilize it (Kamnev and Lelie 2000). The removal of contaminants can be achieved by excavation of the substrate and detoxification and/or destruction of contaminants, however such techniques are expensive and run the risk to further damaging the area by the destruction of the ecosystem or simply the addition of more contaminants needed for the stabilization, solidification, immobilization, incineration or destruction of the first ones (Gerhardt et al. 2017; Oyuela et al. 2017).

The roots of the plants are of vital importance to soil health, not only be-

cause they determine the structure and flows of water and nutrients, but also represent the core of living organisms in the topsoil. The lack of a functional plant community and vegetative cover facilitates the movement of contaminants by erosion and percolation, alters the soil structure and water balance, and eventually can reach the underlying ground water (Vangronsveld et al. 1995), resulting into more complex and wide problems. Therefore, the use of plants is a prominent solution to deal with the contaminants by means of extraction (phytoextraction), immobilization (phytostabilization), destruction and breakdown (phytodegradation) or filtration from the flowing water (rhizo- or phytofiltration) (Fritioff and Greger 2003; Oyuela et al. 2017). Plant growth also affects soil biology: microbial communities in rhizospheres differ greatly from unvegetated soils, and it affects directly the mobility, bioavailability and degradation of soil contaminants (Gerhardt et al. 2017).

Phytoremediation refers to the techniques used to detoxify by means of biological processes, and it has been reported also as phytocleaning or phytocorrection (Oyuela et al. 2017). Robinson et al. (2015) reported over 6500 articles by June 2014 on Web of Science that deal with some aspect of phytoremediation. Some of the most prominent and used techniques for soil restoration are phytoextraction and phytostabilization (Robinson et al. 2015; Yadav et al. 2018). The second is discussed in the following sections and it is the aim of the present work.

Phytoextraction is the use of plants that are able to uptake significant amounts of contaminants (in this case metal(oid)s) to the above-ground parts, with the aim to be removed from the site. The biomass should be removed from the site and burned to reduce its volume afterwards. This technology presents several challenges (Oyuela et al. 2017; Robinson et al. 2015), including:

Time of removal: it requires species with large biomass and high bioaccumulation coefficient to be able to remove contaminants from the soil in a fair amount of time (I.e 25 – 50 years).

Proportions of contaminants in the soil: the amounts of contaminant uptake by the plant decreases as metal concentration in soil decreases.

Weather conditions: the mobility and bioavailability of metal(oid) contam-

inants is highly correlated with the mobility and disponibility of water in the soil.

Soil characteristics: the water, and thus, nutrients and contaminants movement within the soil depend not only on weather conditions, but also chemical and physical characteristics of the soil.

Ecological drawbacks: even when the biomass is fully removed and treated after harvesting, there is an increase in the mobility of the contaminants, specially when additives are applied to facilitate its bioaccesibility, i.e. by wild animals feedstock, microorganisms or to the groundwater.

Species tolerant to metals and metalloids

Spontaneously revegetated sites usually exhibit a high natural value, result of the significant biodiversity: natural selection ensures the establishment of better adapted species (Baba et al. 2016). The identification and selection of resistant species, together with a good understanding of the ecological groups (specially populations and communities) are important factors for the reclamation of the ecosystem (Mench et al. 2010; Wong 2003). Moreover, directed succession of plant communities is inexpensive, and spontaneously revegetated sites reflect a high natural value due to significant biodiversity (Baba et al. 2016).

Different species have developed different strategies to tolerate the excess trace elements in soils. The behavior of selected species in relation to the contaminants might allow us to determine resistant species (Austruy et al. 2013). Plants able to grow on modified grounds reflect special ability of adaptation, developing higher capacity for survival under stress conditions, making the special target for remediation purposes (Machado-Estrada et al. 2013). The responses of plants in relation to the contaminants are complex and vary according to several conditions: as an example, different studies show that low concentrations of As stimulate the growth of plants, however As is generally considered phytotoxic and is expected to affect the plants negatively (Austruy et al. 2013). Thus, particular plant species targeted as potentially resistant should be studied under field conditions to identify the best candidate(s) for

restoration purposes. Physical environmental elements of the ecosystem have strong influence on the plant – contaminant relationship. For instance, the mobile/immobile distributions of metal fractions, controlled by various pedogenic and biogenic processes, are influenced by seasonal changes as well as metal – rhizosphere interactions, thus phytoremediation relies heavily on rhizospheric processes influenced by soil pH and biochemical transformations (Padmavathiamma and L. Y. Li 2012).

Particular plant species must be studied under particular conditions to achieve the best suitable system for the restoration of a given contaminated site. **Table 2.1** presents a list of several species that have been used for phytostabilization purposes in the last years, among the most used ones are *A. capillaris* (Nandillon et al. 2019b), *F. rubra* (Radziemska et al. 2020, 2019, 2018), *L. perenne* (España et al. 2019; Gandarillas et al. 2019; Radziemska et al. 2017b,c; Waterlot and Hechelski 2019), *B. juncea* (Forjan et al. 2018; González et al. 2017) and different species of *Salix* (Lebrun et al. 2018a, 2019; Touceda-González et al. 2017).

Agrostis capillaris L. is a low-growing, rhizomatous, perennial grass that forms dense swards of fine leaves. Occurs mainly in grasslands, but can become adapted to a wide range of other habitats: it can be particularly dominant on poor and acidic soils, invades disturbed areas, heathland, woodland, scrub and sand dune habitats, as well as urban areas where it frequently colonizes roadsides. *A. capillaris* is widely reported in the literature to be suitable for phytoremediation purposes: presents tolerance to contaminated soils, allowing a physiological adaptation to contamination of metallicolous ecotypes (Austruy et al. 2013) and As tolerance (Meharg and Macnair 1991). It has been successfully used as phytostabilizing agent, in support of biochar amendment in Pb-Zn contaminated soils (Houben and Sonnet 2015), and it receives important support from microorganisms, specially AMF (Malcová et al. 2003; Sudova et al. 2008). However, it has been proved that *A. capillaris* develops genetic adaptations to contamination by metals and metalloids, while specimens from uncontaminated sites are not able to tolerate high concentrations of metal(oid)s (Austruy et al. 2013; Doubkova and Sudova 2016).

Festuca rubra L. is a perennial grass widespread along the northern hemi-

sphere from temperate regions to arctic wastelands. It occurs on dry to wet sites in open habitats from sea level to high elevations, it grows on sand dunes, dry beach, coastal headlands, freshwater shores, bogs and marshes. The species present an extensive root system with high binding capacity, quick growing and tolerance to drought, high temperatures and high salinity (Gajic et al. 2016; Mitrovic et al. 2008). It is known to possess multi-metal tolerance, thus it has been used widely for restoration on sites under stress conditions, specially on contaminated sites as phytoextractor or phytostabilizing agent. It has also been report to easily adapt and develop genotypes for different conditions like climate, hydrology and geology of specific regions, providing valuable alternatives for restoration projects (Gajic et al. 2016; Malagoli et al. 2014; Mitrovic et al. 2008; Padmavathiamma and L. Y. Li 2012).

Support of microorganisms

The support by microorganisms play an important role in the effective application of plant-based technologies for the recovery of contaminated soils. As mentioned before, microbial activity enhances plant growth and supports its response to stress. But microorganisms also have an important role on the soil structure, specially aggregation: fungal activity excretes agents like polysaccharides, enmeshes soil particles physically through hyphal network and produces hydrophobic materials which decrease aggregate wettability, whereas the bacterial role is more related with polysaccharides production and stabilizing clay and silt-sized particles (Rahman et al. 2017). On the other hand, studies have demonstrated that the diversity and function of rhizosphere microbial communities have a close relationship with plant community composition, moreover, microbial communities respond to changes in plant growth response to stress and disturbance (Bouasria et al. 2012).

Mycorrhizal associations are known for contributing to the survival of the host plant in stressful conditions, including contamination. Different mycorrhizal types are defined by the symbiosis forming the association, and the morphology of the root structures; the roots of the plants are colonized by numerous different fungi, among the most common ones being ectomycorrhiza, a common association for trees, arbuscular mycorrhiza more common in herba-

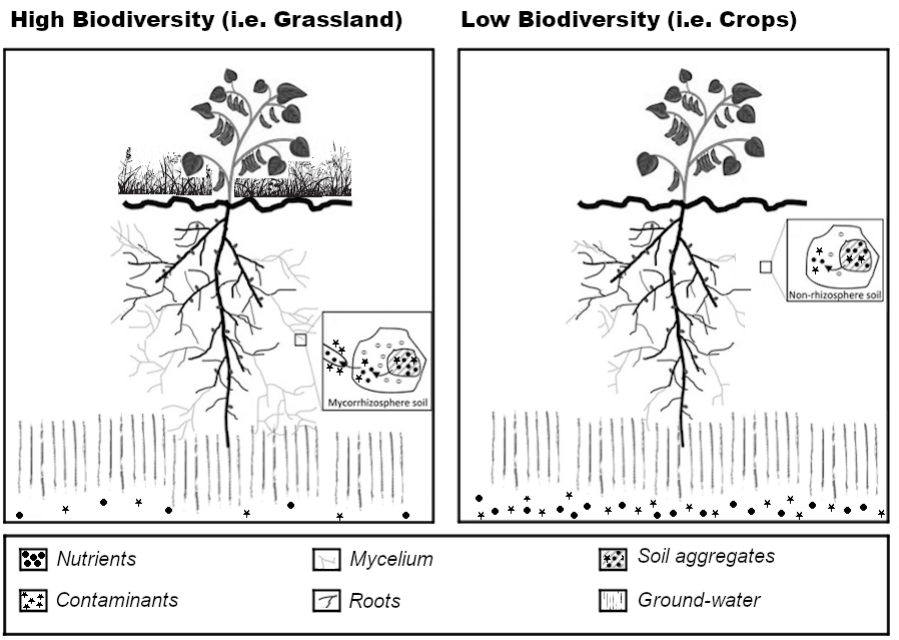

Figure 2.3: Interactions of the mycorrhizosphere involving arbuscular mycorrhizal fungi (AMF) and mobility of nutrients and contaminants in the rhizosphere under high (left) and low (right) biodiversity. Addapted from Azcon-Aguilar and Barea 2015.

ceous species, and dark septate endophytes, an ubiquitous root – associated fungi possibly symbiotic (Ruotsalainen et al. 2007).

Arbuscular mycorrhizal fungi (AMF) are soil fungi that can form symbiosis with more than 80% of terrestrial plants, helping them to obtain mineral nutrients, cope with stress factors such as drought and salt, and enhances plant resistance to contamination (Wu et al. 2018). In the last years it has been a main focus of research to study and promote the beneficial plant – mycorrhizal relations in sites with soils rich in toxic elements such as metal(oid)s, and thus it has been proved the contribution of AMF to the immobilization of such elements beyond plant rhizosphere by exudates, pasive adsorption and chelation (Baba et al. 2016). Metal detoxification is promoted by glomalin compounds and binding on fungal cell walls, and the chelation in the cytosol by various ligands such as pythochelatins and metallothioneins (Doubkova and Sudova 2016).

The interaction plant – AMF – contaminant is very complex and the results

of such interactions vary considerably according to the specific species (both, plant and AMF), specific contaminants, and other environmental conditions, however there is a general tendency to decrease colonization levels with the increase of contaminants in the soil (Baba et al. 2016; Gucwa-Przepiora et al. 2007; Malcová et al. 2003; Ruotsalainen et al. 2007). Some studies have found higher concentrations of the contaminants in the plant tissues due to AMF influence, whereas others have shown reduction in the contaminants concentrations in colonized plants (Malcová et al. 2003). Therefore, information on the specific mechanisms that AMF follows to uptake and tolerate metal(oid)s by particular host plants is important for a proper phytoremediation process (Doubkova and Sudova 2016). For example, adaptation of species growing in contaminated sites is an important factor to take into account: AMF from contaminated soils have been reported to cope better with toxicity than those not exposed to this long – term selection process (Sudova et al. 2008), however there are different interactions, while some of the resistant AMF strains help the plants to uptake more contaminants, others are better for keeping the contaminants at the mycelium levels (Kanwal et al. 2015; Malcová et al. 2003).

Phytostabilization

Phytostabilization is a process in which tolerant plants are used to reduce the mobility of pollutants, reducing the risk of further environmental degradation. These plants immobilize the contaminants at the rhizosphere by sorption onto the root surfaces, precipitation to less soluble forms, complexation with organic products, and metal accumulation in root tissues (Bini 2010; Mench et al. 2003; Mendez and Maier 2008). Metal immobilization aims at changing speciation of trace elements in the substrate, limiting its uptake by plants, and reducing the direct exposure through soil by reducing metal availability to heterotrophic organisms (Bert et al. 2012).

Phytostabilization requires the use of adapted plants with an extensive root system to create a stable vegetative cap that does not accumulate metals into above-ground tissues (Mendez and Maier 2008), thus reducing also the risk to mobilize contaminants to the food chain (Mench et al. 2005). It has been proved that clonally reproducing grasses fulfill this needs due to their rapid

growth and establishment, wide root system, below-ground accumulation of metals, high longevity and easy maintenance (Doubkova and Sudova 2016).

It is important to consider all the elements of the rhizosphere as one whole system in order to achieve a successful reclamation of contaminated sites. In the process of phytostabilization, AMF improve plant nutrition, increase pathogenic resistance and phyto-hormone production and increase water uptake from soils (Baba et al. 2016). It also cope with metal by metal detoxification trough glomalin-compounds and binding on fungal cell walls associated with metal restriction in root tissues, as well as reduced metal uptake across the membrane (Doubkova and Sudova 2016).

The use of metal-tolerant plant ecotypes together with resistant microorganisms still faces important challenges, specially on the chemical aspect due to the limitations caused by metal(oid)s to their physiological functions. In the last years, the use of metal-immobilizing additives is gaining attention as a successful solution to the problem, enhancing immobilization of the metals on the soil complex (Vangronsveld et al. 1995).

2.4 Stabilization of metals and metalloids in contaminated soils

Assisted phytostabilization

Soils contaminated by metal(oid)s are know for its low fertility and water holding capacity and unfavorable soil structure, which results in low fertility and high toxicity for living organisms, including plants (Ali et al. 2019). Therefore, before establishing a strong vegetation cover, it is important to consider treatments by additives that transform chemical characteristics of soils, reducing the mobility and bioavailability of metal(oid)s, thus improving the conditions for the development of the plants and microorganisms (Clemente et al. 2019; González-Chávez et al. 2019; Rinklebe and Shaheen 2015).

In other words, phytostabilization can be improved by amendments that

immobilize metal(oid)s, and at the same time improve the physical, chemical an biological properties of contaminated soils (Kumpiene et al. 2019; Mench et al. 2006; Sigua et al. 2019). In the last years, different kinds of additives have been used to confer chemical stability to the soil and improve its characteristics to enhance plant growth, being the most effective those that can reduce the mobility of contaminants, and at the same time increase soil fertility (Chirakkara and Reddy 2015; Grieger et al. 2010; Komárek et al. 2013; Martínez-Fernández and Komárek 2016; Mi et al. 2016; Michálková et al. 2017; Michálková et al. 2014; Rahman et al. 2017). **Table 2.1** shows a list of publications of the last three years, where plant species with phytostabilizing potential were investigated, growing in contaminated soils, and with the application of amendments. Several other publications were left outside of the already long list, such as exploratory research (i.e. evaluation of several wild-species), toxicological experiments (i.e. application of different concentration of the amendment to selected plants), use of microorganisms, among others. The amount of publications investigating this topic shows the complexity of the problem: specific contaminants in specific kinds of soils react differently according to the plant species growing and the amendments used (Ali et al. 2019). Therefore it is necessary to conduct research targeting a particular problem in order to find the most suitable solution(s).

Table 2.1 also shows several kinds of materials used as amendments, from chemically engineered ones, to biologically created with no or little pre-processing. Among the most popular ones are the use of organic amendments and nano-sized particles of metal oxides (Ali et al. 2019; Clemente et al. 2019; Kim et al. 2018; Lebrun et al. 2019; Martínez-Fernández et al. 2015b; Trakal et al. 2016).

Organic amendments

The addition of organic matter to the soil can improve soil structure, nutrients content and chemical characteristics (Chirakkara and Reddy 2015; Rahman et al. 2017). Different residues from agriculture have been used traditionally to add organic matter to the soil, such as green waste, sewage sludge, biochar, pulp, bagasse, manures, among others, getting as a result an increase in the pool of nutrients, pH neutralization, improvement of the soil hydraulics and

development of the microbiota (Clemente et al. 2019; Lebrun et al. 2019; Sigua et al. 2019).

Compost is the product of conversion of organic materials by microbial degradation to a biologically stable and humified organic material (Beesley et al. 2014; Lebrun et al. 2019). The use of composts enhance plant growth and increase microbial activity (Chirakkara and Reddy 2015) by providing nutrients and increasing humic content, thereby increasing water holding capacity, improving soil structure and microbiological activity (Medina and Azcon 2010). Furthermore, the humified organic matter resulted from composting has a high capacity to interact with ions, thus mobilizing or immobilizing them (Nandillon et al. 2019b).

The sources of organic materials are so diverse with a wide variety of properties, which can include potentially toxic elements. Some materials such as sewage sludge bring numerous benefits to the soil, but they are also one of the main sources of metal contamination (Kumpiene et al. 2019). A good selection of the source and knowledge of its properties is of high importance before its usage.

Biochar

Among other components, soil organic C is a key factor in soil quality assessment. The total contents of organic C have beneficial effects on crop productivity, while labile organic C (a small-proportion fraction of total organic C), which includes dissolved organic C, microbial biomass, particulate organic C and potassium permanganate-oxidizable $KMnO_4$-C can be used as early indicators of changes in the C contents in soils (Mi et al. 2016).

Biochar is a heterogeneous material obtained from pyrolysis of biomass that has been used for phytostabilization due to its capacity to immobilize trace elements and low cost (Ali et al. 2019; Kumpiene et al. 2019). The addition of biochar to contaminated soils is gaining interest because it has been proved that increases crop growth and improves soil structure by decreasing bulk density and reducing mechanical properties. Also has been demonstrated to increase

cation exchange capacity, and the ability of soils as habitat for microbes and fungi (Rahman et al. 2017). The application of biochar increases C stocks and improves C sequestration in the long term in soils, increasing soil fertility and plant growth (Houben and Sonnet 2015). Moreover, recent studies have shown that the use of biochar can remove metal(oid)s and reduce their bioavailability through three main sorption mechanisms: (i) ion exchange, (ii) metal complexation with free functional groups together with physical adsorption, and (iii) surface precipitation (Trakal et al. 2016).

Several authors have proven the use of biochar as stabilizing agent as a support for plants growing in contaminated soils. Lebrun et al. (2019) found an increase in water holding capacity, neutralization of pH and reduction of As, Fe and Pb in a multi-contaminated soil cultivated with *Salix viminalis*. Sigua et al. (2019) proved the support of biochar for the growth of *Zea mays* by neutralizing pH and reducing Pb and Zn mobility. Ali et al. (2019) found an increase of water holding capacity and reduction of the mobility of Zn, Cd, Pb and Cu in different contaminated soils sown with wheat. Rinklebe et al. (2016) found a reduction of several toxic elements in a multi-contaminated soil after application of biochar.

Fe-based materials

The use of Fe-based materials is a promising technology for removal of trace elements from the environment, due to their super paramagnetic properties, great biocompatibility and their reactivity and elevated sorption capacity (Martínez-Fernández et al. 2015b; Michálková et al. 2017). It has been demonstrated that materials such as precipitates of Fe oxides, Fe(II)/(III) sulphates, iron grit, scordite and goethite are good coping with toxic forms of As, Cr, Cd, Cu, Pb and Zn (Hartley et al. 2004; Kumpiene et al. 2008; Zuverza-Mena et al. 2017). Fe oxides are preferred over Fe salts because the former contains three times more iron by weight than the most common Fe salts. Even if oxidation reactions of Fe in soil are not as fast as those of salts, it might be beneficial in a long-term perspective (Kumpiene et al. 2008). Also, the addition of Fe oxides and their precursors increase the sorption capacity of the soil (Kumpiene et al. 2019).

Iron oxides can be found as erosion products in almost all kinds of soil types, in forms like goethite, hematite, maghemite and magnetite, therefore, synthetic materials are interesting candidates for the removal of contaminants in soil and water (Martínez-Fernández and Komárek 2016). Iron oxides are often applied in the form of their precursors or industrial waste, which has advantages such as cost and availability in large amounts, however attention to the composition of the materials is important, as it could be a source of additional contamination (Michálková 2016).

Some of the most promising Fe-based materials are those with good stabilising characteristics: goethite ($\alpha - FeOOH$) the most common stable Fe oxide in the soil; hematite ($\alpha - Fe_2O_3$) which often occurs in association with goethite, predominantly in warm regions; maghemite ($\gamma - Fe_2O_3$), a result of partial or complete oxidation of Fe(II) in magnetite structure; magnetite (Fe_3O_4), inherited from the parent rock, contains both Fe(II) and Fe(III) in contrast with other Fe oxides; and nano zero-valent Fe (nZVI) (Michálková 2016; Zuverza-Mena et al. 2017).

Engineered nano-materials

Chemical stabilisation can support plant enhancement through pH adjusting, adsorption of contaminants, surface precipitation, structural incorporation or ion exchange (Martínez-Fernández and Komárek 2016). In the last years, Nano-sized materials (NM) (materials with at least two dimensions between 1 and 100 nm) have been used as a new generation of remediation technologies that can provide cost-effective solutions to environmental pollution (Grieger et al. 2010; Komárek et al. 2013; Martínez-Fernández and Komárek 2016). These NM act as important scavengers for contaminants, mainly because of their high reactivity, large specific surface area, and a modified surface structure (Michálková et al. 2014; Zuverza-Mena et al. 2017). Nano particles of metal oxides have specific affinities for metal and metalloids adsorption, thus their application has been rapidly extended for environmental tasks (Martínez-Fernández and Komárek 2016). The physico-chemical properties of oxides allow sorption of various chemical species, producing specific (chemical) and nonspecific (physical) adsorption: specific adsorption include surface complexation processes-

reactions between metal ions and surface functional groups (Komárek et al. 2013).

In particular, nZVI have received special attention in the last years due to its larger range of application options and high degrees of reactivity (Grieger et al. 2010). To date, many studies have demonstrated the applicability of nZVI to degrade a wide range of organic and inorganic soil and water contaminants (Fazlzadeh et al. 2016; Grieger et al. 2010; Lefevre et al. 2016; Martínez-Fernández et al. 2015b; Michálková et al. 2017; Michálková et al. 2014). The possible mechanisms by which stabilises metal(oid)s include adsorption and/or surface precipitation, redox reduction and co precipitation in the form of metal iron oxides (Martínez-Fernández and Komárek 2016). In situ use of nZVI may reach areas at contaminated sites not easily accessed by other treatment methods, however, successful injection and distribution of nZVI are among the main technical challenges for its application. Another challenge is the concentration of application, which is very site-dependent due to variations between source zone architecture, contaminant kinds and dimensions, hydrogeological and climatic conditions, etc. (Grieger et al. 2010).

Several studies have demonstrated the advantages of nZVI as amendment for soils contaminated by toxic metals and metalloids such as Cr (Wang et al. 2014), As (S.-H. Lee et al. 2011; Praveen et al. 2017; Vítková et al. 2018), Pb, Zn (Martínez-Fernández and Komárek 2016; Michálková et al. 2014), among others.

Table 2.1: Publications investigating assisted phytostabilization in the last three years

Reference	**Species used**	**Assisted by**
Burges et al. 2020	Tobbaco and sunflower	compost and dolomitic limestone
Radziemska et al. 2020	*F. rubra*	diatomite, dolomite and halloysite
Ali et al. 2019	*T. aestivum*	Wood biochar
Clemente et al. 2019	*S. marianum and P. miliaceum*	pig slurry and paper mill sludge
España et al. 2019	*L. perenne*	pig sludge and sandy loam
Gandarillas et al. 2019	*L. perenne*	pig slurries
González-Chávez et al. 2019	*R. communis*	vermicompost sawdust
Kim et al. 2019	*B. campestris*	P-based fertilizer

Table 2.1 continued from previous page

Lebrun et al. 2019	*Salix viminalis*	biochar, compost and iron grit
Luo et al. 2019	*A. donax, B. papyrifera, C. fortunei, etc.*	Particulate organic matter
Martínez-Martínez et al. 2019	13 Mediterranean native plant species	Marble waste and raw pig slurry
Martínez-Oro et al. 2019	*P. halapensis*	organic amendment
Moradi et al. 2019	*C. sativus*	beeswax biochar
Nandillon et al. 2019b	*A. capillaris*	biochar, compost and iron sulfate
Nandillon et al. 2019a	poplar	garden soil, compost and biochar
Nowak et al. 2019	*M. giganteus, P. virgatum and S. pectinata*	fertilizers
Pogrzeba et al. 2019	*D. glomerata*	granular S
Radziemska et al. 2019	*F. rubra*	chalcedonite, limestone, activated C
Saavedra-Mella et al. 2019	*Acacia chisholmii and A. ligulata*	soluble phosphate
Sigua et al. 2019	*Zea mays*	biochar and manure
Tapia et al. 2019	*A. halimus*	commercial humic substances
Thongchai et al. 2019	*Tagetes erecta*	manure, leonardite and Osmocote (R)
Wasilkowski et al. 2019	*F. arundinacea*	Na-bentonite and green compost
Waterlot and Hechelski 2019	ryegrass	phosphorus fertilizers
J. Zhan et al. 2019	*Athyrium wardii*	biodegradable chelates
Abbaslou et al. 2018	*R. officinalis*	fibrous clay minerals
Castaldi et al. 2018	*A. donax*	iron-rich water and compost
Eissa 2018	*Atriplex nummularia*	Compost and biochar
L. Huang et al. 2018	*C. alata*	biochar
Kim et al. 2018	*L. multiflorum, S. cereale, etc.*	manure and drainage sludge
Lebrun et al. 2018a	*S. alba and S. viminalis*	biochar and garden soil
Lebrun et al. 2018b	*Salix spp.*	biochar
Leclercq-Dransart et al. 2018	Poplar species	fly-ashes
Z.-Y. Li et al. 2018	8 indigenous species	manure, compost, sludge, etc.
Midhat et al. 2018	*M. sativa*	powdered marble
Mu et al. 2018	*V. zizanioides*	alkaline silicon
Radziemska et al. 2018	Mix of grasses	halloysite, diatomite, dolomite
Radziemska 2018b	*F. rubra*	dolomite, halloysite, and chalcedonite
Radziemska 2018a	*F. rubra*	chalcedonite and dolomite

Table 2.1 continued from previous page

Santana et al. 2018	*C. ensiformis*	bovine manure vermicompost
Vítková et al. 2018	*H. annus and L. perenne*	nano zero-valent iron
Xue et al. 2018	willow and indigo	compost plus dolomitic limestone
You et al. 2018	mixed native woody and grass spp	woodchips
Álvarez-López et al. 2017	*S. caprea and N. tabacum*	compost
Ciarkowska et al. 2017	*D. carthusia-norum and B. laevigata*	NPK fertilizer
Elloumi et al. 2017	*N. oleander*	phosphogypsum
Forjan et al. 2018	B. juncea	biochar
Forján et al. 2017	B. juncea	compost and biochar
González et al. 2017	B. juncea	Marble sludge
Grobelak et al. 2017	P. silvestris, P. abies and Q. robur	sewage sludg
Kiran et al. 2017	B. chinensis	cow manure and its derived biochar
Labidi et al. 2017	R. pseudoacacia, A. glutinosa and S. alba	coal fly ashes
Lebrun et al. 2017b	Salix viminalis	Biochar
Lebrun et al. 2017a	S. alba, S. viminalis and S. purpurea	Biochar
Marco et al. 2017	S. multijuga	peat
Michálková et al. 2017	H. annus	Mn oxide and nano zero-valent Fe
Moreno-Barriga et al. 2017	P. miliaceum	marble waste and biochar
Parra et al. 2017	L. dentata, R. officinalis, T. vulgaris etc.	calcium carbonate and pig manure
Placek et al. 2017	P. sylvestris and M. giganteus	Organic and inorganic fertilizers
Radziemska et al. 2017c	L. perenne	food waste compost
Radziemska et al. 2017d	F. rubra	halloysite
Radziemska et al. 2017a	L. perenne	several fertilizers
Tapia et al. 2017	Carpobrotus aequilaterus	Potasium humates
Touceda-González et al. 2017	Salix spp, Populus spp. and A. capillaris	municipal solid wastes
Wasilkowski et al. 2017	F. arundinacea	pulp from coffee and Na-bentonite

2.5 Side effects of stabilizing amendments

The remediation process is complex and it should involve the reduction of the contaminants in the soil, as well as its ecotoxicity. Therefore, the stabilizing

amendment should not be toxic to any biological compartment because the ecosystem should be sustained on the contaminated site afterwards. The stabilization process should also restore soil enzyme activities, reduce plant and microbial toxicity and influence positively the soil ecosystem (Komárek et al. 2013).

The interactions between the amendment material and the contaminants varies with the nature and chemical composition of the amendment, and the particular contaminant(s) involved and its environment. This system becomes more complex when biological activity is involved into the system: roots of different plant species excrete different kinds of exudates, microbial activity influences the chemical composition of the soil, and several factors such as roots and mycorrhiza uptake and/or immobilize different elements in the soil in particular ways. However, very few research projects manage to analyze and understand every aspect of this complex system. Research focusing on the use of additives to amend soil contamination usually target the chemical relationship between the amendment and the particular contaminant. Research focused on phytoremediation targets a small selection of plant species and their interaction with the contaminant. And field experiments give insights of the behavior of the amendment - contaminant system under specific weather conditions and a particular ecological community. There is no recipe for a successful restoration process. However, the information generated through this experiments and research provides useful tools to take action into such processes. Likewise, information about the side effects of the amendments on living organisms is also important for the success of the restoration. One of the main problems that an amendment can represent for the ecosystem is toxicity, which can be caused by its chemical composition directly, excess of the application doses, or as a collateral effect by promoting plant to uptake of the contaminant element(s). Some studies have proved this point and new research is focusing on investigating such side effects. For example, (Forjan et al. 2018) found that pseudo-total concentrations of target contaminants, Cu, Pb, Ni and Zn increased in contaminated soils treated with compost and biochar.

The numerous uses of NM in several areas including industry, agriculture and medicine has given rise to an increase in the research showing the environmental impact of such particles, and particular environmental threads have

been found (Wang et al. 2016a). Nano particles may accumulate in edible parts of plants, have adverse effects on agronomic traits, yield and productivity of crops and transfer to trophic levels (Wang et al. 2016b). i.e. Xiang et al. (2015) tested four kinds of Zn NM and observed a reduction in the shoot and root elongation of cabbage seedlings, and Wang et al. (2016b) found that Zn NM accumulate in shoot and root of maize, and at concentrations of 800 mg per Kg of soil, the growth of both, plant and AMF reduced considerably. The study of toxicity caused by NM is very complicated due to the big number of factors that influence it, for example most of the NM have toxic effects in plants at relatively high concentrations, however the toxicity is species - dependent, and some plants have shown visible signs of recuperation, indicating that the toxicity was temporary (Martínez-Fernández and Komárek 2016). At the microbial level, engineered NM can impact in different ways: (1) direct toxic effects, (2), indirect effects as a result of interaction with natural compounds, (3) enhacing the toxicity of organic pollutants in soil and water and (4) by changing the bioavailability of toxins and nutrients (Hegde et al. 2016).

Nano zero-valent Fe is one of the nano particles more used in environmental remediation: it was first applied at field scale in 2000 and after that its use growth rapidly (Ma et al. 2013). Due to its high reactivity, nZVI accumulates easily in the environment and is vulnerable to oxidation, as a result of aging that takes place in contact with soil constituents, and the extent of the oxidation might determine its ecological influence (Wang et al. 2016a). Transport of NM is essential to determine its ecological impact: nZVI mobility in soil is influenced by interrelated environmental parameters such as ionic strength and composition, pH, O_2 concentration, presence of organic matter and hydraulic conductivity; in groundwater systems, aggregation and adhesion are the main factors. Soil and groundwater are the main entry points to living organisms and thus, a good understanding of its movement within these systems is important to prevent or forecast side effects (Lefevre et al. 2016).

Once inside living systems, possible toxic effects have to be taken into account. To date, there is still scarcity in the research focused on nZVI toxic effects, with most of the works focusing on microorganisms, and in a minor extent, arthropods and plants (Stefaniuk et al. 2016). Most of the studies dealing with nZVI toxic effects in plants have found no or minimum impact, with

exceptions at high doses, a general negative effect on cell viability and integrity when it comes to microorganisms (Lefevre et al. 2016). At concentrations above 200 $mg \cdot L^{-1}$, nZVI caused toxic effects in *Typha latifolia*, and reduced transpiration and growth of hybrid poplars (Ma et al. 2013). Studies have shown that nZVI can be absorbed on cell membranes of bacteria, or penetrate to them, which often leads to an alteration on its functioning. Also, as a result of oxidation, the production of reactive oxygen species may cause peroxidation of lipids and damage to DNA, as well as death of cells (Stefaniuk et al. 2016). However, most of the nZVI remaining particles and its derivations will undergo dilution, aggregation, sedimentation and further oxidation, reducing its toxic impact (Lefevre et al. 2016).

Although nanotechnology has the potential to solve problems that cannot be solved by other products or techniques, the safety regarding its use still represents a barrier due to its innovative application (Martínez-Fernández and Komárek 2016), therefore further studies are necessary to achieve its proper utilization.

CHAPTER

THREE

SEASONAL DEVELOPMENT OF ZN, PB, AS AND CD CONTENTS IN THE BIOMASS OF SELECTED GRASS SPECIES GROWING ON CONTAMINATED SOILS: IMPLICATIONS FOR IN SITU PHYTOSTABILIZATION

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3.1 Abstract

Phytostabilization aims to immobilize contaminants at the rhizosphere level using the root system of adapted plants. In order to exploit wild grasses with potential for phytostabilization, a screening throughout the year was conducted at a site contaminated by Pb and Zn. Three plant species were chosen: *Agrostis capillaris*, *Calamagrostis epigeios* and *Arrhenatherum elatius*. Rhizospheric soil and biomass was used for chemical characterization. Above- and below-ground was analyzed. For each sample, arbuscular mycorrhiza fungi colonization was determined. The highest concentrations of Pb were found in the *A. capillaris* rhizosphere (3,417 mg· kg^{-1}), and in *A. elatius* for Zn (3,876 mg· kg^{-1}). $CaCl_2$-extractable Zn in the rhizosphere of *C. epigeios* was the lowest and Pb was lower for *A. elatius*. $CaCl_2$-extractable Cd was neither species-dependent nor time-dependent. Arsenic was not species-dependent. The fractionation of target elements did not show differences between separate sampling campaigns and Pb was the only element that showed differences during the year. *A. capillaris* showed the best capacity to take up elements. The colonization by AMF did not show significant differences for different sampling times, or interactions between time and species, however differences were found for different species, i.e., *C. epigeios* showed significantly lower colonization by arbuscular mycorrhiza fungi. Our results indicate that *A. capillaris* appears to be a good indigenous candidate for phytostabilization.

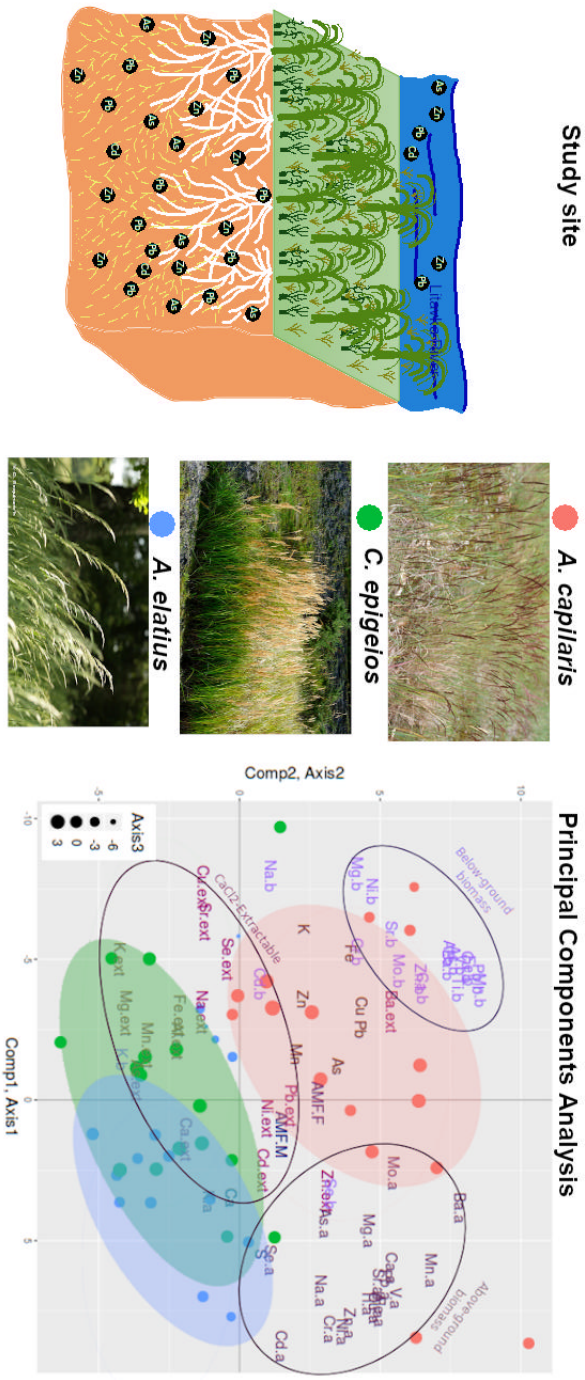

Figure 3.1: GRAPHICAL ABSTRACT

3.2 Introduction

Phytostabilization is a process in which plants tolerant to contamination are used to reduce the mobility of pollutants, thus reducing the risk of further environmental issues. Such plants immobilize contaminants in the rhizosphere by sorption onto the root surfaces, precipitation of less soluble forms, complexation with organic ligands, and metal accumulation in root tissues (Bini 2010; Mendez and Maier 2008). Metal immobilization aims to change the speciation/fractionation of trace elements in the substrate, limiting their uptake by plants and erosion, and to reduce the direct exposure of the soil by reducing metal availability to heterotrophic organisms (Bert et al. 2012). Phytostabilization requires the use of adapted plants with extensive root systems to create a stable vegetative cap that does not accumulate metals into aboveground tissues (Mench et al. 2010; Mendez and Maier 2008), thus reducing the risk of mobilizing contaminants into the food chain. Remediation can be considered successful when the risks associated with a site are removed or considerably reduced; in the case of soil contamination, this means reducing the spread of contaminants by erosion, percolation or bioaccumulation (Mench et al. 2003). It has been proven that clonally reproducing grasses fulfil these needs due to their rapid growth and establishment, wide root systems, belowground accumulation of metals, high longevity and easy maintenance (Doubkova and Sudova 2016; Kucharski et al. 2005).

Most of the research has been focus mainly to commercial, agricultural species, but there is still a need to identify phytostabilization potential in well adapted indigenous plants. The identification and selection of such species, along with a good understanding of their ecological role, are important factors for reclamation of an ecosystem (Kucharski et al. 2005; Wong 2003). Several authors have proved the efficiency of local *Poaceae* species for the phytostabilization process of sites contaminated by metal(oid)s. Kucharski et al. (2005) used indigenous *Deschampsia cespitosa* for a mesocosm experiment in multimetal contaminated soils (Cd, Pb and Zn) in southern Poland, proving the ability of this species to colonize the target soil and to accumulate most of the contaminants in roots, rather than in shoots. Rodriguez-Seijo et al. (2016) found similar results when evaluating the phytoremediation ability of *Agrostis*

capillaris in a Pb-polluted soil in northern Spain, supported by the presence of traces of pyromorphite ($Pb_5(PO_4)_3X$), a phosphate with an extremely low solubility ($10^{-84.4}$) and linked to its rhizosphere.

Support by microorganisms plays an important role in the effective application of plant-based technologies for the recovery of contaminated soils. Particularly, fungi excrete agents such as polysaccharides, enmesh soil particles physically through hyphal networks and produce hydrophobic materials, which decreases aggregate wettability (Rahman et al. 2017). Arbuscular mycorrhizal fungi (AMF) improve plant nutrition, increase pathogenic resistance and phytohormone production and increase water uptake from soils (Baba et al. 2016). Some authors have confirmed that AMF cope with metal(oid)s by detoxification through glomalin compounds and by binding to fungal cell walls associated with metal(oid) restriction in root tissues, as well as by reducing contaminant uptake across the membrane (Doubkova and Sudova 2016), whereas Neagoe et al. (2014) showed that the beneficial effect of inoculations with AMF on plants growing in contaminated substrates is due to an improvement in phosphorus nutrition and, frequently, alleviation of toxic element transfer to plants is due to smaller oxidative stress.

The present research has been proposed under the need to identify indigenous plants with potential for phytostabilization. The aim of the present study is to evaluate wild grasses that are tolerant to metal(loid)s as a function of the chemical characteristics of contaminated soils, to find suitable species for phytostabilization, and to consider the role of AMF symbiosis as a support for the plants. We hypothesize that there is a correlation between the chemical characteristics of soils and the plant communities growing on it, and at the same time, the presence of contaminants influences AMF colonization.

3.3 Methods

Study site

The study site is located in the alluvium of the Litavka River, 7 km north of Příbram, Czech Republic (49°43'10"N, 14°0'47"E, **Figure 3.2**). This site has been severely contaminated due to emissions from the metallurgical industry and flood events by contaminated water from spoil heaps whose barriers have been damaged several times. Smelting of Pb-As ores had been performed in a smelter located in the area from 1786 to 1972 (Ettler et al. 2005). The altitude of the study site is 500 m.s.l., the mean annual temperature is 6.5°C, and the annual precipitation is 700 mm. We selected three plant communities with three different dominant grasses: *Agrostis capillaris* L., *Calamagrostis epigeios* (L.) Roth, and *Arrhenatherum elatius* (L.) P.Beauv. Ex J.Presl & C.Presl. Characterization of the soils is given in **Table S1**.

Collection of samples

From each plant community, four soil monoliths of 0.3 m^3 along with their aboveground biomass were collected in March, May, July and November 2017. Soil samples were extracted from the monoliths and then the belowground biomass was washed with deionized water. The aboveground biomass was separated from the belowground biomass after its separation from the soil to ensure

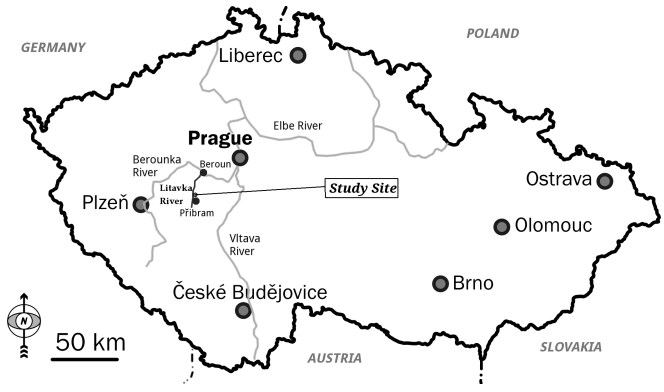

Figure 3.2: Localization of the study site in the Czech Republic

its correct determination. For the case of *C. epigeios*, we extracted the biomass of only this species, as it grew separately from other species. For the *Agrostis* community, the biomass of *A. capillaris* was mixed with that of *Festuca rubra*, in a proportion of approximately 3:2 w/w, while for the case of the *Arrhenatherum* community, the biomass of *A. elatius* was mixed with *Molinia caerulea* in a proportion of approximately 4:1 w/w; in both cases, precise separation of the two species involved was impossible; therefore, to make the samples directly comparable, the same proportions of above- and belowground biomasses were taken for each species mixture. All biomass samples were carefully washed, first by tap and then by distilled water, to ensure full removal of soil particles.

Soil analysis

The soil was air-dried, homogenized and sieved through a 1 mm stainless sieve. Determination of pH was performed using distilled water and KCl at 0.1 M 1:2.5 (w/v) (USDA 2014). For the readily exchangeable fraction of elements, samples of 2 g of soil were extracted with 20 mL of 0.01 M $CaCl_2$ (Lachner), shaken for 3 h at 30 rpm, centrifuged for 10 minutes at 3,000 rpm and filtered through a 0.45 μm nylon filter.

For total concentrations of elements and the sequential extraction analyses, samples were taken during the four seasons and at the four sites and were mixed and homogenized, and the samples were separated by species and by sampling time. Pseudo-total concentrations of elements were extracted by adding 10 mL of aqua regia (2.5 mL HCl and 7.5 mL HNO_3(Penta)) to 0.5 g of dry soil and were digested at 200°C under microwave conditions (SPD-Discover, CEM, USA). The samples were diluted to 25 mL with deionized water and filtered through a 0.45 μm nylon filter.

The sequential extractions were performed according to the methodology described by Quevauviller (1998) improved by (Rauret et al. 1999): 1 g of dry soil was treated with 40 mL of 0.11 M acetic acid (Lachner) as a first step (acid-soluble fraction), in the second step with 40 mL of 0.1 M hydroxylamine hydrochloride (Sigma aldrich) at pH 2 (reducible fraction) and in the third step, the residue was digested in 10 mL of 8.8 M H_2O_2 (Penta) at 85°C for 1 h, and

then 50 mL of 1 M ammonium acetate pH 2 was added (oxidizable fraction). After each step, the samples were shaken at 200 rpm for 16 h, centrifuged at 4,500 rpm for five minutes, and passed through a 0.45 μm nylon filter.

All concentrations of elements were analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES) (720ES, Varian Inc., CA, USA). The standard reference materials used were 2710a Montana Soil I (NIST, USA) and CRM 483 (Institute for Reference Materials and Measurements, EU) were used.

The distribution of metals in the rhizosphere was investigated using a TESCAN VEGA3XMU scanning electron microscope (SEM) (TESCAN Ltd., Czech Republic) equipped with a Bruker QUANTAX200 energy dispersive X-ray spectrometer (EDS) for imaging and semiquantitative chemical analyses of the target particles (accelerating voltage 15 keV, changeable working distance). The rhizosphere soil samples were placed on conductive tape and were carbon-coated before analysis.

Analysis of biomass

The samples of biomass material were dried at 80°C for 24 h and ground. A 0.2 g sample was digested in 2 mL H_2O_2 and 8 mL of concentrated HNO_3 (Penta) at 200°C under microwave conditions and then diluted to 25 mL with deionized water, filtered through a 0.45 μm nylon filter and analyzed by ICP-OES for determination of element concentrations. For each set of samples, the reference plant material, NCS ZC73018 (Bowen's Kale; IUPAC 1979), was also analyzed. The translocation factor (TF) was calculated as the ratio of elements in the aboveground tissue to those in the belowground tissue.

Arbuscular mycorrhiza colonization assessment

Arbuscular mycorrhiza colonization was determined by the methods described by Phillips and Hayman (1970). Fresh roots were rinsed with distilled water and washed by 10% (w/v) KOH (Lachner) at 90°C and then rinsed in 2% HCl (v/v) (Penta) and stained with 0.05% trypan blue (w/v). The intensity of the

mycorrhizal colonization (M%) and the frequency of mycorrhiza colonization (F%) were assessed using the MYCOCALC software (www2.dijon.inra.fr/mychintec/Mycocalc-prg/download; Trouvelot et al. 1986).

Statistical analysis

The intensity and frequency of mycorrhizal colonizations were normalized by extracting the square root of each value and applying the arcsin function. The rest of the variables were analyzed using the Shapiro-Wilk test, and no normalization was needed. One-way ANOVA was performed to find differences between blocks, while two-way ANOVA was used to find interactions between sampling times and plant species. The Tukey Honest Significant Difference method was used with a confidence level of 0.95. All statistical analyses were performed using the R 3.5 software (The R Foundation for Statistical Computing 2018, under the GNU General Public License).

Principal component analysis (PCA) was performed to find correlations between the element concentrations in plant tissues, and in the rhizosphere, extracted by aqua regia and 0.01 M $CaCl_2$. The analysis was done using the R-package "ade4 1.7-13" (Analysis of Ecological Data: Exploratory and Euclidean Methods in Environmental Sciences, Stephane DrayGPL). The data were centered by subtracting the mean of each variable to each point and then reduced by dividing each point by the standard deviation of each variable. Three axes were used for the PCA, which extracted 56.8% of the information (inertia in axis 1= 17.29, axis 2= 14.37, and axis 3= 6.41).

3.4 Results and discussion

Soil characteristics

Differences in metal(oid) concentrations in the rhizospheres of different plant communities were found (**Table S1**); the highest Pb concentrations were found in the *A. capillaris* rhizosphere (3,417 $mg{\cdot}Kg^{-1}$), and in *A. elatius* for Zn (3,876

$mg \cdot Kg^{-1}$) and were lower for *C. epigeios* (2,310 $mg \cdot Kg^{-1}$). Other elements also reflected differences in plant communities; K was lower for *A. elatius*, while P and Cl were higher; Cu was higher in *A. capillaris*; and Mn and Fe were the lowest for *C. epigeios*. The pH also showed significant differences, being higher in the rhizosphere of *A. elatius*. Differences in the chemical characteristics of the rhizospheres of different species have been previously documented, e.g., by Lehmann and Rebele (2004), who found differences in the total contents of Zn and Cd at the same site when evaluating the rhizospheres of different plant communities; Houben and Sonnet (2015) determined that the roots of *A. capillaris* and *L. albus* influenced chemical speciation of Cd, Zn and Pb regardless of the presence or absence of stabilizing amendments (biochar). The differences in the concentrations observed in the present study could also be due to the heterogeneity of the contaminants related to their causes, e.g., flooding events and atmospheric deposition (Ettler et al. 2005; Zak et al. 2009). Studies under controlled conditions (i.e., pot experiments) could help to understand the behavior and differences of the influence of roots to soil contaminants.

The ecotoxicity of metals and metalloids depends on their specific chemical forms or methods binding, therefore toxic effects and biogeochemical pathways can only be studied on the basis of the determination of these forms. To date, several techniques have been developed to evaluate the metal(oid) fractions available to plants and the environmentally accessible trace metals, however the extracted forms should only be related to the extractant used (e.g. $CaCl_2$-extractable element) and not as e.g., "bioavailable", "mobile", etc. Strictly speaking, speciation of elements would cover the determination of well-defined chemical species, e.g. organometals (Quevauviller 1998). Therefore, $CaCl_2$ extraction and three step sequential extraction (Rauret et al. 1999) were used to correlate the behaviour of the contaminants in the soils with its movement to the plants.

Zinc was found to be the most mobile of the studied contaminants, its $CaCl_2$-extractable fraction ranged between 150 and 250 $mg \cdot Kg^{-1}$ (**Table S4**), and its acid-soluble fraction was the highest from the sequential extraction study. In particular, $CaCl_2$-extractable Zn in the rhizosphere of *C. epigeios* was lower than in other species and samples taken in November had the highest concentrations. Potentially available Pb was lower (**Figure 3.3**a and **3.4**) than

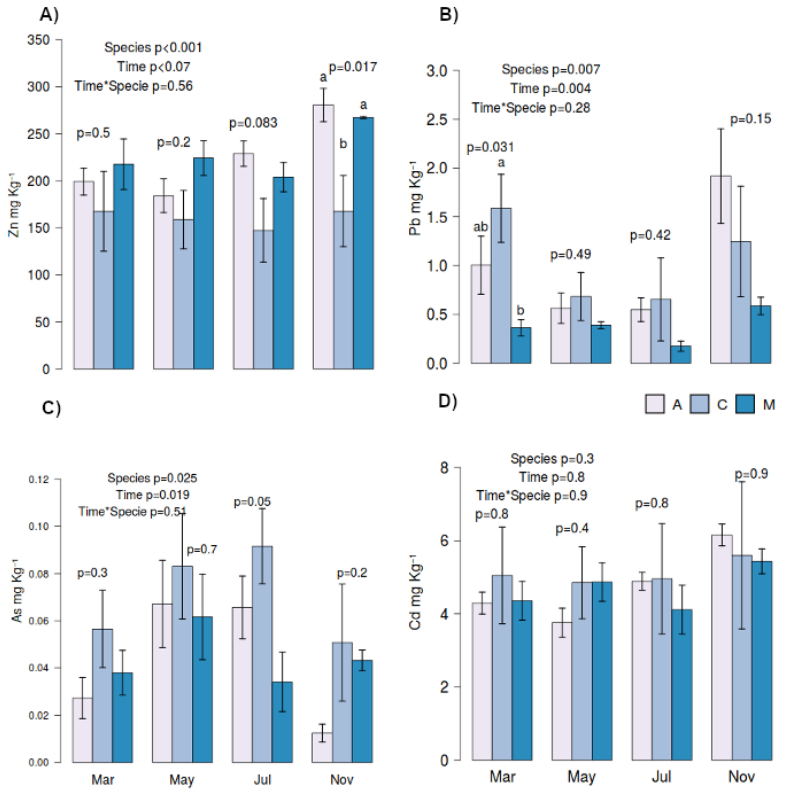

Figure 3.3: Concentrations of Zn (Fig **A**), Pb (**B**), As (**C**) and Cd (**D**) (mean values and standard error) in the $CaCl_2$-fraction of the rhizosphere of *A. capillaris* (A), *C. epigeios* (C) and *A. elatius* (M). One-way ANOVA p value is presented above the bars, one-way ANOVA p in function of species and time and two-way ANOVA for interaction (time*species) are also provided. Letters above the bars represent statistic differences per species by group of time given by Tukey Honest Significant Difference at $p \leq 0.05$.

Zn, as expected, particularly in the rhizosphere of *A. elatius*, while concentrations of the $CaCl_2$ fraction were different compared to the other species. Time of sampling also influenced the results, with March and November being the sampling times with the highest concentrations. Differences in potential availability were statistically insignificant when one-way ANOVA was used; however, two-way ANOVA showed differences among overall sampling seasons and sites with the highest concentrations during May and July, and by species, pointing to the *C. epigeios* rhizosphere having higher contents. $CaCl_2$-extractable Cd was neither species-dependent nor time-dependent, but the acid-soluble fraction (**Figure 3.4**) was lower in the rhizosphere of *C. epigeios*, making its reducible

fraction higher.

Generally, Cd and Zn were more mobile, showing similar trends in the fractionation tests, while As(V) and Pb were less mobile (Austruy et al. 2013; Kucharski et al. 2005; Michálková et al. 2017; Padmavathiamma and L. Y. Li 2012), and this behavior was obvious from our data from the $CaCl_2$ and sequential extractions (**Figure 3.3** and **3.4**). Austruy et al. (2013) found that fractionation of As differed according to the plant species used but did not change considerably when different treatments were applied. Cadmium and Pb often coexist in soils, resulting in interactions for competing (ad)sorption, thus influencing their availability and uptake by plants; high concentrations of Pb enhance Cd solubility in soils and its uptake by plants (F. Zhan et al. 2013). We also observed this behavior in our study as available Pb was higher in the rhizosphere of *C. epigeios* than that in *A. capillaris* (**Figure 3.3**B), explaining why the belowground biomass of *A. capillaris* took up the highest concentrations of Pb and the lowest concentrations of Cd, while the opposite behavior was found for *C. epigeios* (**Figure 3.6**B and D).

Our results from the sequential extractions are consistent with previous studies dealing with the same soil (Michálková et al. 2017). Other authors have found similar fractionations of Pb and Zn; the affinity of Pb for organic ligands explains its low partitioning in the acid-soluble fraction. Humic substances in soil organic matter can reduce metal solubility by forming stable metal chelates; therefore, the roots of different plant species alter the local metal chemistry in the rhizosphere in differing ways, changing the oxidation state of metals, and exerting a direct influence on the mobile/immobile fractions (Padmavathiamma and L. Y. Li 2012). The fractionation of metals in the rhizosphere of *C. epigeios* showed clear differences when compared with the other species, and in comparison with previous studies (Michálková et al. 2017); the acid-soluble fractions of Zn, Pb and Cd were lower, resulting in an increase of the reducible fraction of Cd, and in the oxidizable fractions of Zn and Pb (**Figure 3.4**). This result points to *C. epigeios* as a good indigenous candidate for metal stabilization.

The interactions between time and species have no or limited influence on the available pool of target elements in the studied soils ($p>0.05$). The fractionation of target elements did not show differences between separate sampling

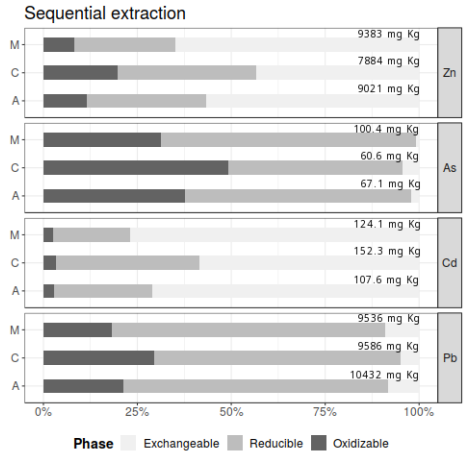

Figure 3.4: Sequential extraction. Percentage of three fractions of target elements in the rhizosphere of *A. capillaris* (A), *C. epigeios* (C), and *A. elatius* (M). Numbers above bars represent total concentration of element (100 %)

campaigns for any of the samples taken (data not shown). Lead was the only element that showed differences in its $CaCl_2$ fraction between the sampling campaigns in the present study, with the highest concentrations observed in samples taken in November. These results are in contrast with those of Padmavathiamma and L. Y. Li (2012), who studied metal fractionation over three seasons and found higher concentrations of $CaCl_2$-extractable Pb in the summer and lower concentrations in the autumn and winter.

Plant uptake of contaminants

Higher contents of Zn were found in the biomass compared to Pb for all species (**Figure 3.5** and **3.6**). The belowground biomass of *A. capillaris* accumulated higher concentrations of Pb, Zn and As (**Figure 3.6**abc) than the other species, as well as Pb in the aboveground biomass (**Figure 3.5**b). The belowground biomass of *C. epigeios* accumulated the highest amounts of Cd (**Figure 3.6**d), but the lowest contents of Zn and Pb in their aboveground biomass were observed, particularly during March (**Figure 3.5**ab). *A. elatius* accumulated the lowest amounts of Zn in its belowground biomass (**Figure 3.6**a) but had the highest amounts in its aboveground biomass along with *A. capillaris* (**Figure 3.5**a), meaning a higher rate of translocation of Zn

from below to the aboveground biomass. Accumulation of Pb and Cd in their belowground biomasses was also lowest for this species (**Figure 3.6**bd) but did not show significant differences in aboveground biomass (**Figure 3.5**bd).

The concentrations of the studied elements in the aboveground biomass showed increases during the year, nevertheless there was a clear trend of gradual increases in their contents (**Figure 3.5**d) only for Cd, while Pb was the only element where the interaction of time and species influenced its translocation; March was the time with the lowest content, in particular for *C. epigeios* and during November the concentrations were higher for *A. capillaris*.

The Zn, Pb and Cd contents in aboveground biomasses increased with time,

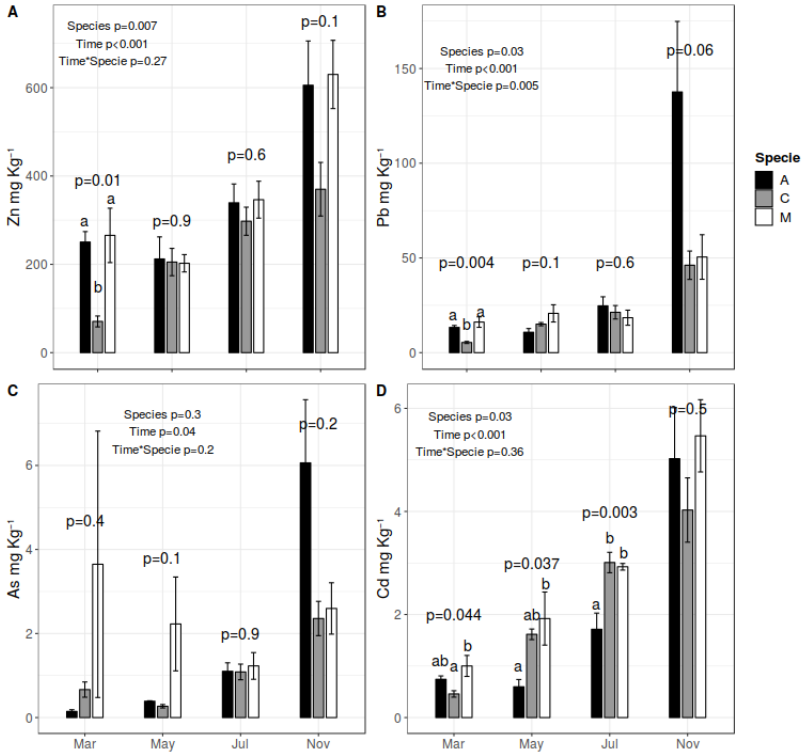

Figure 3.5: Concentrations of Zn (Fig **A**), Pb (**B**), As (**C**) and Cd (**D**) (mean values and standard error) in the above-ground biomass of *A. capillaris* (A), *C. epigeios* (C) and *A. elatius* (M). One-way ANOVA p value is presented above the bars, one-way ANOVA p in function of species and time and two-way ANOVA for interaction (time*species) are also provided. Letters above the bars represent statistic differences per species by group of time given by Tukey Honest Significant Difference at $p \leq 0.05$.

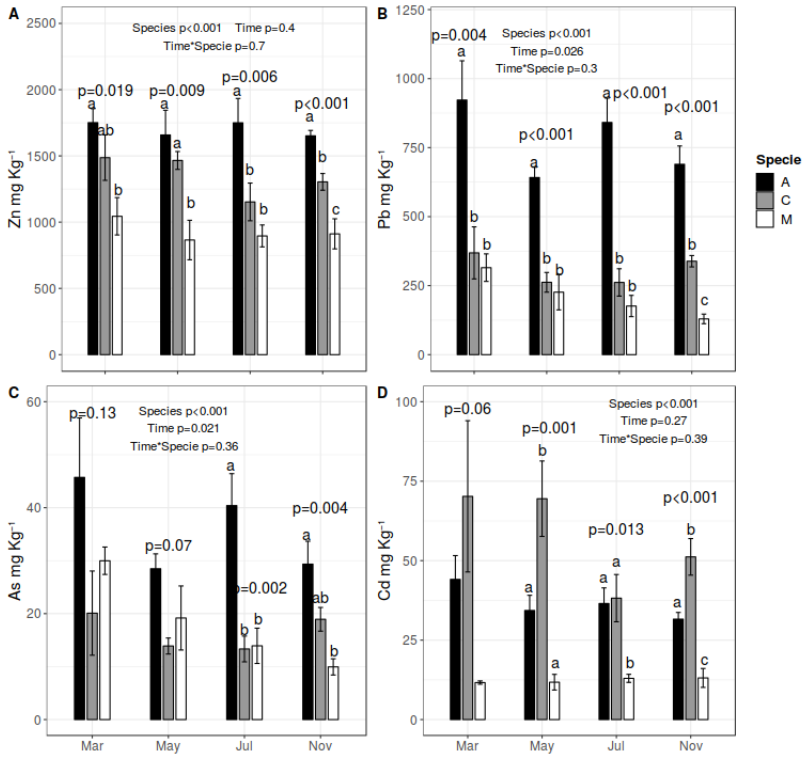

Figure 3.6: Concentrations of Zn (Fig **A**), Pb (**B**), As (**C**) and Cd (**D**) (mean values and standard error) in the below-ground biomass of *A. capillaris* (A), *C. epigeios* (C) and *A. elatius* (M). One-way ANOVA p value is presented above the bars, one-way ANOVA p in function of species and time and two-way ANOVA for interaction (time*species) are also provided. Letters above the bars represent statistic differences per species by group of time given by Tukey Honest Significant Difference at $p \leq 0.05$.

with the highest contents in November. Padmavathiamma and L. Y. Li (2012) investigated *F. rubra* in British Columbia, Canada, and the contents of Zn and Pb in the aboveground biomass were higher in summer and autumn, respectively. These differences may be connected to differences in climatic conditions, and the mobility of nutrients (including trace elements) within the plant highly correlated with environmental factors such as rainfall, humidity, and temperature. *Helianthus annuus* L. has been used in previous experiments growing on the same soil (Martínez-Fernández et al. 2015a; Michálková et al. 2017), and took up higher amounts of contaminants, especially for Zn with up to 1,470 $mg \cdot Kg^{-1}$ in shoots, and 17,700 $mg \cdot Kg^{-1}$ in roots (Michálková et al. 2017), and the plants started to die after 30 days of growth. These results highlight the importance of using local species for the reclamation of contaminated sites;

successful phytostabilization should establish a long-term succession of plant communities that promotes the development of the soil and maintains sustainable soil ecosystem functions (Mench et al. 2010). The benefits of using *Poaceae* species for stabilization of metal(oid) contaminants are mentioned by Kucharski et al. (2005), who compared *Deschampsia cespitosa* (*Poaceae*) to *Cardaminopsis arenosa* (*Brassicaceae*) growing in a multimetal contaminated soil (Cd, Pb and Zn); *C. arenosa* appeared spontaneously in a field experiment and accumulated higher amounts of contaminants in shoot tissues, but lower levels in roots; however, *D. cespitosa* was the dominant species for all treatments. Austruy et al. (2013) found the potential of *A. capillaris* for phytostabilization of As. When compared to other species, *A. capillaris* accumulated the highest amount of As in contaminated soils but accumulated the lowest amounts when grown in unpolluted soil. Previous works suggest that species from the *Poaceae* family are able to store contaminants in the belowground biomass, while a considerably smaller amount is translocated to the aboveground biomass, thus reducing the risk of contaminant transfer to grazing animals and increasing its immobilization at the rhizospheric level (Austruy et al. 2013; Machado-Estrada et al. 2013; Padmavathiamma and L. Y. Li 2012; Rabier et al. 2014).

Different plant species exhibit different mechanisms for coping with different elements in the soil. Rabier et al. (2014) found Zn concentrations of approximately 68 and 165 $mg{\cdot}Kg^{-1}$ in roots of *A. halimus* growing in two contaminated soils, while the concentrations in shoots were higher, at 150 and 161 $mg \cdot Kg^{-1}$, respectively. However, most of the literature reports that higher concentrations occur in roots compared to shoots (Fuksova et al. 2009; Machado-Estrada et al. 2013; Unterbrunner et al. 2007; F. Zhan et al. 2013). Therefore, the species evaluated should be taken into consideration when comparing concentrations of elements in plant tissues.

Among the species evaluated in the present study, *A. capillaris* was the most studied in the literature and is proven to adapt well to contamination stress and to be suitable for phytostabilization by keeping contaminants in the root system, while maintaining low or relatively lower concentrations in the aboveground biomass. Rodriguez-Seijo et al. (2016) reported Pb concentrations of 1,107 $mg \cdot Kg^{-1}$ in roots and 135 $mg \cdot Kg^{-1}$ in shoots; Houben and Sonnet (2015) found 55 $mg \cdot Kg^{-1}$ Cd, 280 $mg \cdot Kg^{-1}$ Pb and 2,500 $mg \cdot Kg^{-1}$ Zn in

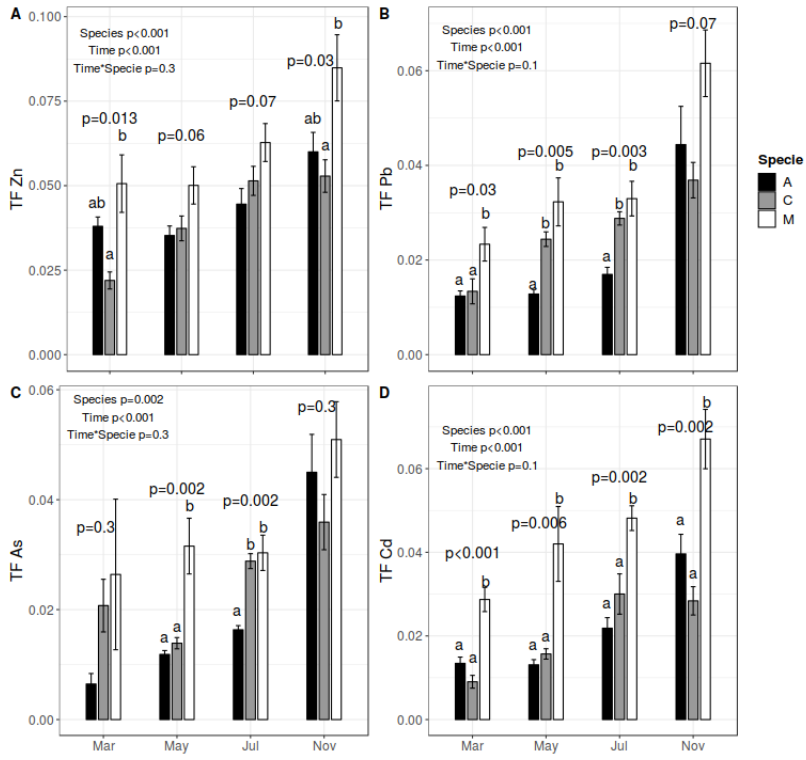

Figure 3.7: Translocation factor of of Zn (Fig **A**), Pb (**B**), As (**C**) and Cd (**D**) (mean values and standard error) of *A. capillaris* (A), *C. epigeios* (C) and *A. elatius* (M). One-way ANOVA p value is presented above the bars, one-way ANOVA p in function of species and time and two-way ANOVA for interaction (time*species) are also provided. Letters above the bars represent statistic differences per species by group of time given by Tukey Honest Significant Difference at $p \leq 0.05$.

roots, while the concentrations in shoots were low. Austruy et al. (2013) tested three plant species growing in contaminated soils (3,162 $mg \cdot Kg^{-1}$ As) and *A. capillaris* proved to be the species best adapted to contamination. This species showed the highest concentrations of As in roots (1,000 $mg \cdot Kg^{-1}$ As), while the lowest concentrations were found in the roots of *V. faba* (250 $mg \cdot Kg^{-1}$).

The TF increased with time of the year and varied considerably among different species. For the four target elements, *A. elatius* presented the highest TF, *A. capillaris* showed similar TF of Pb in May and July, and As in July; for all other elements and sampling times, both *A. capillaris* and *C. epigeios* showed significantly lower TF values. However, the three selected species presented lower values compared with other studies. Austruy et al. (2013) used

A. capillaris in As-contaminated soils, obtaining values between 0.2 and 0.4. Padmavathiamma and L. Y. Li (2012) found TF of Pb between 0.19 and 0.25 and of Zn between 0.69 and 0.77 for three *Poaceae* species when grown under contaminated conditions, and between 0.14 and 0.21 Pb and 0.6 to 0.7 Zn when an amendment was applied. Lower TF are not commonly reported in the literature for the *Poaceae* species, e.g., Rabier et al. (2014) found a low TF of Pb (0.006-0.05) but high levels of Zn (1.1-2.2) in *A. halimus*, a halophytic species that is also resistant to metal contamination. A low TF is important for the phytostabilization process, the lower the TF, the lower the mobilization of contaminants from the belowground to aboveground tissues. The differences in TF between this and other studies could probably be due to climatic and edaphic conditions. Nevertheless, we conclude that all selected species have the potential for phytoremediation.

Microscopic observations of the rhizosphere

Although there were no visible differences among the rhizospheres of the three species, important features were observed (**Figure S2**). In particular, increased Pb concentrations (mean value of 21.5 wt.%) were detected in all samples. In most cases, such high Pb contents were associated with Mn oxides, as recently reported by Vítková et al. (2018) for the same soil type. Moreover, the preferential binding of Pb in the reducible fraction is demonstrated in **Figure 3.4** and a significant correlation of Pb-Mn was determined (**Figure S1**). **Figure S2**c and d clearly show that the residues of organic matter (plant tissues) are covered with Mn oxides containing Pb. In addition, partly-oxidized metallic Pb particles were occasionally observed (Fig. **Figure S2**a).

Role of arbuscular mycorrhizal fungi

The colonization by AMF did not show significant differences between sampling times, or interactions between time and species; however, differences in the frequency and intensity of colonization were found for different plant species (Table **Table S2**), and samples of *C. epigeios* showed significantly lower colonization by AMF. Concentrations of elements in rhizospheres and plants showed

poor correlations with AMF colonization (**Table S3**). Other studies have also found little or no correlations between chemical variables and AMF colonization (Baba et al. 2016; Ruotsalainen et al. 2007). Kanwal et al. (2015) observed a decrease in the colonization of *Medicago sativa* roots by *G. intraradices* after the addition of Cd. Gucwa-Przepiora et al. (2007) observed a negative correlation between AMF colonization parameters and $CaCl_2$-extractable Cd and Zn in soil, but all other chemical parameters were not significantly correlated with AMF variables. While it has been shown that AMF colonization decreases in contaminated soils (Ruotsalainen et al. 2007), it seems that a higher correlation exists with physical parameters, such as particle size and water holding capacity, rather than with the chemical parameters (Baba et al. 2016). Moreover, the plant species acting as hosts seem to be a decisive factor in the colonization of particular AMF species. Malcová et al. (2003) found that addition of Pb at different concentrations does not change the percentage of root colonization by *G. intraradices*; however, there were differences in the colonization levels of the two plant species, *Z. mays* and *A. capillaris*, with the latter being lower. Likewise, the effect of AMF did not influence Pb uptake; thus, the combination of AMF and host plants was a determining factor for the results of their interactions under chemical stress.

We wanted to acquire a better understanding of the soil chemical characteristics playing a role in the AMF-colonization process; however, the only factor that was statistically correlated with the colonization of roots was the plant species, similarly to what has been frequently observed in the literature, e.g., Sudova et al. (2008), after testing six clones of *A. capillaris* under three isolates of *G. intraradices* found that the effect of AMF inoculation on plant growth and metal(loid)s-uptake depended on the particular combination of plant clone and fungal isolate, with no clear differences between tolerant and nontolerant clones. Doubkova and Sudova (2016) found no difference in root colonization by *Rhizophagus irregularis* for two clones of *A. capillaris*, proving that for this particular species, colonization is not determined by the AMF resistance to metal tolerance, but by the particular host plant. F. Zhan et al. (2013) observed a poor correlation of AMF colonization and uptake of Cd, Pb and Zn by wild species growing in contaminated field conditions, only As in both shoots and roots showed a negative correlation with AMF colonization. However, Neagoe

et al. (2014) found that the decrease in As concentrations in plant tissues correlates with a decrease in oxidative stress promoted by AMF symbiosis. Based on the literature data, it is clear that AMF symbiosis improves the resistance of the plant to metal(oid)s stress and that colonization is directly dependent of the plant species acting as the host. Therefore, we can state that *C. epigeios* is not a good choice for phytoremediation where AMF plays a role in supporting the plant or the stabilizing agent. However, further research is needed to observe AMF colonization for the other species, both individually and combined, to find the best candidate(s) for AMF-aided phytoremediation.

Principal component analysis

Principal component (PC) analysis was performed for three axes, representing 56.8% of the cumulative inertia (**Table S4**). PC-1 clearly separates element content in belowground biomass to the left, and aboveground biomass to the right (**Figure 3.8**, contribution of the variables to the components can be found in the **Table S4**), while PC-2 correlates positively to the concentration of all elements in biomass, with the only exception being for K (both above and belowground). PC-2 also separates *A. capillaris* from the other species (**Figure 3.8**), which reflects its ability to take up more elements in general, but an inclination to the left is observed, positioning most of the samples next to the element concentrations in the belowground biomass. This shows, similarly to **Figure 3.6**, the potential of *A. capillaris* to retain contaminants in the rhizosphere. Moreover, its good correlation with element concentrations in general shows the strength of this species to also obtain nutrients, pointing to this species as a strong candidate for phytostabilization.

When rotating the graphic of PC-3 (**Figure 3.8**), *A. elatius* separates from the others and is positioned on the opposite side from the concentrations of elements in the belowground biomass, but closer to aboveground As and Zn, as well as soil and $CaCl_2$-extractable Zn. This correlates well with previous results where we showed that *A. elatius* took up more As and Zn in the aboveground biomass (**Figure 3.5**) even if no significant differences were observable, most likely due to the large standard deviation. For $CaCl_2$-extractable Zn (**Figure 3.3**), *A. elatius* always had the highest concentrations along with *A.*

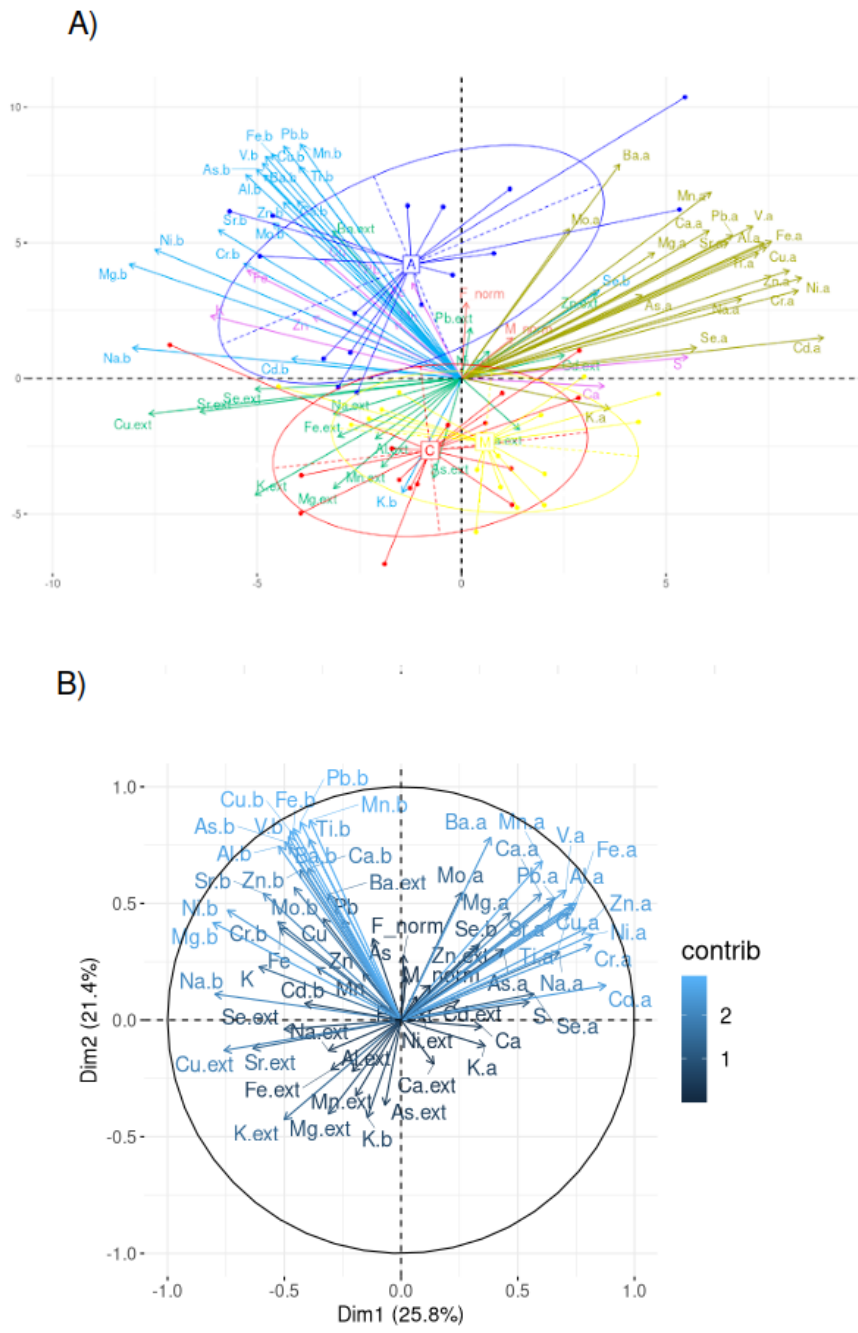

Figure 3.8: Concentration of elements in soil (element symbol), $CaCl_2$-extractable (ext), and plant biomass (a= above-ground, b= below-ground) plotted on the plane of the first three components from Principal Component (PC) Analysis (Fig. **A**) and contribution of the variables (Fig. **B**). Values for AMF colonization (F) and intensity of colonization (M) were normalized and added to the plot. Species: *A. capillaris* (A - blue), *C. epigeios* (C - red), and *A. elatius* (M - yellow)

capillaris. Other parameters defining *A. elatius* are total As and Mn, K in the belowground biomass, and Mo in the aboveground biomass. Its ability to take up As and Zn to the aboveground biomass and to obtain K (unlike the other species evaluated) points to *A. elatius* as a potential phyto-extracting species. These results correlate with the analysis of variance, highlight the importance of chemical characteristics of the soil and the plant communities growing on it.

3.5 Conclusions

Based on our results, we conclude that there is a significant correlation between the chemical characteristics of the rhizosphere, with the plant communities growing on it and that AMF colonization is directly connected with the plant species that function as hosts, while it is poorly correlated with the chemical characteristics of the soil. Our data and data from the literature show that among the evaluated species, *A. capillaris* appears to be a potentially good indigenous candidate for phytostabilization as it correlates well with the concentrations of metal(oid)s in its belowground biomass, while translocating relatively low amounts to the aboveground biomass. This shows potential for reducing the mobility of the contaminants in soil, it develops good symbiosis with AMF, and reduces the acid-soluble and $CaCl_2$-extractable fractions of As in the rhizosphere. *C. epigeios* developed poor AMF colonization, which potentially could make this species more vulnerable to stress, however it translocated lower concentrations of Zn and As in the aboveground biomass. *A. elatius* developed good colonization by AMF and kept the $CaCl_2$-extractable Pb in the rhizosphere at lower levels than the other species; however, it translocated higher amounts of Pb, As and Cd to the aboveground biomass. These characteristics position *A. elatius* as a potential candidate for phytoextraction rather than for stabilization purposes; however, the drawbacks of phytoextraction are well known, and phytostabilization should be the preferred option in general.

Further research is needed to understand the interactions between this species and the metal(loid)s in soils. Aside from studies on *A. capillaris*, little research has addressed interactions of these species with contaminants, and even less research has addressed the interactions of plant communities with soil

contamination. The successful establishment of species such as *F. rubra* and *M. caerulea* can be related to its interactions with *A. capillaris* and *A. elatius*, respectively.

3.6 Supplementary Material

Table S1: Total concentrations of elements in the rhizospheric soil of three species. Element concentrations values are all given in $mg \cdot kg^{-1}$. Significant differences by ANOVA are pointed by $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$

Rhizosphere	**Bulk Soil**[a]	*A. capillaris*	*C. epigeios*	*A. elatius*
Clay	5±0.50	-	-	-
Silt	20±0.50	-	-	-
Sand	75±0.50	-	-	-
pH - H2O	5.95±0.01	5.62±0.27	5.54±0.29	***5.99±0.23
pH - KCl	5.14±0.03	5.37±0.37	5.29±0.32	***5.71±0.29
K	6583±293	5534±716	5444±1241	*4575±894
Ca	1778±104	2463±655	2611±445	2361±556
S	490±5.6	410±154	352±181	422±187
Cu	71.9±3	***84±12	***64±10	*74±13
Mn	4276±28	1857±36728	**1295±544	2170±728
Fe	37408±159	*23930±2684	21759±3803	20646±4320
Zn	4002±55	2910±493	*2310±232	2839±845
Pb	3539±30	**2796±425	2426±552	**2073±719
As	296±5	237±48	206±74	218±44
Cd	39±0.90	36±6	39±7	37±9

[a] Data taken from Vítková et al. 2016

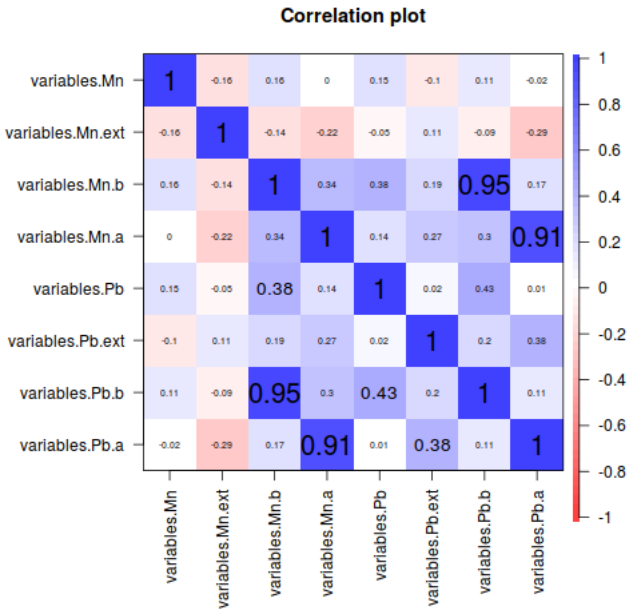

Figure S1: Correlation matrix between variables related to Mn and Pb

Table S2: Mean and standard deviation (SD) of the frequency of mycorrhiza colonization (F) and intensity of colonization (M) by sampling time and specie. A= *A. capillaris*, C= *C. epigeios*, M= *A. elatius*

Specie	Time	F		M	
		Mean	**SD**	**Mean**	**SD**
A	Mar	69.18	29.97	11.03	7.13
	May	88.48	11.54	15.30	4.00
	Jul	48.33	12.29	6.35	6.03
	Nov	58.35	8.81	6.30	7.10
C	Mar	29.17	5.69	1.15	0.59
	May	27.50	17.51	1.61	2.84
	Jul	19.17	14.24	0.57	0.44
	Nov	33.33	13.33	0.94	0.93
M	Mar	59.17	28.20	12.18	8.26
	May	54.68	6.44	11.41	6.72
	Jul	68.33	34.48	22.90	17.61
	Nov	74.17	17.29	18.88	15.76
Anova effect		**P - values**			
Time		0.459		0.953	
Species		<0.001		<0.001	
Time*Spp		0.119		0.258	

Table S3: Correlation of the frequency of mycorrhiza colonization (F) and intensity of colonization (M) with the concentration of elements in the soil by aquaregia extraction (represented by element symbol), $CaCl_2$-extractable fraction (ext), in the above-ground biomass (a) and in the below-ground biomass (b). P value of the linear model and adjusted- R^2

	F		M	
	P	**R^2**	**P**	**R^2**
S	0.703	-0.019	0.417	-0.0071
K	0.404	-0.006	0.1179	0.0317
Ca	0.293	0.003	0.1469	0.0244
Mn	0.012	0.110	0.0083	0.1234
Fe	0.643	-0.017	0.9319	-0.0216
Cu	0.009	0.122	0.0293	0.0795
Zn	0.165	0.021	0.5507	-0.0138
As	0.239	0.009	0.154	0.0229
Pb	0.865	-0.021	0.3993	-0.0059

Na.ext	0.084	0.043	0.3022	0.0019
Mg.ext	0.063	0.053	0.0033	0.1546
Al.ext	0.163	0.021	0.0849	0.0428
K.ext	0.034	0.074	4e-04	0.227
Ca.ext	0.335	-0.001	0.4967	-0.0114
Mn.ext	0.426	-0.008	0.4448	-0.0087
Fe.ext	0.602	-0.016	0.3346	-0.001
Ni.ext	0.207	0.013	0.2191	0.0116
Cu.ext	0.443	-0.009	0.1598	0.0217
Zn.ext	0.209	0.013	0.0667	0.0511
As.ext	0.037	0.071	0.034	0.0743
Se.ext	0.544	-0.014	0.6973	-0.0183
Sr.ext	0.482	-0.011	0.0813	0.0442
Cd.ext	0.201	0.014	0.4666	-0.0099
Pb.ext	0.122	0.031	0.0528	0.0591
Ba.ext	0.016	0.100	0.0981	0.0379
Na.b	0.229	0.010	0.4394	-0.0084
Mg.b	0.629	-0.017	0.675	-0.0178
Al.b	0.620	-0.016	0.584	-0.015
K.b	0.089	0.041	0.2469	0.0079
Ca.b	0.086	0.043	0.9353	-0.0216
Ti.b	0.392	-0.005	0.9455	-0.0216
V.b	0.491	-0.011	0.8683	-0.0211
Cr.b	0.817	-0.021	0.9948	-0.0217
Mn.b	0.416	-0.007	0.9387	-0.0216
Fe.b	0.461	-0.010	0.9194	-0.0215
Ni.b	0.459	-0.010	0.9513	-0.0217
Cu.b	0.051	0.061	0.5879	-0.0152
Zn.b	0.785	-0.020	0.1867	0.0167
As.b	0.179	0.018	0.415	-0.0069
Se.b	0.839	-0.021	0.9537	-0.0217
Sr.b	0.206	0.014	0.7915	-0.0202
Mo.b	0.011	0.113	0.0292	0.0797
Cd.b	0.006	0.136	2e-04	0.2519

Pb.b	0.245	0.008	0.8575	-0.021
Ba.b	0.632	-0.017	0.3253	-2e-04
Na.a	0.478	-0.011	0.6943	-0.0183
Mg.a	0.498	-0.012	0.9334	-0.0216
Al.a	0.765	-0.020	0.8706	-0.0211
K.a	0.227	0.011	0.5105	-0.0121
Ca.a	0.292	0.003	0.4451	-0.0087
Ti.a	0.398	-0.006	0.2989	0.0022
V.a	0.539	-0.013	0.6496	-0.0171
Cr.a	0.564	-0.014	0.4642	-0.0098
Mn.a	0.390	-0.005	0.4155	-0.007
Fe.a	0.674	-0.018	0.6941	-0.0183
Ni.a	0.567	-0.014	0.7463	-0.0194
Cu.a	0.284	0.004	0.2871	0.0034
Zn.a	0.104	0.036	0.0737	0.0476
As.a	0.152	0.023	0.1281	0.0289
Se.a	0.326	0.000	0.9892	-0.0217
Sr.a	0.877	-0.021	0.971	-0.0217
Mo.a	0.017	0.099	0.0989	0.0376
Cd.a	0.997	-0.022	0.5735	-0.0146
Pb.a	0.636	-0.017	0.5868	-0.0151
Ba.a	0.286	0.004	0.6742	-0.0178
AMF.F	0	0.7	0	0.7

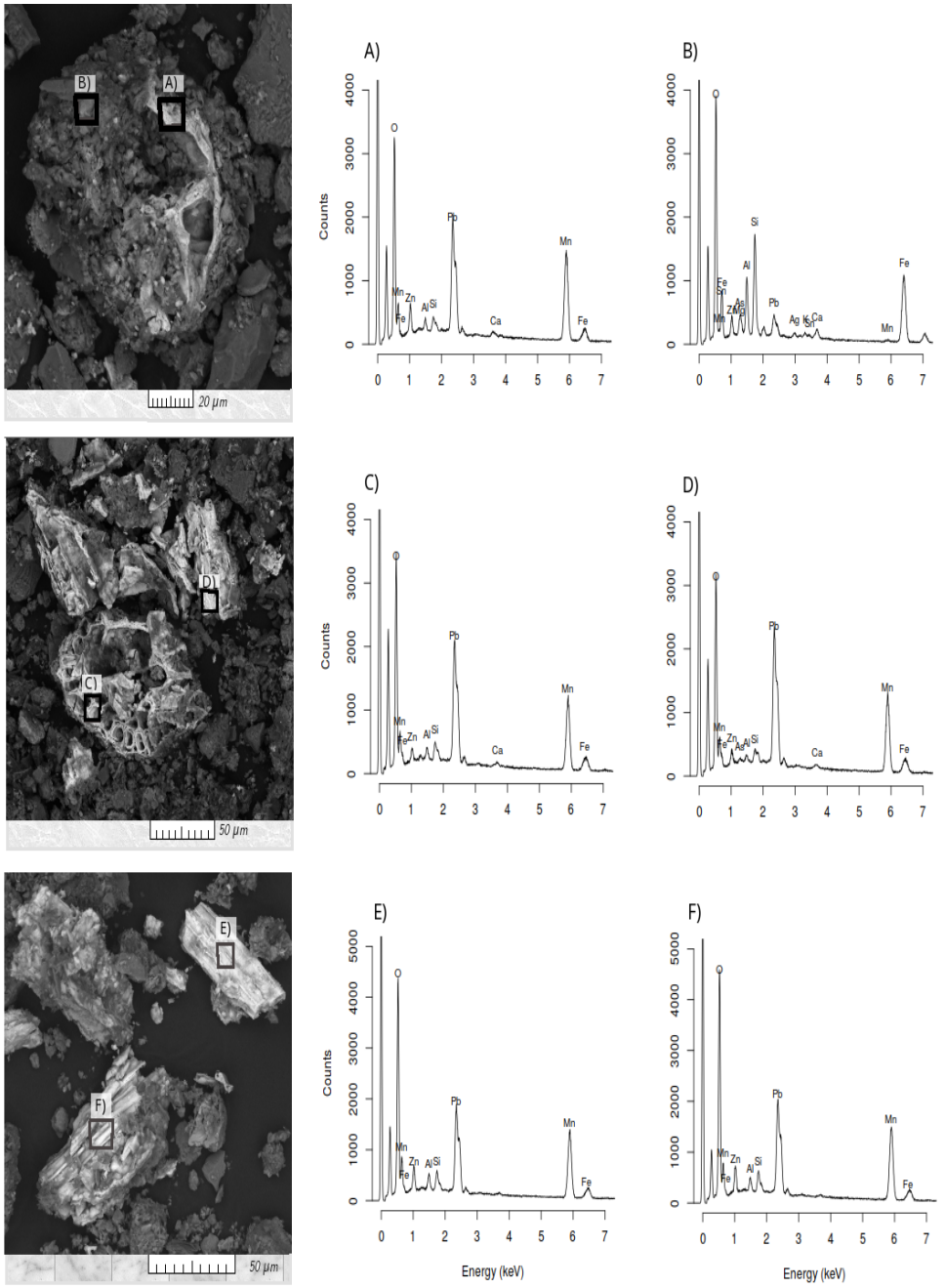

Figure S2: Distribution of metals in the rhizosphere under scanning electron microscope (SEM) with EDS. **A-B** - *A. elatius*, **C-D** - *C. epigeios*, and **E-F** - *A. capillaris*

Table S4: Contribution of the variables to the three components for the PCA. Element symbol represent its concentration in the soil by aquaregia extraction, ext= $CaCl_2$-extractable, a= above-ground biomass, b= below-ground biomass, AMF-F frequency of mycorrhiza colonization, AMF-M intensity of colonization

Variable	PC-1	PC-2	PC-3	Variable	PC-1	PC-2	PC-3
S	0.5481	0.0772	0.2674	Mn.b	-0.3905	0.8560	0.0938
K	-0.6082	0.2284	0.0147	Fe.b	-0.4606	0.8200	0.0858
Ca	0.3458	-0.0291	0.5212	Ni.b	-0.7431	0.4706	-0.2195
Mn	-0.1616	0.2007	-0.5216	Cu.b	-0.4751	0.8102	0.1510
Fe	-0.5194	0.3949	-0.1338	Zn.b	-0.4315	0.6432	0.3887
Cu	-0.3330	0.4344	-0.1868	As.b	-0.4968	0.7641	-0.1106
Zn	-0.3594	0.2247	-0.3827	Se.b	0.3340	0.3229	0.4504
As	-0.1199	0.3488	-0.1457	Sr.b	-0.5892	0.5423	0.3868
Pb	-0.2504	0.4292	0.0988	Mo.b	-0.4569	0.5674	-0.4425
Na.ext	-0.3105	-0.1312	0.4518	Cd.b	-0.4116	0.0719	0.4339
Mg.ext	-0.3100	-0.4019	0.6444	Pb.b	-0.4305	0.8479	0.1751
Al.ext	-0.2084	-0.2200	0.5718	Ba.b	-0.4784	0.7436	0.3676
K.ext	-0.5004	-0.4264	0.4626	Na.a	0.6785	0.2907	0.2614
Ca.ext	0.1403	-0.1878	0.2410	Mg.a	0.4671	0.4591	0.3043
Mn.ext	-0.1938	-0.3248	0.5515	Al.a	0.7322	0.4951	-0.0199
Fe.ext	-0.2993	-0.2135	0.7287	K.a	0.3594	-0.1108	0.6006
Ni.ext	0.0667	0.0997	0.3587	Ca.a	0.5996	0.5405	0.0945
Cu.ext	-0.7579	-0.1300	-0.0986	Ti.a	0.7194	0.4574	-0.1658
Zn.ext	0.3263	0.3116	-0.2739	V.a	0.7055	0.5557	-0.0280
As.ext	-0.0684	-0.3646	0.5499	Cr.a	0.8159	0.3206	0.0172
Se.ext	-0.5006	-0.0397	-0.3031	Mn.a	0.6042	0.6801	0.0358
Sr.ext	-0.6341	-0.1221	0.1384	Fe.a	0.7508	0.5001	-0.0172
Cd.ext	0.2491	0.0851	-0.0099	Ni.a	0.8235	0.3672	0.2293
Pb.ext	0.0216	0.1844	0.1079	Cu.a	0.7391	0.4806	0.2271
Ba.ext	-0.3124	0.5389	0.0311	Zn.a	0.7939	0.3940	-0.1667
Na.b	-0.7970	0.1113	-0.1637	As.a	0.4371	0.3032	-0.3639
Mg.b	-0.8054	0.4172	0.0179	Se.a	0.5687	0.1119	0.1886
Al.b	-0.5231	0.7445	0.1417	Sr.a	0.6455	0.4965	0.1187
K.b	-0.1440	-0.4173	-0.4567	Mo.a	0.2600	0.5467	0.1050
Ca.b	-0.3981	0.6492	0.3417	Cd.a	0.8769	0.1483	-0.1062
Ti.b	-0.3937	0.7748	0.0768	Pb.a	0.6568	0.5229	-0.0814
V.b	-0.4824	0.7861	0.1136	Ba.a	0.3831	0.7824	0.1240
Cr.b	-0.5275	0.4204	-0.4226	AMF.F	0.0112	0.2771	-0.2605
				AMF.M	0.1243	0.1482	-0.4064

CHAPTER

FOUR

APPLICATION OF CO-COMPOSTED BIOCHAR SIGNIFICANTLY IMPROVED PLANT-GROWTH RELEVANT PHYSICAL/CHEMICAL PROPERTIES OF A METAL CONTAMINATED SOIL

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4.1 Abstract

A woody-biochar was added to waste biomass during a composting cycle. The resulting compo-char was amended to a metal contaminated soil and two plant species, *L. perenne* and *E. sativa*, were grown in a pot experiment to determine **1)** plant survival and stress factors, **2)** uptake of metals to plants and, **3)** chemical characteristics of sampled soils and pore waters. Compost supplemented with biochar after the composting process were also tested, as well as a commercially available compost, for comparison. Co-composting with biochar hastened the composting process, resulting in a composite material of reduced odour, increased maturity, circum-neutral pH and increased moisture retention than compost (increase by 3% of easily removable water content). When amended to the soil, $CaCl_2$ extractable and pore water metals were reduced by all compost treatments with little influence of biochar addition at any tested dose. Plant growth success was promoted furthest by the addition of co-composted biochar to the test soil, especially in the case of *E. sativa*. For both tested plant species significant reductions in plant metal concentrations (e.g. 8-times for Zn) were achieved, against the control soil, by compost, regardless of biochar addition. The results of this study demonstrate that the addition of biochar into the composting process can hasten the stability of the resulting compost-char, with more favourable characteristics as a soil amendment/improver than compost alone. This appears achievable whilst also maintaining the provision of available nutrients to soils and the reduction of metal mobility, and improved conditions for plant establishment.

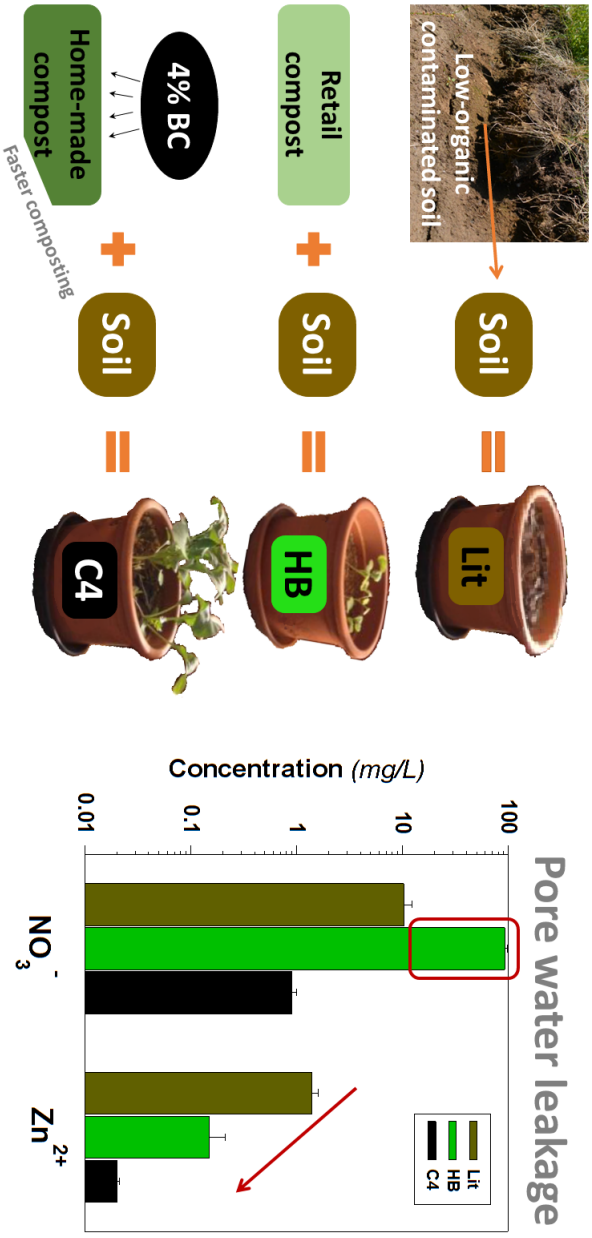

Figure 4.1: GRAPHICAL ABSTRACT

4.2 Introduction

The addition of organic matter to the soil can improve soil structure, nutrients content and chemical characteristics (Chirakkara and Reddy 2015; Rahman et al. 2017). The use of compost (product of spontaneous microbial bio-oxidation of raw wastes to produce a biologically stable, humified organic matter; Beesley et al. 2014) can enhance plant growth and increase microbial activity in soils (Chirakkara and Reddy 2015) by providing nutrients and increasing humic content, thereby increasing water holding capacity, improving soil structure and microbiological activity (Medina and Azcon 2010). Compost also has properties advantageous to the remediation of metal-contaminated soils because immobilized complexes are able to form between organic ligands and metals assisted by changes in soil pH (Oustriere et al. 2017).

For similar reasons the use of biochar as an amendment for contaminated soils has also gained attention in recent years. Biochar is the solid product from pyrolysis of waste biomass residues, under anoxic conditions using temperatures ranging from 350 to 900 °C (Forjan et al. 2018; Novak et al. 2019). The addition of biochar to contaminated soils has been demonstrated to increase cation exchange capacity, and act as an enhanced habitat for microbes and fungi (Rahman et al. 2017) due to the high surface areas of biochar, in comparison to, for example, composts. The application of biochar, similarly to compost can also increase C stocks in soils, increasing soil fertility and plant growth (Houben and Sonnet 2015). Three main sorption mechanisms exist upon biochar application to soils, which are favourable to its application to metal contaminated soils: (i) ion exchange, (ii) metal complexation with free functional groups together with physical adsorption, and (iii) surface precipitation (Trakal et al. 2016).

Several authors have proven that the combination of compost with biochar may be more suitable than biochar alone to promote immobilization metal(loid) contaminants and buffer nutrient depletion in contaminated soils. Compost combined with biochar may improve total soil C, N, and P, stabilize soil aggregates, and stimulate microorganisms (Forjan et al. 2018; Novak et al. 2019; Oustriere et al. 2017; Radin et al. 2018; Rodriguez-Vila et al. 2016; Ye et al. 2019). Potential benefits of using biochar as a compost amendment can be sum-

marized as i) increasing microbial activity by enhancing aeration, ii) reducing soil bulk density, iii) increasing compost temperature, iv) decreasing ammonia volatilization, v) enhancing water holding capacity, vi) reducing nutrient losses by leaching, vii) reducing greenhouse gas (GHG) and odour emissions, and viii) increasing the degree of humification (Akdeniz 2019; Godlewska et al. 2017; El-Naggar et al. 2019; Wang et al. 2019). As the application of compost has been shown to affect positively the above-mentioned soil properties, the use of biochar as an amendment during composting has the potential to improve these benefits even further (Wang et al. 2019).

The aims of the present study were to compare retail compost against home-made compost and the compost amended by biochar in the context of 1) soil water and nutrients retention; 2) the metal(loid)s stabilization; as well as 3) the consequent responses to two plants species (*Lolium perenne* L. and *Eruca sativa*) when grown in a metal-contaminated soil amended with compost/biochar.

4.3 Materials and methods

Experimental soil, biochar and compost

The soil "Litavka" used for the experiment was collected from a site located near a lead smelter (Pribram, Czech Republic). The soil used was taken from several sampling locations at various depths up to half a meter deep in order to collect a representative bulk of sample. Bulk soil was dried and sieved (< 2 mm). Initial soil analysis are presented in **Table 4.1**.

The biochar used in the experiment was a mixture of soft wood, mostly spruce made in a CHP plant in Kozomín (Czech Republic) in atmospheric, fixed-bed, multi-stage gasifier, which uses air as a gasifying medium (GP 750). The biochar was heated for 6 hours between 500 – 600 °C. Initial biochar characteristics are in Table S1 while its chemical composition is in **Table 4.1**.

Compost was prepared from a grove of oak and maple trees, small sticks, and freshly cut grass. The leaves, twigs and grass were chopped up with shears

and added to a 200-liter spinning plastic drum at a ratio of 5:1. Three different drums were prepared: C0 with simple compost, and C4-C10 with compost mixed 4 and 10%wt. of biochar. The drums were placed in a greenhouse at approximately 20°C and spun to mix and aerate 3 times per week during 16 weeks. Moisture content and pH were measured during the composting and co-composting processes from the start until 126 days. Samples were obtained weekly from each treatment (C0, C4 and C10) and 5g was used to measure pH in a H2O suspension at 1:5 (w:v) ratio. The remaining sample was weighed and dry at 60°C until constant weight, moisture content was calculated as the difference between wet and dry weight. Details of the compost preparation can be found in the Supplement.

Soil water retention and available water content

Four treatments were used for the measurement of soil water retention (SWRC): control Litavka soil without any addition of organic substrate (Lit.), soil mixture with pure compost (C0), soil mixture with compost containing 4% of co-composted biochar (C4), and soil mixture with compost and 4% of biochar added after composting (FC4). Percentages of biochar are by weight of dry matter. All treatments were filled into sample rings and pre-prepared (fully saturated and later kept at given suction pressure head) for consequent testing according to the preparation procedure introduced in the Supplement.

Thereafter the soil plus amendments were tested: (1) in the Sandbox up to the suction pressure of 1 m (up to pF2, which was used as estimate of field capacity) using the standard method Eijkelkamp 2019; (2) in the 5 Bar Ceramic Plate Extractor 1600 (Soilmoisture, United States of America) in suction pressure from 10 to 50 m (pF3 – pF3.7) were measured using the standard method (Soilmoisture 2008).; and (3) in the 15 Bar Ceramic Plate Extractor 1500 (Soilmoisture, United States of America) in suction pressure by 150 m (pF4.18, wilting point) was measured using the standard method (Soilmoisture 2015). Available soil water content for plants (AWC) was calculated as the difference between volumetric water content at pF2 and pF4.18. Easily (readily) available water content for plants (EAWC) was calculated as the difference between pF2 and pF3.7.

Table 4.1: Pristine characteristics of the soil and all used amendments

	Litavka	**Biochar**	**HB**	**C0**	**C4**	**C10**
Clay (%)	8.7[a]	/	/	/	/	/
Silt (%)	34.8[a]	/	/	/	/	/
Sand (%)	56.5[a]	/	/	/	/	/
pH (-)	5.90	11.4	6.66	7.59	7.33	7.67
C ($g \cdot Kg^{-1}$)	2.87	868	197	377	451	503
N ($g \cdot Kg^{-1}$)	0.2	5.8	11.7	17.5	19	17
K ($g \cdot Kg^{-1}$)	6.58	3.15	9.84	14.5	11.8	10.6
Mg ($g \cdot Kg^{-1}$)	0.68	2.82	3.62	2.91	2.43	2.27
Fe ($g \cdot Kg^{-1}$)	37.4	/	8.47	2.47	1.28	2.01
Mn ($g \cdot Kg^{-1}$)	4.28	/	0.26	0.32	0.22	0.35
Cu ($mg \cdot Kg^{-1}$)	71.9	6.86	27.3	14.1	11.8	12.3
Zn ($mg \cdot Kg^{-1}$)	4002	651	167	133	110	249
Pb ($mg \cdot Kg^{-1}$)	3539	12.9	25.87	9.12	13.31	22.6
Cd ($mg \cdot Kg^{-1}$)	39	0.13	0.63	0.25	0.12	0.21

[a]Data obtained from Jacka et al. 2018

Growing pot experiment

Seeds (up to 100 pieces) of yard grass (*Lollium perenne* L.) and arugula (*Eruca sativa* Mill.) were sown directly into 1L pots containing 1440g of soil and composts in a ratio 2:1 (w/w). Seven different treatments were prepared, each in four replicates: Litavka soil without compost (Lit), retail compost (HB) (Agro Zahradnicky Kompost, Agro CS, CZE), prepared compost and compochar (C0, C4, C10), and a composition of finished C0 mixed with 4 (FC4) and 10% of biochar (FC10). Each pot was watered by distilled water to 60% WHC for germination; this was maintained over the whole period of the experiment (checked and adjusted, if necessary, 2 – 3 times per week). The growing experiment was conducted inside a greenhouse for 30 days, with an average temperature of 20° and a 12-hours period of light, ensured by high-pressure sodium lamps. The plants were grown for a period of 35 days.

Characterization of metal(oid)s in amended soil and pore water

After 35 days soil sub-samples were taken from each pot, air dried and sieve at 2 mm. Pseudo total concentration of elements were extracted by microwave-assisted aqua-regia extraction, according to EPA 3051A: 0.250g of sample was digested under microwave oven (Multiwave PRO microwave reaction system SOLV, Anton Paar, Germany), in 9mL HNO_3 + 3mL HCl + H_2O_2 in order to enhance the organic matter decomposition. After cooling down, the samples were diluted to 50mL with demineralised water and filtered using 0.45 μm pore nitro-cellulose syringe filters.

For the directly available metal pool, samples of 4 g of soil were treated with 40 mL of 0.01 M $CaCl_2$ (Quevauviller 1998), shaken for three hours at 300rpm, centrifuged for 10 minutes at 3000rpm and filtered through a 0.45 μm nylon filter (VWR, Germany). The pH was measured after centrifuge using an inoLab®pH metre (pH 7310, WTW, Germany).

Pore water was also collected after 35 days from each pot using 10cm long rhizons (Eijkelkamp, NED) extracted by removable plastic syringes and stored at 10°C until analysis. Electric conductivity (EC) and pH in the extractant were measured using a multi-meter and pH meter (Multi 3420 and pH 7310 respectively, WTW, Germany). Major inorganic anions were determined using Dionex ICS-5000 ion chromatography system (Dionex, USA) and Total organic/inorganic C was determined using the carbon analyser TOC-L CPH for liquids, both after diluting 1 mL of the extractant into 49 mL of deionized water.

Total concentrations of elements in all the analysed solutions were obtained by inductively-coupled plasma optical emission spectrometry (ICP-OES) (720ES, Varian Inc., CA, USA).

Plant analysis

At harvest, leaves of the plants were cut at the base and 3 cuts were made on 3 different leaves at different heights to obtain representative 3g samples,

which were then frozen at -80°C for further analysis. The precise extraction procedures and analyses are presented in the Supplement (S1).

For the extraction of proteins, frozen plant material was homogenized with a mortar and pestle in cold 0.1M Tris/HCl buffer (Roth) (supplemented with 5mM EDTA (Ing. Petr Švec – PENTA Ltd., Czech Republic), 1% PVP K30 (Carl Roth GmbH + Co. KG), 5mM DTE (Roche), and 1% Nonidet P40 (Roche)) at pH 7.8 (10mL of extraction buffer per 1g FW). The homogenate was centrifuged at 20,000rpm at 4°C for 30min. The sample was filtered with Miracloth Filter (Calbiochem), and the supernatant volume was measured. For the first precipitation, 40% ammonium sulfate (LachNer, Czech Republic) was added, and the solution was agitated for 30min. The suspension was centrifuged at 20,000rpm at 4°C for 30min using Ultracentrifuge L7-55 (Beckman, USA). The sample was filtered through Miracloth Filter, and the supernatant volume was again measured. For the second precipitation, 80% ammonium sulfate was added, and the solution was agitated for 30min. The suspension was centrifuged at 20,000rpm at 4°C for 30min. The pellet was resuspended in 2.5mL of 25mM Tris/HCl buffer (pH 7.8), placed in PD 10 columns, eluted with 3.5mL of 25mM Tris/HCl buffer (pH 7.8) and stored at -80°C.

The protein samples were used for the measurement of enzymes. All enzyme assays were performed using a TECAN Infinite M200 microplate reader (Tecan Group Ltd., Switzerland). Peroxidase (POX) activity was detected based on the colour reaction with ABTS or guaiacol substrate (modified from Drotar et al. 1985). Glutathione-S-transferase (GST) activity was detected based on the reaction with five different substrates (DCNB, fluorodifen, CDNB, pNBC or pNBoC) based on a methodology modified from Habig et al. (1974). Catalase (CAT) activity was detected based on the disappearance of hydrogen peroxide (modified from Verma and R. Dubey 2003. Ascorbate peroxidase (APX) activity was detected by a decrease in the absorbance of ascorbate (Vanacker et al. 1998). Superoxid dismutase (SOD) activity was detected by using xanthine-oxidase system based on the methodology of El-Shabrawi et al. (2010). Details of the modifications to the methodologies can be found in supplements (S4).

The enzyme activities were expressed in ncat mg-1 protein. The protein concentration was determined according to the Bradford assay (Bradford 1976)

using bovine serum albumin as a standard (BIO-RAD). All reported values for enzyme activities were the means of the determinations of eight separate extracts for each sampling point. Each extract was prepared from both parts (root, leaf) of the plant.

Leaf frozen samples (10–20mg FW) were extracted with 10mL methanol in the dark at 4°C. The absorbance of methanolic extract was evaluated using 470, 652.4, and 665.2nm and it was monitored in a Tecan Infinite 200 PRO microplate reader (Tecan, Switzer-land) and the chlorophyll and carotenoid contents were calculated using a formula according to Lichtenhaler (1987). All samples were analysed in triplicate.

The remaining plant material was dried at 80°C and ground. The amount of 0.2g of dry material was digested in 2:8 mL H_2O_2 : HNO_3 at 200°C in microwave oven, then diluted to 25mL with deionized water, filtered through a 0.45 μm nylon filter and analysed by ICP-OES for determination of elements. For each set of samples, the reference plant material NCS ZC73018 (Bowen's Kale; IUPAC 1979) was also analysed.

Statistics

Analysis of variance (ANOVA) was performed to find differences between treatments and Tukey Honest Significant Difference method was used with confidence level of 0.95, the results are presented for $p < 0.05$. The statistical analyses were performed using the software R 3.5 (The R Foundation for Statistical Computing 2018, under the GNU General Public License).

4.4 Results and discussion

All material characteristics and (co-)composting process on amendment characteristics

The biochar used in this study has very high reactive surfaces and porosity as well as carbon purity (e.g. low amount of volatile matter), which deems it

suitable for co-composting (due to potentially high capacity to retain water from the fresh biomass during the process). The retail compost (HB) has slightly acidic pH in comparison to all home-made composts with pH = 7.33 – 7.67.

Total nitrogen content in HB was lower in comparison to all home-made composts. Additionally, C content is significantly lower in HB and the presence of biochar in home-made compost increased C amount even further, due to its own high C content (86.6%).

As shown in **Figure SD1** the presence of biochar enhanced the composting process. After one month of composting, the grass was still visible in C0 and C4, but hardly present in C10. The C0 variant was clearly the least decomposed and the most pungent in aroma; C4 was less pungent and C10 had no unpleasant smell as the compost materials had broken down the furthest. Co-composting biochar indeed hastened the composting process, confirming the findings of (Y. W. Chen et al. 2016; Fischer and Glaser 2012; Lohri et al. 2016). Both C4 (4% BC) and C10 (10% BC) reached a stable state 3 and 2 weeks earlier than C0 (0% BC), respectively. This could be explained by an increased water and nutrients holding capacity of the biochar presented which logically created better conditions for co- composting process.

Moisture content during co-composting process was kept be- tween 65 and 75% during the first 80 days (**Figure SD2**). After this period of time the moisture content started decreasing in accordance with the amount of BC in the compost: C0 decreased to 25%, C4 to 52% and C10 remained stable at 67% moisture content at the end of the monitoring period. During the first two weeks of co-composting pH varied considerably between all the treatments and samples, but after the third week it reached neutral values between 6.9 and 7.6 (**Figure SD2**) in the three prepared composts and remained stable until the end of the sampling. The compost with highest dosage of bio- char (C10) showed slightly higher (alkaline) pH in comparison to C0 and C4, respectively.

Based on the observations of the experiment, it can be concluded that the ideal percentage of BC needed to aid decomposition here lies somewhere between 4 – 10 %. However, this is also most likely dependent on the initial composition of the feedstock materials for the compost. Co-composting chicken

manure, for instance, requires a larger amount of biochar in order to see results; Agegnehu et al. (2017) found a 20 % addition of BC decreased nitrogen loss by 52 %. Decreasing the time necessary for a stabile finished compost has obvious advantages practically and economically, as long as there is no sacrifice in the quality of the material.

Implications for soil water retention

The quality of the resulting amendments produced by the co-composting process, in terms of suitability as a soil amendment, can be indicated by soil water retention. **Figure 4.2**a shows distinct differences in easily available water content for plants (EAWC) ac- cording to soil amendment; the lowest EAWC was observed for control soil. Pure compost addition (C0) significantly increased mean EAWC value from 21% to 23%. Addition of biochar to compost further significantly increased EAWC value from 23% to approx. 26%. This increase was noted independently of the timing of biochar addition (before (C4) or after composting process (FC4)). The increase of EAWC in soil after the compost application is the result of the increased amount of organic matter. When the biochar was added into the compost the amount of easy-available water increased significantly due, in all likelihood, to high biochar porosity and significant swelling effect explained as a loading of H_2O molecules on the biochar surface through the hydrogen bonds (Jacka et al. 2018).

Without amendment control soil (Lit.) also exhibited significantly lower available water content for plants (AWC) than following amendment (see **Figure 4.2**b). Pure compost addition (C0) distinctly increased mean AWC value from 23% for control to 29%. Addition of biochar to compost (independently on timing of biochar addition) only slightly increased mean value of AWC from 29% to approx. 30% and this difference in mean AWC value was not statistically significant. This suggests that the amount of strongly bonded water (difference between pF 3.7 and 4.18) increased in the compost treatment from 2% to 6%. Conversely, both biochar + compost treatments increased the amount only by 2%. This means that biochar presence in compost enhanced mainly the amount of water ranging between field capacity (pF = 2.0) and the drought stress zone (pF = 3.7; Mikolajczak et al. 2016. Thus the application of compost and espe-

cially, compost-biochar mixture could be beneficial for plants during period(s) of agricultural drought.

Trace elements and nutrients according to amendments and plants

As expected, the different treatments affected the pseudo-total concentrations of elements in soils, however plant species also influenced differently the total pool of elements, with the exception of Mg, Cu and Cd (**Figure SD3**). The concentration of K was increased by the co-composting of BC (both, C4 and C10), while significantly decreased in the soils treated with retail compost under *E. sativa* growth; for all the other treatments, concentrations of K were higher in soils with *E. sativa* growth. Unlike the pseudo-total concentrations, the $CaCl_2$-extractable fraction shows clear influences by the treatments, with a general increase of the nutrients and reduction of contaminants (**Figure 4.3**). Rhizosphere soil of *E. sativa* showed higher concentrations of extractable K, Mg, Fe, Mn and organic C compared with that of *L. perenne*. In particular, $CaCl_2$-extractable K was the highest for FC10 treatment, mainly for the rhizosphere soil of *E. sativa*, although the same treatment shows statistical differences with

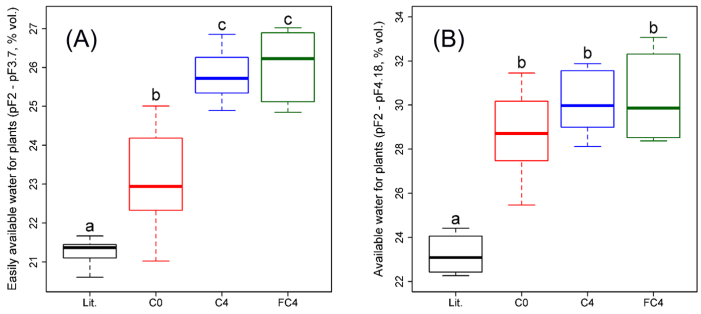

Figure 4.2: Boxplot (sample minimum, first quartile, median, third quartile, maximum, n=7) of easily available soil water for plants (A) and available soil water for plants (B): **Lit** = Litavka soil (Control); **C0** and **C4** = co-compost with 0%, 4% and 10% BC, respectively; **FC4** = compost with addition of 4% BC at the end of composting process. Different letters represent statistic differences by Tukey HSD at $p < 0.05$.

C4 and C10 under *L. perenne* growth. Magnesium was the highest under retail compost, and the lowest under C0 treatment. Extractable Fe under *L. perenne* growth did not show differences between different compost treatments, but for *E. sativa* there is a clear increased under C0 treatment. Extractable Mn was significantly decreased due to the effect of home-made compost, while there are no statistical differences between Litavka variant and retail compost (higher for both). Dissolved organic C was the highest for retail compost under *E. sativa* growth (together with FC1 under same specie), and the lowest under *L. perenne* (together with Lit., same specie) also under HB treatment. The increase in the $CaCl_2$-extractable concentrations for all amended soils reflects the contribution of the composts to the pool of nutrients in the contaminated soil. This resulted in an increase of the uptake of nutrients by *L. perenne*, which reflects its ability to adapt well to contamination stress; despite of the reduction in the mobile fractions of the contaminants, the pseudo-totals remain high for all the treatments, at the same time, nutrients are added to the soil through the compost, resulting in a higher uptake by the plant only when its physiological mechanisms are adapted to overcome stress. Radin et al. (2018) found no differences in nutrients uptake by oil palm under different mixtures of biochar and compost.

Potassium concentration in pore water was the lowest in control soil without amendment, with no statistical differences between the other treatments. Concentration of Mg in pore water was highest under retail compost whereas Mn in pore water was significantly reduced with home-made compost treatments. Total C in pore water was significantly lower under control whilst chloride was the highest under retail compost. Nitrate was considerably greater under retail compost treatment, followed by the control, while significantly reduced under all other treatments. Concentration of sulfate was the only variable in the pore water that was affected by both plant species and by the interaction of treatment with plant species: the highest concentrations were found under retail compost, particularly under *L. perenne*, while *E. sativa* caused a reduction in concentration in pore water. In summary, the HB compost may simply have had more nutrients in available forms easily leached into pore water, because it was aged further than the home-made compost it was compared against here. Although it is not stated on the packaging of HB used in this study, it

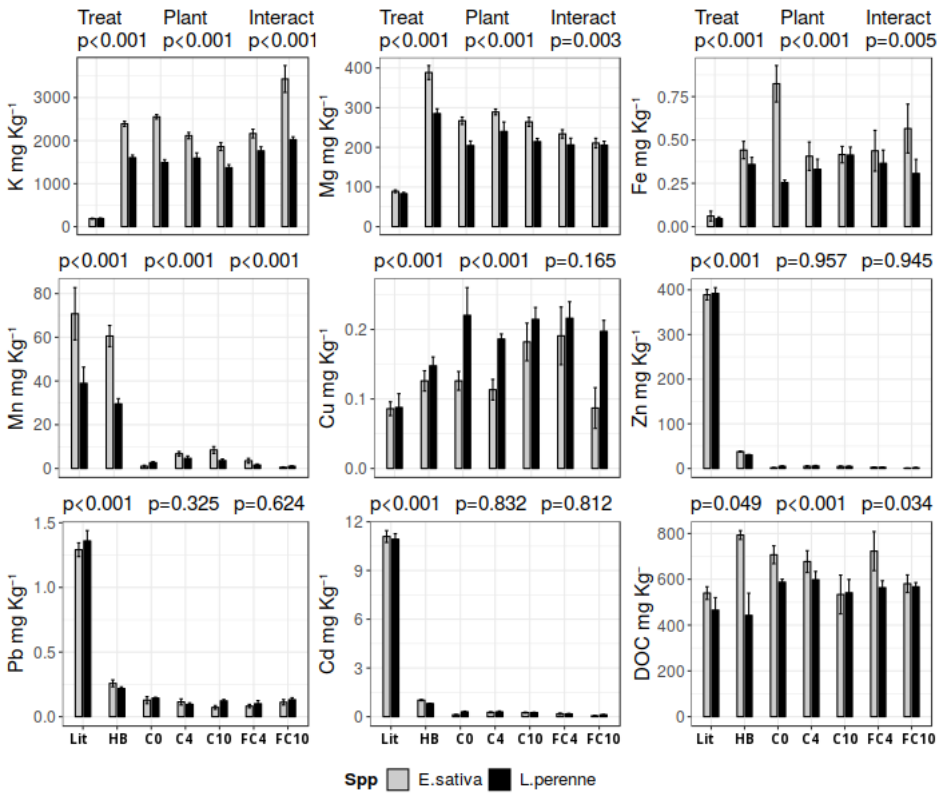

Figure 4.3: Mean and standard error values of the concentrations $CaCl_2$-extractable elements in soil. **Lit** = Litavka soil (Control); **HB** = retail compost; **C, C4** and **C10** = co-compost with 0%, 4% and 10% BC, respectively; **FC4** and **FC10** = compost with addition of 4 and 10% BC at the end of composting process. The p-values are provided for effect of treatment (Treat), plant species (Plant) and the interaction of both (Interact).

is also possible that soluble nutrients are artificially added to the compost to improve growing performance, as could be the case for other retail purchased soil amendments.

The uptake of nutrients by plants (**Table 4.3**) was lowest for plants grown in control soil (Lit. soil), as expected, but different treat- ments showed different interactions with nutrient uptake. Potas- sium in *L. perenne* has lowest concentration for plants in control soil, and there are no meaningful differences between other treatments, while in *E. sativa*, the opposite behaviour appeared: plants under Lit. soil showed the highest concentrations, while treatments C0 and C4 show the lowest. Calcium showed no statistical differences for *E. sativa*,

but for *L. perenne* the highest uptake was found under retail compost and Lit. soil, however only the addition of 10% of BC (both, co-composted and final) considerably reduced the uptake of Ca by the plant. Magnesium showed no meaningful differences for *L. perenne*, but for *E. sativa* there was considerably higher uptake in plants under control soil than plants growing under the addition of compost. Sulphur in *E. sativa* showed the same behaviour as Mg: significantly higher for Lit. soil, and no other differences for the composts. *L. perenne* also showed the highest uptake of S under Litavka treatment, but more interactions are shown in **Table 4.3**: the lowest concentration was found under the 10% BC co-composted treatment (C10); retail compost reflects the highest uptake of S, and there is no statistical difference between this and the compost without BC (C0) nor the composts where BC was added at the end (FC4, FC10). Uptake of Mn was also higher in Lit. soil, while no differences were found between treatments for *E. sativa*, for *L. perenne* there is a clear decrease in the plants under C10 treatment. Copper in *L. perenne* showed no differences, while in *E. sativa* was higher only in Lit. soil.

Metals concentration and availability in soil

Pseudo-total concentrations of Cu, Cd, Zn and Pb in soils were generally higher in the soils under *L. perenne*, whilst the lowest concentrations were that of the retail compost under *E. sativa* growth (**Figure SD3**). Application of home-made compost and/or biochar to the contaminated soil had also significant effects on the pseudo-total concentration of metals, as can be seen in **Figure SD3**, where despite of statistical differences of the treatment, the variation between particular amendments for some of the metals is minor. Ye et al. (2019) logically highlighted that the pseudo-total concentrations of amended soils mirror dilution effects of compost on the soil it is amended to. This is likely to have been the case in the present study, due to the 2:1 ratio, by mass, of soil to composts added. Additionally, Forjan et al. (2018) found that the pseudo-total concentration of Cu, Pb, Ni and Zn increased in contaminated soils treated with compost and biochar, due to the high concentration of this elements in the compost, however the percentage of $CaCl_2$-extractable concentrations in relation to the pseudo-total concentration decreased with the

application of the amendment due to the ability of the compost and biochar to fix these metals. Rodriguez-Vila et al. (2016) found a decrease in the $CaCl_2$-extractable Al, Co, Cu, Fe and Ni after application of compost and biochar to contaminated soils vegetated by *B. juncea*, together with increase of nutrients and pH to neutral values. Such studies highlight the relative insensitivity of assessing the impacts of organic amendments in soils via the use of pseudo-total concentrations only; essentially, they are a "blunt-instrument" in this situation. The $CaCl_2$-extractable fraction of all the contaminants (Zn, Pb and Cd) was significantly reduced under all the treatments in the present study, better reflecting the expected outcome of adding organic amendments to metal rich soil (**Figure 4.3**). Concentrations of Zn and Cd in the pore water showed the same effect (**Figure 4.4**). Concentration of contaminants in both extractions were not affected by plant species, with the only exception of Pb in pore water which was poorly influenced by the different treatments (**Figure 4.4**).

Concentrations of Cd, Pb and Zn in pore water were reduced in all amended soils compared to the control and, according to the literature, this seems to be a general response when organic amendments with low concentrations of contaminants are applied to contaminated soils (Forjan et al. 2018; Novak et al. 2019; Oustriere et al. 2017; Rodriguez-Vila et al. 2016; Trakal et al. 2017; Z. Yang et al. 2018). Oustriere et al. (2017) found a decrease in the concentration of Cd, Pb and Zn in pore water of soils treated with compost and biochar under different combinations, and concluded that the interactions between biochar-amended soils and metals are complex and it could be due to a combination of the following factors: (1) increase in the pH activates electrostatic interactions with negatively charged surfaces on soils, (2) specific metal-ligand complexation involving surface functional groups of biochars and (3) sorptive interactions between cations and aromatic p electronic systems from C==C bounds of biochars. Novak et al. (2019) found a decrease of H_2O- and $CaCl_2$-extractable Cd and Zn with addition of compost and biochar at different rates, partly due to an increase of the pH from acidic to more neutral values, but also due to sorption by mineral ash constituents present in the poultry litter biochar used, however, similar to the present study, no differences were found between different treatments (compost and biochar ratios), but only between control and treated soils. This generalised trend has often been seen in studies where

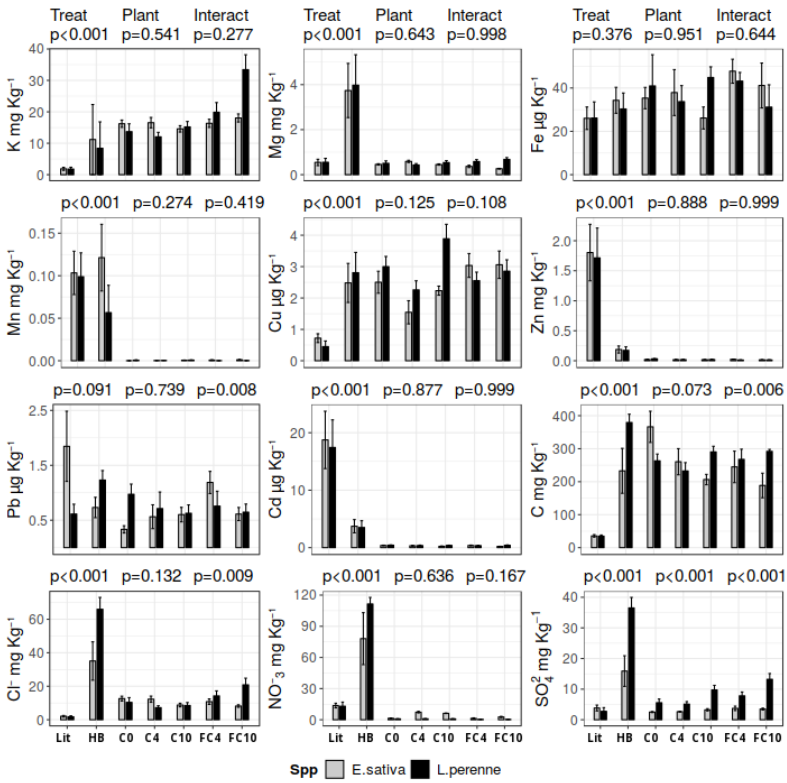

Figure 4.4: Concentration of elements and main anions in the pore water (Mean and Standard Error). **Lit** = Litavka soil (Control); **HB** = retail compost; **C, C4** and **C10** = co-compost with 0%, 4% and 10% BC, respectively; **FC4** and **FC10** = compost with addition of 4 and 10% BC at the end of composting process. The p-values are provided for effect of treatment (Treat), plant species (Plant) and the interaction of both (Interact).

biochars have been amended to metal-rich soils, as summarised by Beesley et al. (2015).

Uptake of metals by plants

Metals (Cd, Pb and Zn) uptake by plants showed similar behaviour as that found for most of the nutrients; plants growing in Lit. soil had greater concentrations of these elements than soil with compost addition (regardless of biochar presence). This is true for Zn in both plant species, and Cd and Pb in *L. perenne*. In *E. sativa*, Pb has no statistical differences regardless of the

treatment, while Cd was also higher for plants in Lit. soil, and considerably lower for the soils with 4% of BC (both C4 and FC4).

All treatments decreased the availability of the target contaminants (Cd, Pb and Zn) in the soil, resulting in a decreased of the plant uptake (with the exception of Pb for *E. sativa*), with the home-made compost being more efficient; however no difference between addition or absence of biochar was found, nor between the different combinations. Cadmium and Pb are not essential elements for plant growth, but they are taken up by plants into their tissues nonetheless, resulting in adverse effects in the physiological function of plants when the concentrations are high (phytotoxic). Zinc is considered as a micronutrient important for cellular enzymatic functions, protein production and membrane integrity (Novak et al. 2019), but is phytotoxic at excessive concentrations in plants. Forjan et al. (2018) found that the effect of applying compost with biochar reduced available concentrations of Ni over time, due to an increase in the pH and organic matter content, aided by the uptake of the element by the roots of the plants. Longer term experiments are necessary to understand and possibly predict the effect of biochar for stabilization of metallic contaminants. Novak et al. (2019) found that, after different combinations of compost and biochar applied to contaminated soils, the lowest Cd concentrations in plant tissue were found in plants growing in soils amended by a mixture of compost and biochar. An additional explanatory factor behind the general reduction

Figure 4.5: *L. perenne* (A) and *E. sativa* (B) 5 weeks after germination. Left to right: Lit, HB, C0, C4, C10, FC4 and FC10.

in plant metal uptake regardless of amendment type, was the dilution factor of soils by amendments already discussed. At the pot scale, as used here, the concentration of rooting in the confines of the pot will likely exacerbate the effects of amendment addition, compared to field conditions where plant roots could spread. This is especially the case here as the amendments were added at relatively high volumes to the pots, when considering their likely much lower bulk densities compared to soil and the mass added to the soil. The importance of verification of the results seen here in field conditions is worthy of note, especially considering the relative lack of field trials verifying the impacts of biochars and their derivatives on metal polluted soils (Sizmur et al. 2015).

Plant growth and stress

Visual differences were observed between treatments during the growing period, as shown in **Figure 4.5**. Five weeks after germination *E. sativa* did not germinate in the control-contaminated soil (Lit), while application of HB and FC10 treatments produced small specimens where necrosis was observed; application of home-made compost with and without biochar co-composted produced strong plants growth. For *L. perenne* the response was different, due to its relative insensibility to high soil metal contents, plants under control established successfully but with poor biomass production. Plants under HB developed better and plants with the application of home-made compost produced the greatest biomass. Only few days before harvest, signs of chlorosis were observed in the plants growing under control (Lit). The poor development of plants growing in the control soil was likely due to phytotoxicity of metals; this was particularly acute with *E. sativa*, where even the addition of retail compost did not help alleviate toxicity stress. Exposure to metals creates reactions with the oxygen in the metabolic system of plants, producing more reactive forms of oxygen which induce alterations in protein expression and carbohydrate metabolism; to cope with such damage plant cells produce antioxidant enzymes.

Application of compost increased production of total chlorophyll and carotenoids in *L. perenne*, with no statistical difference between the compost treatments (**Table 4.2**). Stress-related enzymes produced by plants did not show statis-

Table 4.2: Mean values ± standard deviation of enzymes activity (POX, GST, CAT, APOX and SOD) given in $ncat{\cdot}mg^{-1}$, total chlorophyll (Chlor), and carotenoids (Car), both in in $mg \cdot g^{-1}$ DW and total protein (Prot) in plant ($mg \cdot ml^{-1}$). **Lit** = Litavka soil (Control); **HB** = retail compost; **C0**, **C4** and **C10** = co-compost with 0%, 4% and 10% BC, respectively; **FC4** and **FC10** = compost with addition of 4 and 10 % BC at the end of composting process. The **p**-value obtained after one-way ANOVA is provided in the last column. Different letters represent statistic differences by Tukey HSD at $p < 0.05$.

	L	**HB**	**C0**	**C2**	**C5**	**FC2**	**FC5**	**P**
				L. perenne				
POX	NA	0.29±0.11	0.29±0.04	0.46±0.26	0.29±0.07	0.57±0.52	0.4±0.12	0.511
GST	NA	0.93±0.78	0.58±0.43	0.5±0.19	1.57±2.62	0.46±0.24	0.6±0.4	0.735
CAT	NA	619±279	472±225	891±424	645±257	873±485	1018±410	0.305
APOX	NA	3.05±0.69	3.02±0.79	4.86±2.43	3.29±1.84	4.94±2.77	2.54±0.21	0.275
SOD	NA	48.15±11.35	58.9±11.11	45.3±22.42	39.17±16.62	58.15±15.5	56.67±12.39	0.395
Chlor	^{a}640±140	^{b}1264±286	^{b}1212±115	^{b}1541±129	^{b}1463±102	^{b}1279±66	^{b}1383±170	<0.005
Kar	^{a}159±38.3	^{b}308±73.3	^{b}296±34.8	^{b}389±30.8	^{b}367±25.1	^{b}324±16.4	^{b}339±55.8	<0.005
Prot	NA	1.71±0.39	1.22±0.17	1.66±0.62	1.78±0.47	1.3±0.27	1.28±0.21	0.190
				D. tenuiflora				
POX	NA	NA	0.2±0.14	0.07±0.02	0.05±0.02	0.06±0.03	NA	0.039
GST	NA	NA	1.05±0.66	0.88±0.54	1.61±1.42	1.68±2	NA	0.769
CAT	NA	NA	502±155	461±438	791±1089	361±305	NA	0.779
APOX	NA	NA	3.22±0.91	4.64±1.61	3.54±0.55	2.99±1.17	NA	0.223
SOD	NA	NA	32.98±4.77	43.07±16.11	34.7±18.1	37.55±14.43	NA	0.767
Chlor	NA	810±312	1116±132	1487±72	1585±728	1311±110	NA	0.059
Kar	NA	197±91.6	293±34.1	379±19.9	394±186.3	332±20.5	NA	0.066
Prot	NA	NA	1.98±0.07	1.77±0.6	1.64±0.19	2.02±0.53	NA	0.552

tical differences caused by treatments, with the only exception of peroxidase produced by *E. sativa*, where the co-composting with 10% BC (C10) shows the lowest production, while the compost without BC (C0) shows the highest. However, not enough plant material was produced in the control soil to obtain enzymes production, which could suggest differences between control and compost addition, like chlorophyll and carotenoid production. In general, the results showed that the addition of compost improved the growth and development of plant considerably, regardless of the type of compost or BC addition.

Sensitivity in plants to metal stress generally depends upon metal concentration, plant species, exposure time and age (Sidhu et al. 2017). The only difference found under the statistical analysis was POX activity in *E. sativa*, which was higher in the compost without biochar. It has been proved that POX activity increases with the exposure to high concentration of metals in the growing sub- strate. Drazkiewicz et al. (2004) found significant increase of POX in exposure to Cu, and Sidhu et al. (2017) with exposure to Pb. The difference in the influence of POX activity between *E. sativa* and *L. perenne*

observed in our study could be due to photosynthetic oxygen evolution in C_3 plants, which produce large amounts of reactive forms of oxygen in the form of H_2O_2 (Drazkiewicz et al. 2004).

Jia et al. (2019) found that different concentrations of biochar applied in hydroponics resulted in differences of enzymatic activity for *Amarantus mangostanus*, showing a general increase of stress with medium concentrations of the BC (2.6e13.3 $g \cdot L^{-1}$) and absence of stress (compared to the control) with low (1.3 $g \cdot L^{-1}$) and high (26.6 $g \cdot L^{-1}$) concentrations. It seems clear that different source material for biochar variously affects the physiological response of the plants: the biochar used for the present study appears to be suitable as an amendment for metal-contaminated soils, such as the one tested here.

4.5 Conclusion

The application of biochar hastened the maturing of compost made of woody and green material. As a consequence of improved moisture and nutrients status of the resulting compost-char hybrid the pool of $CaCl_2$-extractable nutrients was enhanced upon addition to a soil contaminated by Pb and Zn. This also corresponded with a decrease in metals extractable from amended soil (pore water and $CaCl_2$ fraction). The plant species grown in amended soil reacted differently to the more favourable conditions induced by the amendments; *L. perenne* was more resistant to contamination stress, therefore no physiological differences were observed regardless of the amendment type and dose applied to the soil. On the other hand, *E. sativa* was more sensitive to metal contamination in the tested soil, though oxidative stress (reflected in POX activity) was reduced furthest with the addition of biochar. The addition of biochar co-composted with, or added at the end of composting process, had no effect on the development of the plant (enzymes activity, chlorophyll production, uptake of elements) during the experiment but did positively impact on some characteristics of the soil. Our next studies will focus on 1) testing different application rates of the compost-char to a variety of contaminated soils and 2) conducting small field trials to validate the results of greenhouse and laboratory studies.

Table 4.3: Mean values and standard deviation of the concentrations of elements in plant tissue, given in $^{\alpha}g \cdot Kg^{-1}$ or $^{\epsilon}mg \cdot Kg^{-1}$. **Lit** = Litavka soil (Control); **HB** = retail compost; **C0, C4** and **C10** = co-compost with 0, 4 and 10% BC, respectively; **FC4** and **FC10** = compost with addition of 4 and 10% BC at the end of composting process. The **p**-value obtained after one-way ANOVA is provided in the last column. Different letters represent statistic differences by Tukey HSD at $p < 0.05$.

	L	**HB**	**C0**	**C4**	**C10**	**FC4**	**FC10**	**p**
				L. perenne				
K^{α}	^{a}18.2±3.1	^{b}37.4±3.6	^{b}37.9±13.2	^{b}35.7±0.35	^{b}32.4±4.2	^{b}43.4±2.6	^{b}44.2±1.1	<0.005
Ca^{α}	^{ab}2.8±0.43	^{b}3360±346	^{ac}2143±543	^{ac}2265±81	^{c}1857±325	^{ac}2523±277	^{c}2026±114	<0.005
Mg^{α}	1.34±0.19	1786±183	2092±1539	1447±85	1230±171	1557±144	1514±47	0.519
S^{α}	^{a}2.8±0.14	^{b}1941±344	^{bcd}1723±296	^{cd}1420±138	^{c}1187±171	^{bd}1954±254	^{bd}1931±83	<0.005
Fe^{ϵ}	115±88.8	42±6.2	37±4.9	200±272.8	44±7.7	74±5.9	107±28.4	0.305
Mn^{ϵ}	^{a}185±96.5	^{ab}154±20.9	^{ab}92±97.8	^{ab}59±7.8	^{b}56±10.6	^{ab}60±8.4	^{ab}66±8.3	0.013
Cu^{ϵ}	6.25±3.26	7.27±0.82	8.64±7.87	5.92±0.41	5.48±0.82	7.14±0.45	7.57±0.26	0.865
Zn^{ϵ}	^{a}1668±155	^{b}212±23	^{b}159±36	^{b}133±11	^{b}114±13	^{b}155±16	^{b}133±4	<0.005
Pb^{ϵ}	^{a}17.5±10.7	^{b}4.9±0.3	^{b}2.7±0.6	^{b}3.5±0.2	^{b}3.3±0.8	^{b}3.7±0.5	^{b}7.6±3.9	0.001
Cd^{ϵ}	^{a}16.7±3.5	^{b}2.3±0.1	^{b}4.8±6.3	^{b}1.3±0.3	^{b}0.8±0.2	^{b}1.4±0.3	^{b}0.9±0.1	<0.005
				D. tenuiflora				
K^{α}	^{a}62.7±7.7	NA±NA	^{bc}42.4±2.1	^{bc}43.2±8.3	^{ab}46.8±8.5	^{abc}44.2±2.1	54.6±NA	0.037
Ca^{α}	19.7±1.3	NA±NA	12.9±3.1	12.8±3.8	12.8±2.6	10.9±1.4	8.9±NA	0.059
Mg^{α}	^{a}4.7±0.67	NA±NA	^{b}2.7±0.5	^{b}2.7±0.64	^{b}2.8±0.45	^{b}2.4±0.29	2.3±NA	0.006
S^{α}	^{a}29.9±2.3	NA±NA	^{b}11.6±2.1	^{b}10.7±2.6	^{b}11.1±1.9	^{b}12.1±1.6	13.2±NA	<0.005
Fe^{ϵ}	59.53±4.21	NA±NA	54.67±9.2	51.02±7.31	51.07±16.45	54.82±1.85	49.73±NA	0.916
Mn^{ϵ}	^{a}304.45±14.05	NA±NA	^{b}21.8±3.75	^{b}25.79±8.97	^{b}28.28±10.21	^{b}25.95±5.57	24.53±NA	<0.005
Cu^{ϵ}	^{a}24.7±4.71	NA±NA	^{b}6.14±1.08	^{b}5.39±1.03	^{b}5.99±2.72	^{b}5.85±0.7	3.64±NA	<0.005
Zn^{ϵ}	^{a}713±8	NA±NA	^{b}283±44	^{b}199±48	^{b}237±93	^{b}245±29	243±NA	<0.005
Pb^{ϵ}	5.14±2.3	NA±NA	3.97±1.71	2.81±1.67	1.97±1.23	2.5±0.2	2.62±NA	0.232
Cd^{ϵ}	^{a}13.73±2.01	NA±NA	^{ab}8.56±2.01	^{b}7.06±1.73	^{ab}7.47±2.91	^{b}7.12±0.96	7.47±NA	0.041

4.6 Supplementary Data

Biochar analyses

The following analyses were conducted on the biochar as produced: water content (W) determined by the standard ČSN EN 15414-3; the ash content (A) according to the standard ČSN EN 15403 at the temperature 550°C; the volatile content measured on the basis of ČSN EN 15148 standard at 900°C; the higher heating value (HHV) and the lower heating value (LHV) determined according to the standard ČSN EN 15400. Texture properties (SBET, Smeso, Vmicro, Vtot) were performed by automated volumetric gas adsorption instruments ASAP 2020 and ASAP 2050. (Micromeritics, USA). Next, pH was measured in the solutions prepared according to Czech/European standard ČSN EN 12457-2. Ultimate analysis (C, H, N and S) was performed in Flash EA 1112 device in CHNS/O configuration.

Compost preparation – additional information

This ratio freshly cut grass and leaves (1:5 vol.) was determined to be approximately follow the 25:1 carbon to nitrogen ratio necessary for optimal breakdown of organic materials into compost (USDA 2014). Specifically, the three drums (C0, C4 and C10) were filled with 25kg of leaves and 5kg of grass. Into the drums (C4) and (C10) 4 and 10%wt. of the biochar was mixed with the waste material; no biochar was added to drum C0. All drums were placed in a greenhouse at approximately 20°C and spun to mix and aerate materials 3 times per week for the duration of 16 weeks. Composts, were then sieved individually ($<$ 2mm) and the compost (C0) was at the end of composting amended by 4 and 10% of the biochar in order to prepare the finished compost + 4% BC (FC4) and compost + 10% BC (FC10) treatments.

Moisture content and pH were measured during the composting and co-composting processes from the start until 126 days. Samples were obtained weekly from each treatment (C0, C4 and C10) and 5g was used to measure pH in a H2O suspension at 1:5 (w:v) ratio. The remaining sample was weighed and dry at 60°C until constant weight, moisture content was calculated as the difference between wet and dry weight.

Pre-preparation of samples for water retention analyses

Collected Fluvisol was air-dried, sieved (< 2 mm) and homogenised (Lit.). Organic substrates (compost and compost-biochar mixtures) were air-dried, milled and sieved (< 4 mm). Litavka soil was mixed with organic substrates to create C0, C4, and FC4 treatments. Ratio of soil to each organic substrate was 2:1 (by weight of dry matter). Thereafter, each prepared soil treatment was in seven replicates repacked into the two connected standard steel rings, which were taped together at the edges (according to Stock and Downes 2008). Synthetic permeable cloth membrane was installed at the bottom of the two connected rings using a rubber band. The two connected rings were filled with soil to a height of 6.72 cm (the free space 1.4 cm was designed due to the expected swelling effect; Jacka et al. 2018). All 28 filled connected rings were placed on to a Sandbox (Eijkelkamp, Netherlands) and slowly gradually saturated for one-week. After the saturation, suction pressure of 50 cm was established in sandbox for period of four weeks in order to stabilize soil treatments under the same controlled conditions. Next, only the lower ring (separated with the aid of the fishing line and knife) was weighted and used for SWRC measurement.

The extraction procedure of proteins and enzymes related to plant stress

Protein extraction

Plant tissues were homogenized with a mortar and pestle in cold 0.1 M Tris/HCl buffer (Roth) (supplemented with 5 mM EDTA (Ing. Petr Švec – PENTA Ltd., Czech Republic), 1% PVP K30 (Carl Roth GmbH + Co. KG), 5 mM DTE (Roche), and 1% Nonidet P40 (Roche)) at pH 7.8 (10 mL of extraction buffer per 1 g FW). The homogenate was centrifuged at 20,000 rpm at 4°C for 30 min. The sample was filtered with Miracloth Filter (Calbiochem), and the supernatant volume was measured. For the first precipitation, 40% ammonium sulphate (LachNer, Czech Republic) was added, and the solution was agitated for 30 min. The suspension was centrifuged at 20,000 rpm at 4°C for 30 min using Ultracentrifuge L7-55 (Beckman, USA). The sample was filtered through

Miracloth Filter, and the supernatant volume was again measured. For the second precipitation, 80% ammonium sulphate was added, and the solution was agitated for 30 min. The suspension was centrifuged at 20,000 rpm at 4°C for 30 min. The pellet was resuspended in 2.5 mL of 25 mM Tris/HCl buffer (pH 7.8), placed in PD 10 columns, eluted with 3.5 mL of 25 mM Tris/HCl buffer (pH 7.8) and stored at -80°C.

Enzyme assays

The protein samples were used for the measurement. All enzyme assays were performed using a TECAN Infinite M200 microplate reader (Tecan Group Ltd., Switzerland). Peroxidase (POX) activity was detected based on the colour reaction with ABTS or guaiacol substrate. The reaction mixture used for ABTS assay contained ABTS (Sigma) (100 mM, 0.54 mL), phosphate buffer (50 mM K_2HPO_4 (LachNer), pH = 7.0, 27 mL) and H_2O_2 (Ing. Petr Švec – PENTA Ltd., Czech Republic) (4.5 mM, 0.57 mL). The reaction mixture used for guaiacol assay contained guaiacol (Sigma) (3.4 mM, 0.6 mL), Tris/HCl buffer (Roth) (50 mM, pH = 6.0, 27 mL) and H_2O_2 (Ing. Petr Švec – PENTA Ltd., Czech Republic) (9 mM, 0.6 mL). The supernatant (0.01 mL) was added to 0.19 mL of one of the reaction mixtures in a microplate, and POX activity was measured at 414 nm (ϵ = 35 mM-1 cm-1) or 420 nm (ϵ = 26.6 mM-1 cm-1), respectively (modified from Drotar et al. 1985).

Glutathione-S-transferase (GST) activity was detected based on the reaction with five different substrates (DCNB, fluorodifen, CDNB, pNBC or pNBoC). The reaction mixture used for CDNB, pNBC or pNBoC assay contained CDNB (1-chloro-2,4-dinitrobenzene, Sigma) (30 mM, 1 mL) or pNBC (4-nitrobenzyl chloride, Fluka) (30 mM, 1 mL) or pNBoC (4-nitrobenzoyl chloride, Fluka) (15 mM, 1 mL), Tris/HCl buffer (Roth) (100 mM, pH = 6.4, 23.7 mL) and GSH (Sigma) (0.12 mM, 0.5 mL). The reaction mixture used for DCNB or fluorodifen assay contained DCNB (1,2-dichloro-4-nitrobenzene, Sigma) (30 mM, 1 mL) or fluorodifen (Fluka) (30 mM, 0.25 mL), Tris/HCl buffer (Roth) (100 mM, pH = 6.4, 23.7 mL or 19.7 mL for fluorodifen assay) and GSH (Sigma) (0.12 mM, 0.5 mL).The supernatant (0.04 mL) was added to 0.15 mL of the reaction mixture in a microplate, and GST activity was mea-

sured at 345 nm (ϵ = 8.5 mM-1 cm-1) for DCNB, at 400 nm (ϵ = 17.2 mM-1 cm-1) for fluorodifen, at 340 nm (ϵ = 9.6 mM-1 cm-1) for CDNB, at 310 nm (ϵ = 1.8 mM-1 cm-1) for pNBC or at 310 nm (ϵ = 1.9 mM-1 cm-1) for pNBoC (modified from Habig et al. 1974).

Catalase (CAT) activity was detected based on the disappearance of hydrogen peroxide. The reaction mixture used contained phosphate buffer (100 mM KH_2PO_4 (LachNer, Czech Republic), pH = 7.0, 30 mL) and H_2O_2 (Ing. Petr Švec – PENTA Ltd., Czech Republic) (200 mM, 12 mL). The supernatant (0.01 mL) was added to 0.14 mL of reaction mixture in a microplate, and CAT activity was measured at 240 nm (ϵ = 0.036 mM-1 cm-1) (modified from Verma and R. Dubey 2003).

Ascorbate peroxidase (APX) activity was detected by a decrease in the absorbance of ascorbate. The reaction mixture contained sodium ascorbate (Sigma) (60 mM, 0.01 mL), phosphate buffer (55.56 mM K_2HPO_4 (LachNer, Czech Republic), pH = 7.0, 36 mL) and H_2O_2 (Ing. Petr Švec – PENTA Ltd., Czech Republic) (3%, 0.041 mL). The supernatant (0.02 mL) was added to 0.18 mL of the reaction mixture in a microplate, and APX activity was measured at 290 nm (ϵ = 2.8 mM-1 cm-1) (modified from Vanacker et al. 1998).

Superoxid dismutase (SOD) activity was detected by using xanthine-xanthine oxidase system. The reaction mixture contained phosphate buffer (50 mM K_2HPO_4 (LachNer, Czech Republic), pH = 7.5, 16.8 mL), NTB (nitroblue tetrazolium, Sigma) (2.24 mM, 1.2 mL), xanthine oxidase (Sigma, 0.1 U, 1.2 mL) and catalase (Sigma, 0.1 U, 1.2 mL). The supernatant (0.02 mL) was added to 0.17 mL of the reaction mixture in a microplate and reaction was initiated by xanthine (Sigma, 2.36 mM, 0.01 mL). Change in absorbance was read at 560 nm up to 2 min. A blank reaction was performed using all the components except supernatant (sample). SOD activity was measured using inhibition of reduction of NTB by the superoxide radicals, produced in the system xanthine-xanthnie oxidase. Amount of enzyme required to inhibit NBT reduction by 50% under specified conditions of the assay correspond to 1U SOD activity. (modified from El-Shabrawi et al. 2010).

The enzyme activities were expressed in $ncat \cdot mg^{-1}$ protein. The protein

concentration was determined according to the assay proposed by Bradford (1976) using bovine serum albumin as a standard (BIO-RAD). All reported values for enzyme activities were the means of the determinations of eight separate extracts for each sampling point. Each extract was prepared from both parts (root, leaf) of the plant.

Chlorophyll measurement

Leaf frozen samples (10–20 mg FW) were extracted with 10 mL methanol in the dark at 4°C. The absorbance of methanolic extract was evaluated using 470, 652.4, and 665.2 nm and it was monitored in a Tecan Infinite 200 PRO microplate reader (Tecan, Switzer-land) and the chlorophyll and carotenoid contents were calculated using a formula according to Lichtenhaler (1987). All samples were analysed in triplicate.

Table SD1: Pristine biochar characteristics

ρ	S_{BET}	S_{meso}	V_{micro}	V_{tot}	**W**	**A**	**h**	**V**	**FC**	**HHV**	**LHV**	**H/C**	**O/C**
$kg \cdot m^{-3}$	$m^2 \cdot g^{-1}$	$m^2 \cdot g^{-1}$	$mm^3_{liq} \cdot g^{-1}$	$mm^3_{liq} \cdot g^{-1}$	wt. %	wt. %	wt. %	wt. %	wt. %	$MJ \cdot kg^{-1}$	$MJ \cdot kg^{-1}$	$mol \cdot mol^{-1}$	$mol \cdot mol^{-1}$
142	615	187	202	363	3.75	5.68	94.3	3.8	90.5	30.7	30.5	0.005	0.059

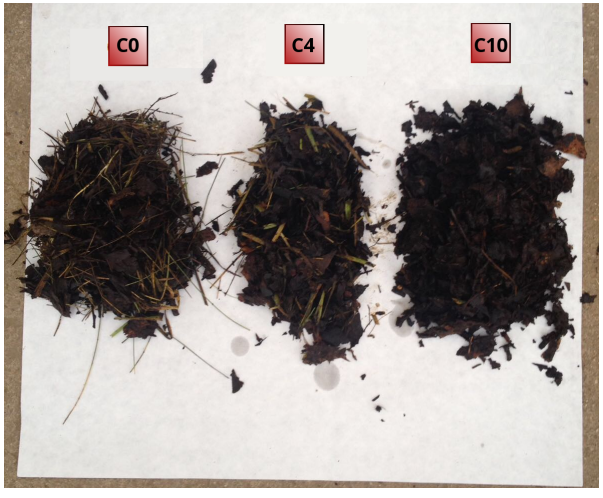

Figure SD1: The biomass after one month of composting with the presence of 0, 4 and 10 % of the biochar (C0, C4 and C10).

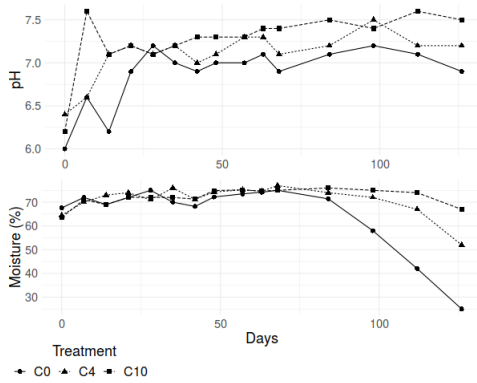

Figure SD2: Variation in time of pH and moisture content during the composting process of compost (C0) and co-composting with 4 and 10 % of biochar (C4) vs. (C10).

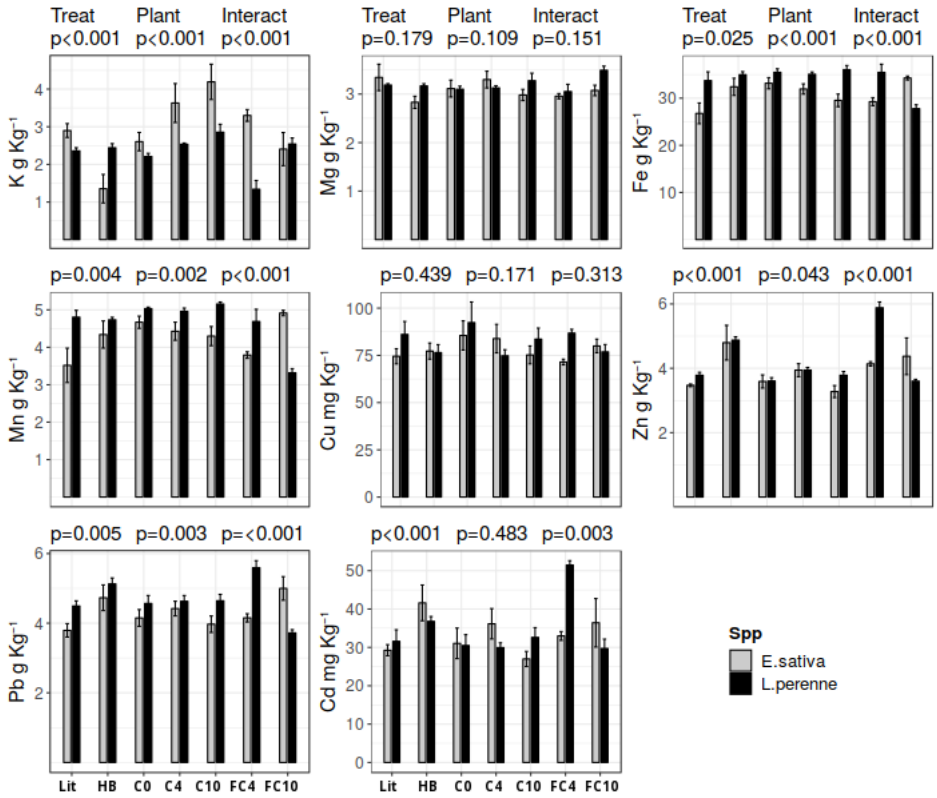

Figure SD3: Mean and standard error values of the pseudo-total concentrations of elements in soil. **Lit** = Litavka soil (Control); **HB** = retail compost; **C, C4** and **C10** = co-compost with 0%, 4% and 10% BC, respectively; **FC4** and **FC10** = compost with addition of 4 and 10% BC at the end of composting process. The p-values are provided for effect of treatment (Treat), plant species (Plant) and the interaction of both (Interact).

CHAPTER

FIVE

NANO ZERO-VALENT IRON MEDIATED METAL(LOID) UPTAKE AND TRANSLOCATION BY ARBUSCULAR MYCORRHIZAL SYMBIOSES

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5.1 Abstract

Nano zero-valent iron (nZVI) has great potential in the remediation of metal(loid)-contaminated soils, but its efficiency in metal(loid) stabilization in the plant-microbe continuum is unclear. This study investigated nZVI-mediated metal(loid) behavior in the arbuscular mycorrhizal (AM) fungal-maize (*Zea mays* L.) plant association. Plants with AM fungal inoculation were grown in metal(loid)-(mainly Zn and Pb) contaminated soils (Litavka River, Czech Republic) amended with/without 0.5% (w/w) nZVI. The results showed that nZVI decreased plant metal(loid) uptake but inhibited AM development and its function in metal(loid) stabilization in the rhizosphere. AM fungal inoculation alleviated the physiological stresses caused by nZVI and restrained nZVI efficiency in reducing plant metal(loid) uptake. Micro-proton-induced X-ray emission (μ-PIXE) analysis revealed the sequestration of Zn (possibly through binding to thiols) by fungal structures in the roots and the precipitation of Pb and Cu in the mycorrhizal root rhizodermis (possibly by Fe compounds originated from nZVI). XRD analyses further indicated that Pb/Fe mineral transformations in the rhizosphere were influenced by AM and nZVI treatments. The study revealed the counteractive effects of AM and nZVI on plant metal(loid) uptake and uncovered details of metal(loid) behavior in the AM fungal-root-nZVI system, calling into question nZVI implementation in mycorrhizospheric systems.

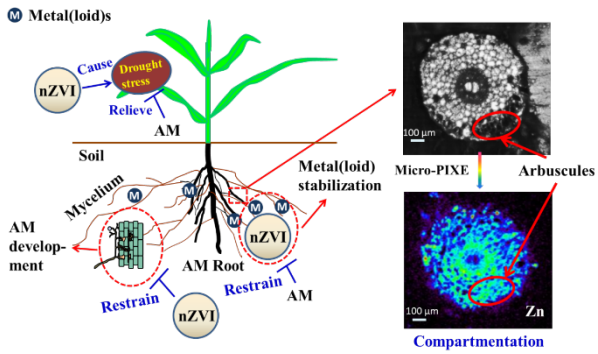

Figure 5.1: GRAPHICAL ABSTRACT

5.2 Introduction

Nano zero-valent iron (nZVI), which has been successfully introduced for groundwater remediation, also has great potential for the remediation of soils contaminated with metal(loid)s (e.g., Zn, Pb, Cr, As) due to its large specific surface area, high surface reactivity and strong capability for reducing oxidized metal(loid)s species (e.g. Cr(VI)) (Komárek et al. 2013; Lai and Lo 2008; Lefevre et al. 2016; Ponder et al. 2000; Sun et al. 2006). In the soil, nZVI can usually decrease metal(loid) bioavailability through adsorption, reduction etc. (Komárek et al. 2013; Michálková et al. 2017), and promote subsequent plant growth and associated microbial development, leading to the restoration and revegetation of metal(loid) contaminated soils. However, nZVI is easily oxidized into Fe (oxy)hydroxides in the environment,(Greenlee et al. 2012; Kumar et al. 2014) and its functions of metal(loid) stabilization can be influenced by plant and soil microbe activities (Kumar et al. 2016), which can transform nZVI through direct interactions with nZVI by their exudates (or metabolites) such as low-molecular-weight organic acids (LMWOAs) (Adeleye et al. 2016; Ma et al. 2013; Vítková et al. 2015). The transformation of nZVI may further influence its adsorption capacity (Komárek et al. 2013; Lefevre et al. 2016). Moreover, despite the fact that metal(loid)s are adsorbed by nZVI, they may be re-dissolved due to plant and soil microbe activities (Kumar et al. 2016; Martino and Perotto 2010), which can influence metal(loid) transformation through altering redox and pH value (Kumar et al. 2015), or complexing metal(loid)s with their exudates (Y. Huang et al. 2017). Additionally, nZVI can also cause toxicity to plants and microbes through the generation of reactive oxygen species (ROS), leading to cell membrane disruption and oxidative stress (Libralato et al. 2016; Ma et al. 2013). Therefore, it is essential to address the effects of plants and microbes on nZVI efficiency in metal(loid) stabilization, as well as the potential influence of nZVI on plant and microbial activities.

Arbuscular mycorrhizal (AM) fungi are ubiquitous soil fungi that can form symbiosis with more than 80% terrestrial plants. The fungi obtain carbohydrates from plant partners and, in return, provide plants with mineral nutrients (e.g., phosphorus and nitrogen) (Smith and Read 2008). AM fungi can also help plants to adapt to various stresses, e.g., drought, salt (Evelin et al. 2009; T. Li

Table 5.1: Basic physico-chemical characteristics of metal(oid) contaminated soil (Metal(oid) concentration and speciation in the soil are included)

	pH (1:2.5)	**pH_{zpc} (1:40)**	**CEC (cmol kg^{-1})**	**Soil organic matter (g kg^{-1})**	**Extractable P (mg kg^{-1})**	**Eh (mV)**
	5.95	5.45	9.08	21.5	1.53	426
			Metal concentration (mg kg-1)			
	Total	**Acid-ext**	**Reducible**	**Oxidizable**	**$CaCl_2$-ext**	**EDTA-ext**
Fe	37400	12.4	1690	8.36	0.06	793
Mn	4280	111	240	545	9.3	1410
Zn	4000	167	541	307	440	1660
Cu	68.3	8.08	n.d.	28.5	n.d.	32.9
Pb	3540	207	892	596	0.89	2040
Cd	39.1	21.3	8.44	2.13	7.49	24.6
Cr	61.3	n.d.	n.d.	0.92	n.d.	n.d.
Ni	24.4	1.79	n.d.	n.d.	0.31	1.32
As	296	n.d.	n.d.	2.02	n.d.	5.18

Table 5.2: Acid-extractable metal(loid)s were extracted by 0.11 mol L^{-1} acetic acid; reducible metal(loid)s were extracted by 0.5 mol L^{-1} hydroxylammonium chloride; and oxidizable metal(loid)s were extracted by 1.0 mol L^{-1} ammonium acetate after being digested using 30% (w/w) hydrogen peroxide. The pH ratio is given as proportion of solid:solution. The soil was also separately extracted by 0.01 mol L^{-1} $CaCl_2$ and 0.1 mol L^{-1} EDTA. Concentrations of all metal(loid) species were determined by inductively coupled plasma-optical emission spectrometry (ICP-OES).

et al. 2014). Additionally, it has been well-established that AM symbiosis can enhance plant resistance to metal(loid)s, mostly through the stabilization of metal(loid)s in the roots or rhizosphere, implying their promising potential in the phytoremediation and revegetation of metal(loid) contaminated soils (B. D. Chen et al. 2007b; Hutchinson et al. 2004; Subramanian et al. 2009; Wu et al. 2015). In fact, a recent field trial study indicated that AM fungi, together with Fe bearing phyllosilicate amendments could help plant survive in metal-contaminated soils (Sprocati et al. 2014). AM fungi can usually interact with metal(loid)s through the direct complexation of metal(loid)s by their exudates and cell wall or by indirectly influencing metal(loid) speciation through the alteration of the micro-environment in the mycorrhizosphere (Subramanian et al. 2009; Wu et al. 2015). Similarly, AM fungi may also interact with nZVI and its aging products (Fe oxides) or directly interact with metal(loid)s adsorbed on the nZVI surface, thus influencing the nZVI efficiency in metal(loid) stabilization. In addition, nZVI may also influence AM formation and development and thus affect its functions towards metal(loid) stabilization. However, little information is available about the metal(loid)-nZVI-AM fungi interactions in the plant-soil system, which would be of critical importance in the implementation

of nZVI and AM fungi in the phytoremediation and revegetation of metal(loid) contaminated soils. Therefore, it is urgent to investigate whether and how AM fungal inoculation influence nZVI functions towards metal(loid) stabilization in plant-soil system.

The present study investigated the influence of AM symbiosis on the efficiency of nZVI in metal(loid) stabilization in alluvial metal(loid)-contaminated soil (originating from mining and smelting) in the Příbram District of the Czech Republic, which has been heavily contaminated with Zn and Pb (as well as Cd, As and Cu) (Ettler et al. 2005). The study was based on a natural metal(loid)-contaminated soil, which is different from many previous studies that used artificial metal(loid)-contaminated soils (Carvalho et al. 2006; Sudova et al. 2007; Wu et al. 2014) and the results would be thus more environmentally relevant. The AM fungus *Rhizophagus irregularis* and maize (*Zea mays* L.) were selected for the study, as they have been proven to be resistant to heavy metal stress in previous studies (B. D. Chen et al. 2004; Malcová et al. 2003). The plants inoculated with/without AM fungus were cultivated in soils amended with/without nZVI. Plant physiological characteristics and metal(loid)s uptake, translocation and transformation were investigated. In particular, cellular distributions of metal(loid)s and mineral nutrients in plant roots were investigated by employing scanning electron microscopy equipped with electron dispersive X-ray spectroscopy (SEM-EDS) and micro-proton-induced X-ray emission (μ-PIXE) analysis, which proved to be a powerful tool for the analysis of elemental distribution in plant tissues (Vogel-Mikuš et al. 2014). In addition, solid phases of metal(loid)s (including Fe) in the mycorrhizosphere soil were analyzed by X-ray diffraction (XRD) to track their transformation. We hypothesized the following: (1) nZVI amendment can decrease metal(loid) uptake by plants but negatively affect AM development and functions in metal(loid) immobilization; (2) AM fungal inoculation can restrain nZVI efficiency towards reducing plant metal(loid) uptake and regulate metal(loid) translocation in roots; and (3) AM symbiosis can influence nZVI functions through altering metal(loid) bioavailability and Fe mineral transformation in the rhizosphere.

5.3 Materials and methods

Materials

The soil was collected in the alluvium of the Litavka River (Příbram District, Czech Republic), which has been contaminated with Zn, Pb, Cd, As and Cu. The soil physico-chemical characteristics are shown in **Table 5.1**. The methodologies of soil characterization are summarized in the Supporting Information (SI). The soil was sterilized by gamma radiation (20 kGrey) one week before the experiments. To increase soil porosity, clean quartz sand (with 1-2 mm diameter) was mixed with the soils (soil: sand = 5:1 w/w) before use.

Maize (*Zea mays*) seeds were obtained from REIN SAAT (Austria). The seeds were surface sterilized in 10% H_2O_2 for 15 min, washed with Milli-Q water, and pre-germinated on moist filter paper until the emergence of radicles. The AM fungus *Rhizophagus irregularis* was isolated from metal-contaminated soils (pH 5) in the same area (Příbram, Czech Republic) (Malcová et al. 2003). AM fungal inoculum included mycorrhizal roots, mycelium, spores (80 spores g^{-1}) and quartz sand. Nano zero-valent iron was provided by Nano Iron (Czech Republic), with a size of 50-100 nm (Figure S1), and the particles had a smooth sphere shape coated with a thin layer of Fe oxides that protected nZVI from further oxidation (further characteristics can be found in SI).

Experimental Design

The soil was amended with/without 0.5% (w/w) nZVI (the level was based on a previous study (Vítková et al. 2016)), and the plants were inoculated with/without AM fungi. The amendment of nZVI was carried out through homogeneously mixing dry powder air-stable nZVI particles with soils. As nZVI becomes very reactive in aqueous environments, the nZVI-amended soil was watered daily with distilled water (keeping the soil moisture at 55% of the water holding capacity (WHC)) to activate the nZVI and facilitate its functions on metal(loid)s stabilization for one month before plant and AM fungal cultivation. AM fungal inoculation was performed as follows: 700 g of soils amended

with (or without) nZVI were first put into a pot, and 300 g of soil containing 30 g AM inoculum were then added. For non-inoculated controls, 30 g gamma radiation (20 kGrey) sterilized AM inoculum was added along with 10 mL of AM inoculum filtrate to reintroduce microbes other than AM fungi in the AM inoculum. Additionally, soil filtrate (without AM fungi but with other indigenous microbes) was also reintroduced into the soil for all treatments to reintroduce the native soil microbial communities. Five germinated seeds were transplanted into each pot and 100 g soil was amended to support the seedlings. Seedlings were thinned to 2 per pot 1 week after emergence of cotyledon. To investigate the influence of plant growth on the efficiency of nZVI in soil metal(loid) stabilization, two bare soils with/without nZVI amendment were also employed. The experimental design is shown in Figure S2. There were 6 treatments, each with 6 replicates, totaling 36 pots. The experiment was conducted in a controlled greenhouse environment at 14/10h (light/dark) and 25/20°C (light/dark). The light intensity was 500-1100 μmol m^{-2} s^{-1}, which was provided by natural light and supplementary lights from high-pressure sodium lamps. Each pot was watered daily to maintain a water content of 55% WHC. Basal nutrients were added to the soil twice per month to give a final level of 60 mg·kg^{-1} N (in the form of NH_4NO_3), 60 mg·kg^{-1} K (KNO_3), and 15 mg·kg^{-1} P (KH_2PO_4) in the soil to support plant growth.

Harvest and Chemical Analysis

Plants grew for 65 days before harvest. One day before harvest, leaf water potential (ψw, MPa) was determined to evaluate the plant water balance. The third leaf from the top of the plant shoot was collected for ψw determination using the Scholander pressure chamber (model 600; PMS instruments Co., Corvallis, Oregon, USA) (Martínez-Fernández et al. 2015a). At harvest, plant shoots and roots were washed carefully with distilled water and weighed (total fresh weight, M_{ft}). Several fresh root segments were immediately prepared for μ-PIXE analysis, and a small amount of fresh roots and shoots were kept for malondialdehyde (MDA), chlorophyll determination and AM colonization assessment (the fresh samples were kept at -80°C until analysis). The remaining fresh plant samples were weighed (M_{fr}), freeze-dried at -50°C for 48 h, and

weighed again (M_{dr}). The dry weight of the whole tissues (roots or shoots) (M_{dt}) were calculated as follows:

$$M_{dt} = M_{ft} \cdot (M_{dr}/M_{fr}) \tag{5.1}$$

assuming that the water content of the extracted tissues (for μ-PIXE, MDA, Chl and AM colonization assessment analysis) were the same with that of the remaining fresh tissues. The remaining dry tissues were then ground for macro-nutrients and metal(loid) determination.

AM Colonization Assessment

AM colonization was determined following the methods of Phillips and Hayman (1970). In brief, fresh root sections (1-cm) were cleared by 10% (w/v) KOH, rinsed in 2% (v/v) HCl, and stained with 0.05% (w/v) Trypan blue. The intensity of the mycorrhizal colonization (M%) and the arbuscule abundance (A%) in the root system was assessed using MYCOCALC software (www2.dijon.inra.fr/mychintec/Mycocalc-prg/download) (Trouvelot et al. 1986)

Plant Physiological and Chemical Analysis

Malondialdehyde (MDA) contents usually represent the level of lipid peroxidation and were measured using a modified thiobarbituric acid (TBA) method (Du and Bramlage 1992). The chlorophyll content in plant leaves was determined according to Wellburn and Lichtenthaler (1984). Details on MDA and chlorophyll determination are in the SI. The lyophilized and homogenized plant samples were digested in HNO_3/H_2O_2 at 210°C on an electric heating plate (HE1 TH1, Harry Gestigkeit GmbH, Germany) for 12h, diluted to 50-mL with Milli-Q water and filtered through a 0.22-μm membrane. Macro-nutrients (P, K, Ca, Mg) and metal(loid) concentrations (e.g., Zn, Pb, Fe, Cu, As) were determined by inductively coupled plasma-optical emission spectrometry (ICP-OES, 720ES, Varian Inc., Palo Alto, California, USA).

Micro-PIXE Analysis

The sample preparation for μ-PIXE was performed according to Vogel-Mikuš et al. (2014) (see details in the SI). The μ-PIXE analysis was performed using a nuclear microprobe (voltage 3 MeV, beam size 0.8x0.8 μm, current 200 pA) at the Jožef Stefan Institute (Vogel-Mikus et al. 2009, 2007). The quantitative element-distribution maps were generated from the initial raw data using the GEOPIXE II software package (http://nmp.csiro.au/Geopixe.html) (Ryan et al. 1990). Element concentrations in different part of the roots (rhizodermis, cortex (including AM fungal structures), endodermis and vascular bundle) were calculated from the numerical matrices after the quantification of PIXE spectra by GEOPIXE II and extracted from the selected regions using ImageJ (https://imagej.nih.gov/ij/index.html).

SEM-EDS Analysis

The thin sections of freeze-dried plant roots were analyzed by scanning electron microscopy (SEM, TESCAN Brno, Czech Republic), which was equipped for electron dispersive X-ray spectroscopy (EDS, Bruker-nano, Berlin, Germany) to detect the elemental distribution across the roots of different treatments.

Soil Analysis

One week before harvest, soil solution samplers (mean pore volume size 0.15 μm, Rhizosphere Research Products, Netherlands) were carefully inserted into the soils, and the soil solution was collected at harvest. Phosphorus and metal(loid) concentrations in soil solutions were analyzed by ICP-OES.

At harvest, soil samples (50g each; stones were removed by sieving) were lyophilized and ground into a fine powder in an agate mortar in liquid nitrogen for X-ray powder diffraction (XRD) analysis. XRD analysis was conducted using a Bruker D8 Discover diffractometer coupled with Johansson-type focusing Ge primary monochromator and a LynxEye linear silicon strip detector. Soil pH and redox potential (Eh) were analyzed as described in the SI.

Table 5.3: Mycorrhizal status and physiological traits of *Zea mays* plants grown under different treatments.

Treatment	F%	M%	A%	Shoot dry weight (g pot^{-1})	Root dry weight (g pot^{-1})	Leaf water potential (MPa)	Leaf Chl content (mg g^{-1})	Leaf MDA content (nmol g^{-1})	Root MDA content (nmol g^{-1})
-M -nZVI	1.7±1.8b	0.5±0.5c	0.1±0.1c	0.89±0.30	0.21±0.05	-0.53±0.05a	1.49±0.73b	29.6±5.84a	5.79±1.35
-M +nZVI	1.1±2.7b	0.3±0.8c	0.03±0.1c	0.81±0.17	0.19±0.03	-0.68±0.06b	1.78±0.22b	22.9±5.90ab	6.48±0.79
+M -nZVI	88±8.9a	29.4±10.0a	18.8±6.2a	0.88±0.29	0.19±0.04	-0.55±0.07a	1.53±0.80b	25.5±6.57a	5.20±0.87
+M +nZVI	80±11.0a	13.6±3.9b	4.9±2.1b	0.67±0.20	0.17±0.05	-0.51±0.06a	2.82±0.35a	16.5±6.90b	6.60±2.01
				Significance of					
M	**	**	**	ns	ns	*	*	ns	ns
nZVI	ns	**	**	ns	ns	*	**	*	ns
M*X*nZVI	ns	**	**	ns	ns	**	ns	ns	ns

These include the frequency of mycorrhiza in the root system (F%), intensity of mycorrhizal colonization (M%) and arbuscule abundance (A%) in the whole root system, shoot and root dry weight, leaf water potential, leaf chlorophyll (Chl) concentration, and leaf and root malondialdehyde (MDA) concentrations. **+/-M** represents with/without arbuscular mycorrhizal (AM) fungal inoculation, and **+/-nZVI** represents with/without nano zero-valent iron amendment. The AM fungal strain for inoculation was Rhizophagus irregularis, isolated from metal-contaminated soils (pH 5). The nZVI addition concentration was 0.5% (w/w). Data are presented as means ± SD (n=6). For the two-way ANOVA, M: AM fungal inoculation; nZVI: nano zero-valent iron amendment; ns: not significant; $^{*}P < 0.05 and ^{**}P < 0.01$. Different letters show significant differences based on the Duncan multiple range test ($P < 0.05$)

Statistical Analysis

The mycorrhizal metal(loid) response (%MMeR) of roots was calculated using the individual metal(loid) content (determined as a product of metal concentration and plant dry weight) of AM roots and mean metal(loid) content of nonAM roots (Eq. **5.2**) (Watts-Williams and Cavagnaro 2012) at different nZVI treatments.

$$\%MMeR = \frac{Me(AMroot) - MeanMe(nonAMroot)}{MeanMe(nonAMroot)} \cdot 100 \qquad (5.2)$$

Because there was a significant difference in A% between the control and nZVI-treated AM plants, we also took into account the A% weights, namely the "arbuscule-weighed %MMeR" (%AMMeR) (Eq. **5.3**).

$$\%AMMeR = \%MMeR \cdot A\% \qquad (5.3)$$

The mycorrhizal metal(loid) response data were analyzed using an inde-

pendent t-test ($P<0.05$) to show the significance of nZVI addition. Plant dry weights, physiological traits, AM colonization rates, and macro-nutrient and metal(loid) concentrations were subjected to a two-way analysis of variance (ANOVA) to examine the effects of mycorrhizal status and nZVI amendment using the statistical package SPSS (Ver 18, IBM, Armonk, NY). Additionally, a two-way clustering analysis with heat-chart (program HemI http://hemi.biocuckoo.org/) was used to investigate the influence of nZVI and AM on nutrients and metal(loid) concentrations in shoots and roots (the data were z-standardized in advance). A Pearson correlation analysis was conducted to investigate the relationship between the concentrations of metals and nutrients in different parts of the roots using SPSS.

5.4 Results and discussion

AM Development and Physiological Functions under Metal(loid) Contamination as Influenced by nZVI

In the inoculated treatments, typical AM fungal structures (e.g., arbuscules, vesicles, mycelium) were found in plant roots (Figure S3), and the thin sections of mycorrhizal roots (prepared for μ-PIXE and SEM analysis) (Figure S4), while no plant roots were colonized in the non-inoculated treatment (**Table 5.3**, Figure S3). The frequency of mycorrhiza in the root system (F%), intensity of mycorrhizal colonization (M%) and arbuscule abundance (A%) were 88%, 29% and 18% (**Table 5.3**), respectively, which were lower than previous reports (B. D. Chen et al. 2004). This may be because the soil used in the current study was contaminated by various metal(loid)s (i.e., Zn, Pb, As, Cd), which can exhibit the toxic effects. Additionally, P bioavailability was much lower in the present study, which can also influence AM development (Treseder and M. F. Allen 2002). As assumed, nZVI amendment restrained mycorrhizal development, which decreased the M% from 29% to 14% (and A% from 19% to 5%) (**Table 5.3**). This effect can be attributed to the oxidative stress caused by nZVI (Ševců et al. 2011), which may restrain plant-AM fungal association development. The decreased AM development may further influence its functions towards nutrient acquisition and metal(loid) stabilization.

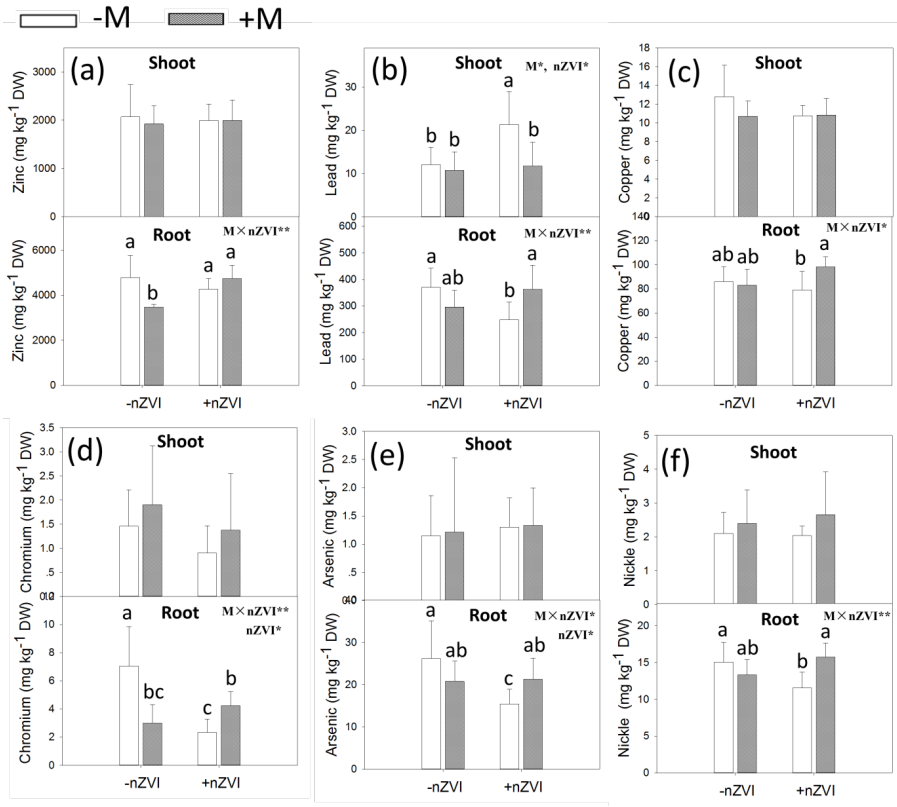

Figure 5.2: Concentrations of Zn (a), Pb (b), Cu (c), Cr (d), As (e) and Ni (f) in shoots and roots of *Zea mays* plants grown in metal(loid) contaminated soils with different treatments. Note: +/**-M** represent with/without arbuscular mycorrhizal (AM) fungal inoculation, +/**-nZVI** represent with/without nano zero-valent iron amendment. For two-way ANOVA, M: AM fungal inoculation, nZVI: nano zero-valent iron amendment, $^{*}P < 0.05$, $^{**}P < 0.01$. Different letters above columns show significant difference based on Duncan multiple range test ($P < 0.05$)

Consistent with a previous study on *Cynodon dactylon* under Cr stress (Wu et al. 2014), AM fungal inoculation also had no influence on plant growth and P uptake in our specific experimental setup (**Table 5.3**, Table S1). AM function towards P acquisition depends on mycorrhizal colonization status, plant species and environmental conditions (Smith et al. 2011). For the current situation, the presence of metal(loid)s and/or nZVI in the soils may restrain AM formation and development and consequentially restrain P acquisition. The deficiency of AM on plant P acquisition thus resulted in lower plant growth improvement by AM symbiosis.

Similar to recent studies (Martínez-Fernández et al. 2015a; Martínez-Fernández and Komárek 2016), nZVI amendment decreased leaf water potential (**Table 5.3**), and green leaves became curled after nZVI addition for plants without AM fungal inoculation (Figure S5). This may indicate that nZVI restrained plant water uptake, as leaf water potential represents water uptake efficiency. The reason may lie in the fact that nZVI amendment may aggregate on the root surface and restrain root water acquisition (Martínez-Fernández et al. 2015a) or change soil mechano-hydrological characteristics and reduce soil water potential. However, when plants were inoculated with AM fungi, leaf water potential increased, and the negative effects of nZVI decreased (**Table 5.3**). This may be because that AM fungal mycelium may directly interact with nZVI in both hyphosphere or the fungal cells and oxidize Fe^0 (from nZVI) to Fe^{2+} or Fe^{3+} (Ševců et al. 2011), thus reducing the direct interactions of nZVI particles with plant roots. Additionally, AM fungi can directly take up water through aquaporins and transport it to the plant partners (T. Li et al. 2013) or regulate the abundance of aquaporins in the plants (T. Li et al. 2016), thus alleviating plant drought stress.

The AMF inoculation together with nZVI significantly increased leaf chlorophyll content, while decreasing leaf MDA content compared with other treatments (**Table 5.3**). This indicates that only AM fungi together with nZVI can alleviate oxidative stress in leaves caused by metal(loid)s. This can be explained by the fact that AM symbiosis can alleviate the negative effects of nZVI on plants while enhance metal(loid)s stabilization in roots together with nZVI, thus reducing plant leaf metal(loid) stress. AM and nZVI did not influence root MDA content, which may result from the balance of the complex effects of AM and nZVI on plant metal(loid) uptake, toxicity and homeostasis.

AM and nZVI Restrained Each Other's Functions towards Plant Metal(loid) Uptake

As expected, plant Zn concentrations were the highest among all metal(loid)s (**Figure 5.2**). Both AM fungal inoculation and nZVI separately decreased metal(loid) (Zn, Pb, Cr, As and Ni) concentrations in plant roots, while metal(loid) concentrations returned to the same level as the control treatment (-M-nZVI)

when they worked together (**Figure 5.2**). The clustering heat-chart analysis of elemental profiles demonstrated well that treatments -M-nZVI and +M+nZVI formed one group, while treatments -M+nZVI and +M-nZVI formed another group based on root metal(loid) concentrations, but no similar trends were found on shoot metal(loid) concentrations (Figure S6). The results are consistent with our recent study (Michálková et al. 2017), and support our assumption that nZVI can reduce plant metal(loid) uptake, which may result from the direct adsorption or co-precipitation of metal(loid)s by nZVI in the soils (Komárek et al. 2013; Vítková et al. 2016), modification of metal(loid) speciation to other forms with lower bioavailability (e.g., reduction of Cr(VI) to Cr(III) (Ponder et al. 2000)), or the aggregation of nZVI on the root surface (Martínez-Fernández and Komárek 2016). However, when plants were inoculated with AM fungi, the nZVI efficiency of reducing plant metal(loid) uptake decreased (**Figure 5.2**), confirming our hypothesis that AM symbiosis can restrain nZVI functions towards metal(loid) stabilization. The AM symbiosis may influence nZVI functions in the following ways: (1) AM fungi may directly interact with nZVI and Fe oxides, and influence their ability to adsorb metal(loid)s; (2) AM hyphae may directly take up metal(loid)s from nZVI amended soils; (3) AM may enhance the complexation of metal(loid)s with root and fungal exudates (e.g., citric acid, amino acids, phenolic acids) (Y. Huang et al. 2017; Nichols 2003) and facilitate metal(loid) dissolution from Fe minerals; and (4) AM symbiosis changes nZVI function and metal(loid) bioavailability through changes in soil pH and Eh (further discussion can be found below).

It is interesting to find that AM function on plant metal(loid) uptake was also dramatically affected by nZVI. When there was no nZVI amendment, the AM fungal inoculation generally decreased metal(loid) concentrations in roots. This may not have resulted from growth dilution effects (B. D. Chen et al. 2007a), as AMF inoculation did not increase plant growth in the present situation, but it may have been due to the stabilization of metal(loid)s by extraradical mycelium (Janouskova and Pavlikova 2010; Wu et al. 2015). Similarly, AM symbiosis also decreased metal(loid) uptake by roots, as the mycorrhizal metal(loid) responses (%MMeR) (on roots) for most metal(loid)s were below zero (**Figure 5.3**). However, when nZVI was amended, AM symbiosis generally increased metal(loid) (Pb, Cu, Cr, As, Ni) concentrations in plant roots

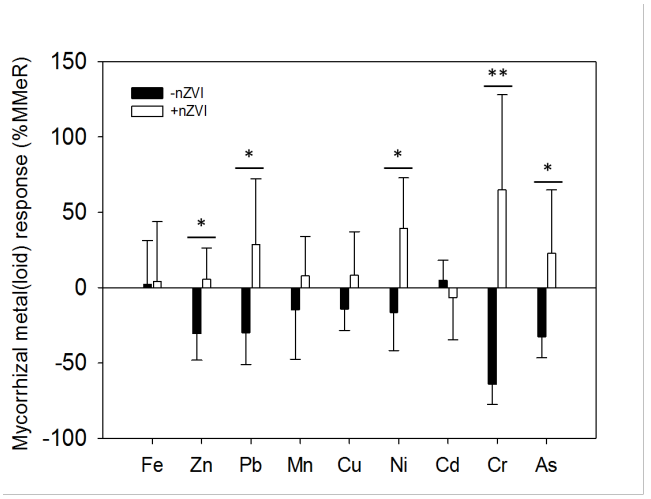

Figure 5.3: Mycorrhizal metal(loid) (i.e., Fe, Zn, Pb, Mn, Cu, Ni, Cd, Cr, and As) responses (% MMeR) on roots of *Zea mays* L. grown in metal(loid)-contaminated soils amended with/without nZVI. Note: +/**-nZVI** represents with/without nano zero-valent iron amendment. Data are presented as means ± SD (n=6). For the independent t-test, $^{*}P < 0.05$ and $^{**}P < 0.01$

(**Figure 5.2**), and %MMeR (on roots) values for most metal(loid)s were above 0 (**Figure 5.3**). The nZVI-regulated AM functions towards plant metal(loid) uptake may have resulted from its effects on AM formation and development, as M% and A% values decreased upon nZVI amendment (**Table 5.3**). This was partially confirmed by the fact that when A% weight was considered in the calculation of mycorrhizal metal response, %AMMeR values decreased under nZVI amendment (Figure S7). However, %AMMeR values under nZVI amendment were still higher than that under non-nZVI amendment, indicating that nZVI may also influence AM functions through other pathways. In fact, nZVI may stimulate AM symbioses to take up more metal(loid)s from the soils through direct interaction with AM roots or fungal mycelium, which leads to the alteration of the root/fungal member structure (Kotchaplai et al. 2017).

Similar to previous studies (B. D. Chen et al. 2007b; Wu et al. 2014), AM fungal inoculation decreased (or had no influence on) metal(loid) concentrations in shoots under nZVI amendment (**Figure 5.2**), which may be because AM may stabilize metal(loid)s in fungal structures (mycelium, spores) and reduce metal(loid) translocation to shoots (Wu et al. 2015, 2016b).

Cellular Metal(loid) Translocation in Roots as Influenced by AM and nZVI

Metal(loid)s and macro-nutrient distributions, as revealed by μ-PIXE and SEM-EDS, are shown in **Figure 5.4** and **Table 5.4**, as well as Tables S2-S5 and Figures S8-S9. Zinc was mainly accumulated in the endodermis of nonmycorrhizal roots (**Figure 5.4**, Table S2 and S3), while it was accumulated in cell walls and AM fungal structures (e.g., arbuscules) in mycorrhizal roots (**Figure 5.4**, **Table 5.4**), which was consistent with our assumption that AM fungal inoculation can regulate Zn translocation in roots. In fact, the retain of metal(loid)s by AM fungal structures in mycorrhizal roots (namely metal(loid) compartmentation) is one of the key strategies for AM symbioses to deal with metal(loid)s stress (González-Guerrero et al. 2009). Similar to Zn, the compartmentation of Cd and Cr in AM fungal structures was also reported previously (Nayuki et al. 2014; Orlowska et al. 2013; Wu et al. 2016a). Additionally, Cu was also found to be accumulated in fungal structures in mycorrhizal roots, while it was mainly located in the rhizodermis upon nZVI amendment whether AM fungi was present or not (**Table 5.4**, **Figure 5.4**). This indicates that nZVI may facilitate Cu precipitation on the root surface, which is not influenced by AM fungal inoculation. Similarly, AM fungal inoculation did not influence Pb distribution, which was mainly located in the rhizodermis of roots especially under nZVI amendment (**Figure 5.4**, **Table 5.4**), indicating that most of the Pb was precipitated on the root surface rather than being taken up. These are reasonable as the immobilization of metal(loid)s by rhizodermal cell walls is usually considered to be an important strategy for plants to alleviate metal toxicity (Hall 2002), and nZVI seemed to enhance this metal(loid) stabilization process. Compared with Pb, more Ni was detected in the cortex, which may be because Ni is taken up as an essential nutrient for plants (DalCorso et al. 2014). Chromium, Cd and As were below the detection limits and are not shown.

Iron and Mn showed different distribution pattern in roots irrespective of AM fungal inoculation. Manganese was mainly concentrated in the cortex, while Fe was mainly located on the root surface, which was revealed by both SEM-EDS and μ-PIXE analysis (**Figure 5.4** and S8). The Fe concentrations in the rhizodermis were positively correlated with Zn, Pb and Cu in mycorrhizal

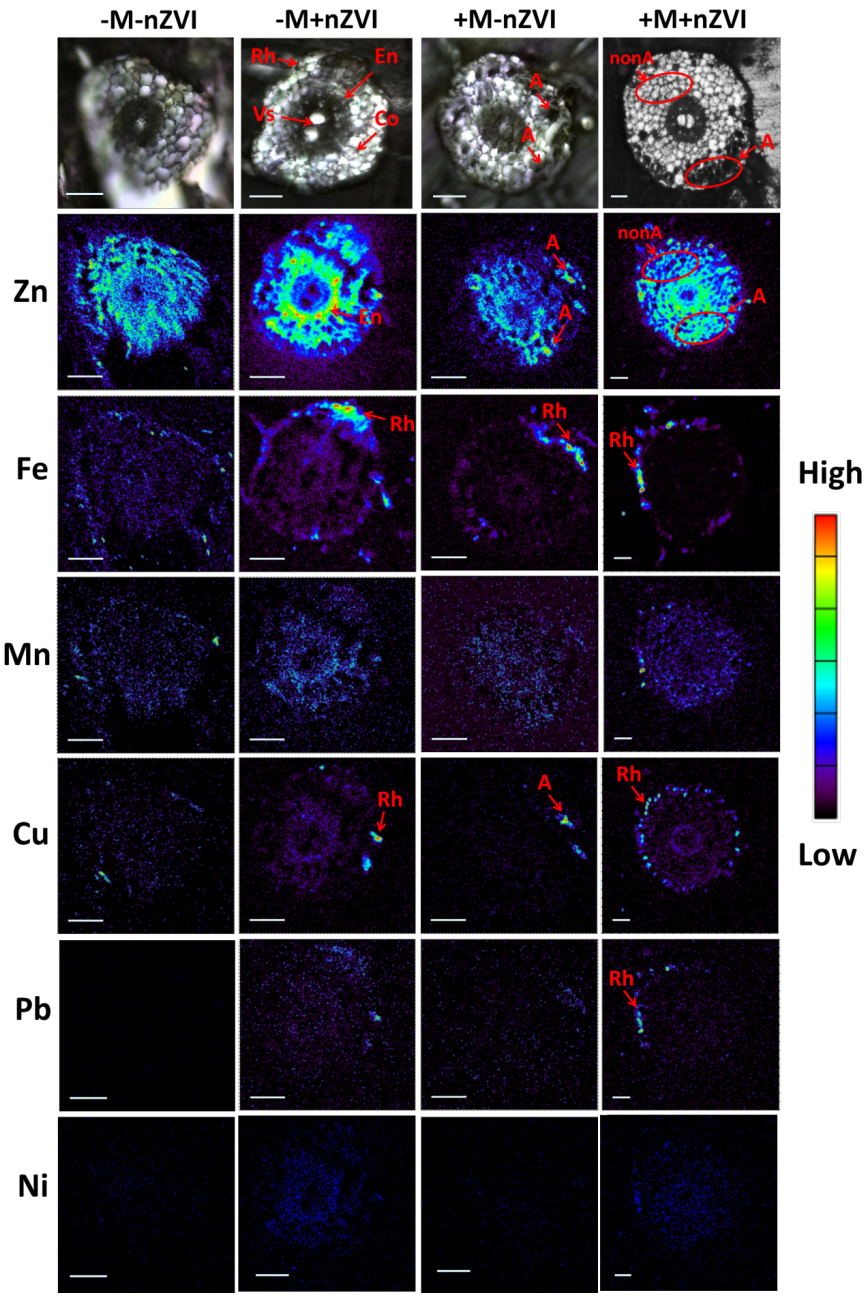

Figure 5.4: Micro-PIXE imaging showing the cellular distribution of key metals (Zn, Fe, Mn, Cu, Ni and Pb) in roots of *Zea mays* plants grown in metal(loid)-contaminated soils with different treatments. Note: The photos of the original root sections are shown in the first line and were taken with a stereo microscope (DP25, Olympus, Japan). +/**-M** represents with/without arbuscular mycorrhizal (AM) fungal inoculation, and +/**-nZVI** represents with/without nano zero-valent iron amendment. Scale bars=100 *mu*m; Rh: rhizodermis; Co: cortex; En: endodermis; Vs: vascular bundles; A: arbuscules; and nonA: cortex tissues without arbuscules inside.

Table 5.4: Concentrations (means ± SD; N=15) of metal(loid)s and macronutrients (mg kg^{-1}) in arbuscular mycorrhizal (AM) fungal structures (arbuscules) and non-AM fungal structures within the cortex of *Zea mays* roots inoculated with *Rhizophagus irregularis* grown in metal(loid)-contaminated soils with/without nZVI amendment.

	+M+nZVI			+M-nZVI		
Element	**AM fungal Structures** $mg \cdot Kg^{-1}$	**NonAM fungal Structures** $mg \cdot Kg^{-1}$	**Significance of independent t-test**	**AM fungal Structures** $mg \cdot Kg^{-1}$	**NonAM fungal structures** $mg \cdot Kg^{-1}$	**Significance of independent t-test**
Zn	12900±1300	3790±866	**	11900±1170	1380±354	**
Pb	287±109	151±151	ns	1480±1480	198±181	ns
Fe	136±48	55±27	ns	231±92	45±44	*
Mn	1780±264	297±75	**	1690±401	296±104	**
Cu	165±70	45±42	*	1760±806	41±40	**
Ni	116±57	40±40	ns	13±13	5±4	ns
P	209±80	101±57	ns	915±337	367±164	ns
S	32000±4900	11500±2090	*	11800±2670	1580±505	**
Ca	1940±714	860±219	ns	3450±799	635±376	*
Cl	2480±441	1230±368	ns	1030±354	876±369	ns
K	18300±3340	12000±2830	ns	7280±1750	5140±1620	ns
Na	13400±1680	4020±997	**	18600±2860	2570±713	**

The independent t-test is also shown for the comparison of elemental concentrations in AM fungal structures and non-AM fungal structures. Fifteen points (with areas of $1 \times 1\mu m$) were randomly selected from each part of the fungal structures and nonfungal structures, and the element concentrations were extracted from PIXE spectra using ImageJ software. Note: for the t-test, $^{*}P < 0.05$ and $^{**}P < 0.01$; ns, represents not significant; "+/-M" represents with/without AM fungal inoculation; and "+/-nZVI" represents with/without nano zero-valent iron amendment.

roots under nZVI amendment (**Figure 5.5**). Particularly, there was a strong correlation between Fe and Pb concentrations, with a relatively high correlation coefficient (r) value of 0.66. This indicates that Fe-bearing compounds may take part in Pb (and/or Zn and Cu) precipitation in root rhizodermis as Fe plaque (consisting of ferrihydrite and goethite) on the root surface usually controls metal(loid) behavior in the plant-root system (Hansel et al. 2001). However, this only occurred in mycorrhizal roots under nZVI treatment, and there was no significant correlation between Fe and metals (Zn, Pb and Cu) in nonmycorrhizal roots (Figure S10). This indicates that AM can favor formation of Fe bearing compounds with high capability for metal(loid) immobilization in root rhizodermis under nZVI presence. The Fe bearing compounds may be generated from the transformation of nZVI (or derived Fe oxides) by AM symbioses.

Both μ-PIXE and SEM-EDS showed that P was mainly in the endodermis and vascular bundles (**Figure 5.4**, S8-S9, Table S2-S5), indicating P translocation from roots to shoots. However, in contrast to Orlowska et al. (2013), P was not accumulated in fungal structures (e.g., arbuscules) in the present study. This was in accordance with the fact that AM did not increase P concentra-

tions in roots (Table S1). Metal(loid) contamination and low soil P availability may have restrained P acquisition and transport by AM fungal structures as described above. On the contrary, AM fungal structures accumulated more S in roots (Figure S9, **Table 5.4**), indicating that AM symbiosis may facilitate S uptake by directly taking up and transporting S to plants (J. W. Allen and Shachar-Hill 2009). Those S-compounds may also contribute to metal(loid) sequestration, as Zn is usually combined with thiols in organisms (Caldelas and Weiss 2017; Cobbett 2000; Medas et al. 2017). This was also partially confirmed by the fact that Zn concentrations in arbuscules were positively correlated with S concentrations irrespective of nZVI treatment (**Figure 5.5**, Figure S11). Similar to previous studies (De Giudici et al. 2015; Medas et al. 2015, 2017), Al and Si mainly located in the rhizodermis of the mycorrhizal roots (Figure S12). However, for the nonmycorrhizal plants, Al and Si could hardly be detected. Besides, different from previous studies (De Giudici et al. 2015; Medas et al. 2015, 2017), Zn was mainly located in the cortex, endodermis and vascular bundles, which was not consistent with the distribution pattern of Al and Si. It seems that Zn cannot be mineralized together with Al/Si minerals on the maize root surface under the present experimental conditions, indicating that plant strategies for controlling Zn uptake depends on the species. Specifically, native pioneer plants like *Euphorbia pithyusa* (Medas et al. 2015), *Pistacia lentiscus* (De Giudici et al. 2015), and *Juncus acutus* (Medas et al. 2017) may form Al/Si barriers on root surface to immobilize Zn, while maize plant (*Zea mays* L.) as an acclimated plant species does not develop such strategies. However, this hypothesis needs further investigations.

Micro-PIXE also indicated the distribution of other macroelements (Figure S9, Table S2-S5). Mg was mainly detected in the cortex. Potassium and Ca mainly accumulated in the vascular tissues and cortex, while Cl was mainly present in vascular tissues. AM fungal inoculation increased the accumulation of the nutrient elements Mg, K, Ca and Cl in the cortex under nZVI amendment, thus systematically improving plant nutrition status under metal(loid) contamination and nZVI amendment.

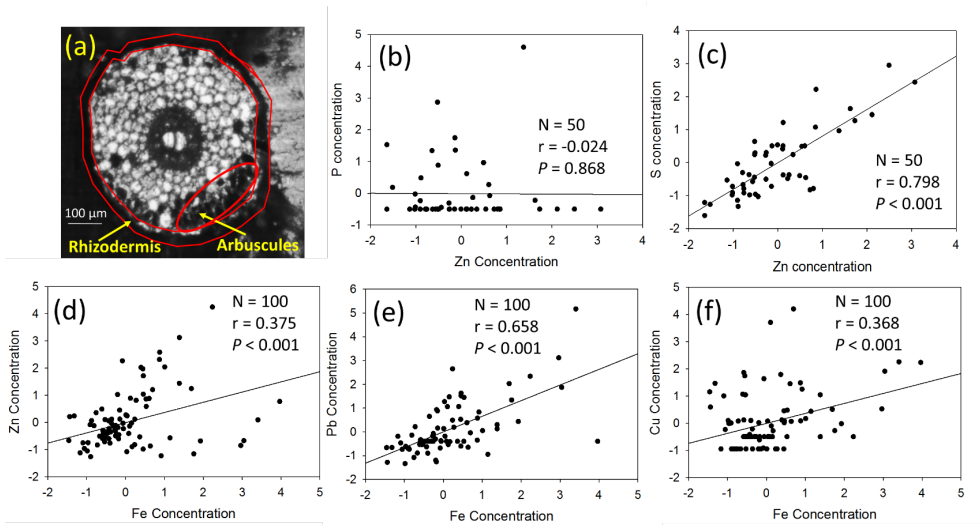

Figure 5.5: Pictures of rhizodermis and fungal structures (arbuscules) in the roots (a), and the Pearson correlation between Zn concentrations and P (b), S (c) concentrations in arbuscules, and the Pearson correlation between Fe concentrations and Zn (d), Pb (e), and Cu (f) concentrations in the rhizodermis of roots in the **+M+nZVI** treatment. One hundred points were randomly selected from the rhizodermis and 50 points were selected from arbuscules, and element concentrations were calculated from PIXE spectra using ImageJ software. All data were standardized for the Pearson correlation analysis using SPSS. Note: **+/-M** represents with/without arbuscular mycorrhizal (AM) fungal inoculation, and **+/-nZVI** represents with/without nano zero-valent iron amendment. Scale bars=100 μm.

Linking Soil nZVI and Metal(loid) Transformation with Mycorrhizal Plant Metal(loid) Uptake

At harvest, Fe^0 was detected in the soil amended with nZVI ($2\theta = 44.8°$), indicating that part of the added nZVI was not oxidized after plant cultivation (**Figure 5.6**, Figure S13). There were also other Fe bearing minerals in soils such as muscovite 2M1 (ferrian), chamosite 1M, and richterite (**Figure 5.6**), which may have resulted from both the original soils and nZVI aging (Dong et al. 2016). Iron concentrations in soil pore water increased upon plant cultivation (Figure S14). This may be due to the functions of maize root exudates such as siderophores, which can draw Fe from Fe minerals and facilitate Fe dissolution (Morrissey and Guerinot 2009). During Fe dissolution, metal(loid)s adsorbed on Fe minerals can be dissolved (Saad et al. 2017) and taken up by plants. However, we did not observe any changes of Fe bearing minerals upon plant

growth by XRD analysis (**Figure 5.6**), indicating that only a small portion was transformed due to plant root activities or transformed to amorphous phases (e.g. ferrihydrite) that can hardly be determined by XRD analysis (Kahle et al. 2002).

Consistent with Vítková et al. (2016), metal(loid) concentrations in pore water were not affected by nZVI addition (Figure S14). Many factors such as soil pH, soil Eh, organic matter and other elements can influence the adsorption/desorption of metal(loid)s on nZVI and its oxidation products. Soil Eh can influence metal(loid) bioavailability in various ways, e.g., Eh-dependent pH changes, co-precipitation with Fe and/or Mn minerals, precipitation as sulfides (Charlatchka and Cambier 2000; Popenda 2014). In the present study, nZVI addition decreased the soil Eh value (Table S6) due to the high reducibility of nZVI (Sun et al. 2006). The lower Eh may have further decreased metal(loid) solubility through stimulating microbial mediated sulfate reduction, or restraining the oxidation of reduced sulfur compounds to sulfate, which is known to facilitate metal(loid) dissolution in the soil (Popenda 2014). However, these processes may occur in the micro-environment of the rhizosphere and can barely be identified by detecting metal(loid) concentrations in soil pore water.

Further XRD analysis showed that Zn was in the form of easily soluble zincite (ZnO) (Escorihuela et al. 2017) in the soils (at $2\theta = 31.8°$, $34.6°$ and $36.1°$), while Pb was in the form of insoluble Pb-jarosite (plumbojarosite, $Pb(Fe_3(OH)_6(SO_4)_2)_2$) (at $2\theta = 33.9°$) and Pb-carbonate (cerussite, $PbCO_3$) (at $2\theta = 24.9°$) (**Figure 5.6**, Figure S13), which were more stable than Pb oxides under natural conditions (Ruby et al. 1999). This can explain why Zn solubility in soil pore water and uptake by plants were much higher than Pb, although their total concentrations in the soils were at the same level (**Table 5.1**). The abundances of Pb-jarosite-like minerals increased upon plant growth for nonmycorrhizal treatments (**Figure 5.6**, Figure S13), indicating that plant root activities may facilitate Pb-jarosite precipitation under current situation. This may be because that root activity may increase dissolved Fe^{3+} and Pb^{3+} levels through pH decrease or organic acid chelation mediated by root exudates (e.g., citric acid, amino acids, phenolic acids etc) (Y. Huang et al. 2017), and thus facilitate Pb-jarosite precipitation, as higher concentrations of Fe^{3+} and Pb^{2+} (together with SO_4^{2-}) usually favor Pb-jarosite formation under acidic condi-

tions (pH 4-6) (Dutrizac et al. 1980; Forray et al. 2010). However, when the plants were inoculated with AM fungi under no nZVI treatment, Pb-jarosite abundance decreased. This may be due to the complexation of Fe^{3+} or Pb^{2+} with AM exudates like glomalin (a glycoprotein with stronger metal binding capability compared with organic acids) (Nichols 2003), which may reduce Fe^{3+} or Pb^{2+} ion concentrations and prevent Pb-jarosite precipitation. Besides, AM symbiosis can usually enhance SO_4^{2-} uptake by plants (J. W. Allen and Shachar-Hill 2009) and thus decrease SO_4^{2-} level in soils for Pb-jarosite precipitation. Interestingly, the nZVI amendment increased Pb-jarosite abundance in soils with AM fungal inoculation (**Figure 5.6**, Figure S13), which should be due to the negative influence of nZVI on AM development and fungal exudate production. It is essential to point out that although the XRD analysis could provide some information on the solid speciation of the metal(loid) phases, some species may have not been identified due to their low abundance or poor crystallinity. Therefore, the metal(loid) speciation in soils detected by XRD analysis cannot fully predict metal(loid) bioavailability, and explain metal(loid) concentration changes in soil pore water. This can also explain why AM together with nZVI enhanced plant metal(loid) uptake, but did not influence metal(loid) phases (or even facilitate mineral precipitation (i.e., Pb-jarosite)) in soils as revealed by XRD analysis.

In summary, the present study has supported our assumptions that AM can restrain nZVI efficiency towards reducing plant metal(loid) uptake, regulate cellular metal(loid) translocation in roots, and alter metal(loid) mineralogy and bioavailability in the rhizosphere. In return, nZVI could also restrain AM development and its function towards metal(loid) immobilization (or even stimulated AM symbioses to dissolve and take up metal(loid)s). The study also confirmed the protective functions of AM symbiosis towards plant physiology upon nZVI and metal(loid) stress, which includes the improvement of plant water and nutrition, the sequestration of Zn by fungal structures within roots, and the precipitation of Pb on root rhizodermis, as revealed by μ-PIXE. Overall, the current study has elucidated the nZVI-mediated metal(loid) uptake and translocation in AM fungal-plant symbioses, contributing to the understanding of nZVI functions in the plant-microbe-metal(loid) continuum. Further studies should be performed to investigate the mechanisms of interactions between AM

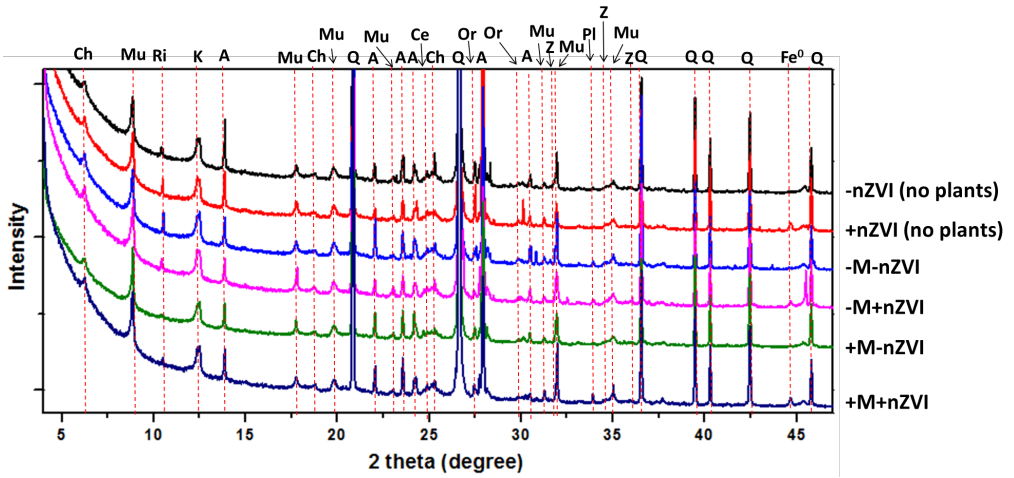

Figure 5.6: XRD pattern of soils obtained from different treatments. Q: quartz (SiO_2); A: Albite $Na(AlSi_3O_8)$; Or: Orthoclase (KSi_3AlO_8); K: Kaolinite ($Al_2Si_2O_5(OH)_4$); Ch: Chamosite 1M ($(Mg_{5.036}Fe_{4.964})Al_{2.724}(Si_{5.70}Al_{2.30}O_{20})(OH)_{16}$); Mu: Muscovite-2M1, ferrian ($K_{0.92}Na_{0.08}Al_{1.78}Fe_{0.22}Mg_{0.1}(Al_{0.83}Si_{3.17}O_{10})(OH)_{1.56}O_{0.25}F_{0.19}$); Ri: Richterite ($Na_2Ca(Mg,Fe)_5Si_8O_{22}(OH)_2$); Z: Zincite ($ZnO$); Fe0: Zero-valent iron; Ce: Cerussite ($PbCO_3$); Pl: Plumbojarosite ($Pb(Fe_3(OH)_6(SO_4)_2)_2$). Note: **+/-M** represents with/without arbuscular mycorrhizal (AM) fungal inoculation, and **+/-nZVI** represents with/without nano zero-valent iron amendment

fungi and nZVI under metal(loid) contamination.

5.5 Associated content

The supporting information is available on the ACS Publications website at DOI: 10.1021/acs.est.7b05516. Methodologies for determining soil/nZVI physico-chemical characteristics, plant malondialdehyde (MDA) and chlorophyll content, as well as μ-PIXE and XRD analysis. Tables showing concentrations of macronutrients (P, K, Ca, Mg, Na), Fe and Mn in *Zea mays* plants; concentration of metal(loid)s and macronutrients in different structures of plant roots from different treatments; soil redox potential and pH under different treatments. Figures showing TEM pictures of fresh nZVI; a graph of the experimental design; photos of nonmycorrhizal/mycorrhizal roots and plant shoots under different treatments; two way clustering heat-chart depicting the

relationships among element concentrations in plants; arbuscule-weighed mycorrhizal metal(loid) response (% AMMeR) on plant roots; SEM image and selective EDS spectra of plant root sections from different treatments; μ-PIXE imaging showing cellular distribution of macroelements in plant roots; the Pearson correlation between Fe and metals (Zn, Pb and Cu) in root rhizodermis in -M+nZVI treatment; the Pearson correlation between Zn concentrations and P, S concentrations in arbuscules of roots in the +M-nZVI treatment; μ-PIXE imaging showing cellular distribution of Al and Si in plant roots; XRD pattern of soils from different treatments; Phosphorus, Fe, Zn, Mn, Pb, Cu, Cd and Ni concentration in soil solution under different treatments.

CHAPTER

SIX

NANOSCALE ZERO-VALENT IRON HAS MINIMUM TOXICOLOGICAL RISK ON THE GERMINATION AND EARLY GROWTH OF TWO GRASS SPECIES WITH POTENTIAL FOR PHYTOSTABILIZATION

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6.1 Abstract

Two *Poaceae* species, *Agrostis capillaris* and *Festuca rubra*, were selected for their potential as phytostabilizing plants in multicontaminated soils. These species are resistant to contamination, develop extensive root systems, and maintain high contaminant concentrations at the root level. Nanoscale zero-valent iron (nZVI) is an engineered nanomaterial with the ability to stabilize metal(loid)s in soils, for example, in so-called aided phytostabilization. Therefore, the potential toxicological effects of nZVI were studied using two possible candidates for in situ phytostabilization, *A. capillaris* and *F. rubra*, in a germination test using (i) filter paper as a control, (ii) soil contaminated with Pb and Zn and (iii) contaminated soil amended with 1% nZVI as well as in an hydroponic experiment with the addition of 0, 25, 50 and 100 mg L^{-1} nZVI (T0, T25, T50 AND T100). nZVI had no negative effects on seed germination or seedling growth but was associated with an increase in shoot growth and reduction of the elongation inhibition rate (root-dependent) of *F. rubra* seedlings. However, applications of nZVI in the hydroponic solution had no effects on *F. rubra* but *A. capillaris* developed longer roots and more biomass. Increasing nZVI concentrations in the growing solution increased Mg and Fe uptake and reduced the Fe translocation factor. Our results indicate that nZVI has few toxic effects on the two target plant species and suggest that *A. capillaris* has mechanisms to assimilate and cope with nZVI; however, these results require further investigation.

6.2 Introduction

Some of the adverse effects caused by soil contamination can be decreased by chemical stabilization, which implies the addition of materials to soil to decrease the mobility and bioavailability of metallic and metalloid contaminants (Ali et al. 2019; Rinklebe and Shaheen 2015). In past years, engineered nanoparticles (ENP), materials with at least two dimensions between 1 and 100 nm, have played a role in newly developed remediation technologies (Grieger et al. 2010; Komárek et al. 2013; Stefaniuk et al. 2016). These ENPs can function as scavengers for contaminants mainly because of their high reactivity and large specific surface area (Lefevre et al. 2016). Nanosized zero-valent iron (nZVI) has recently become popular for use as a stabilizing agent for metal and metalloid contaminants due to its strong reducing properties and high reactivity, which affect contaminant mobility through adsorption, redox reactions and surface precipitation and/or co-precipitation in the form of metal iron oxides (Ma et al. 2010; Michálková et al. 2017; Wu et al. 2018; Zuverza-Mena et al. 2017).

Stabilization of metal(loid) contaminants can be considered as one of the most realistic and viable alternatives for the recovery and conservation of heavily contaminated soils (Martínez-Fernández et al. 2015b). The use of immobilizing amendments (e.g., nZVI) can decrease contaminant solubility (e.g., risk of ground water contamination) and bioavailability (Kumpiene et al. 2008). This is an important factor for establishing vegetation cover that can lead to ecological restoration. Phytostabilization is an efficient remediation approach that includes establishing plant cover by using species that can stabilize pollutants in the root zone (e.g., roots and rhizosphere) by accumulation or precipitation and thus reducing their mobility and bioaccessibility (Lebrun et al. 2019; Sigua et al. 2019). Hence, species that are tolerant to high contamination levels and, at the same time, present an extensive fasciculated root system, rapid growth and establishment, high longevity, easy maintenance, good adaptation to contamination and below-ground accumulation of metals are the best candidates for phytostabilization (Doubkova and Sudova 2016; Kucharski et al. 2005; Sigua et al. 2019). The selection of the appropriate amendment or combination of amendments is also crucial for the success of the remediation process (Ali et al. 2019; Kumpiene et al. 2019; Sigua et al. 2019). The interactions between the

selected amendment and plant species and the subsequent relationship with the target contaminants will condition the restoration process; therefore, understanding of such interactions has recently gained substantial attention (Ali et al. 2019; Clemente et al. 2019; Kim et al. 2018; Radziemska et al. 2018).

In this regard, the use of ENPs may lead to their accumulation in edible parts of plants and the appearance of adverse effects on agronomic traits, yield productivity of crops and transfer to other trophic levels (Wang et al. 2016b). Most environmentally relevant ENPs have toxic effects in plants at relatively high concentrations and the toxicity is usually species-dependent (Wang et al. 2016b; Xiang et al. 2015). However, some plants have shown signs of recuperation, thus indicating that the toxicity was temporary (Martínez-Fernández and Komárek 2016).

Due to its high reactivity, nZVI accumulates easily in the environment and is vulnerable to oxidation (Wang et al. 2016a). Once inside living systems, nZVI can be absorbed on the cell membranes of bacteria (Stefaniuk et al. 2016) and can cause negative effects on cell viability and integrity (Lefevre et al. 2016). Most studies related to nZVI interactions with plants have shown no or minimal (usually at high concentrations) effects on their functions, e.g., Ma et al. (2013) found a reduction of transpiration and growth of poplars after the application of 200 mg L^{-1} nZVI; Martínez-Fernández and Komárek (2016) found reductions in the root hydraulic conductivity of tomato plants growing at concentrations of 100 mg L^{-1} nZVI due to aggregation on the root surfaces. These works suggest that alterations of plant functions may not be due simply to direct chemical phytotoxicity but are also a result of physical interactions between ENPs and plant cell transport paths (Ma et al. 2010; Martínez-Fernández et al. 2015b).

Inhibition of seed germination and seedling growth are among the most evident effects of toxic compounds on plants (Visioli et al. 2014). Hydroponic systems provide a certain degree of control on nutrient concentrations and other molecules provided to the plant and they allow less invasive separations of root and shoot tissues for analysis (Nguyen et al. 2016). To investigate the potential toxicity caused by nZVI, a germination test and hydroponics experiment were conducted using two plant species. Previous studies (Teodoro et al. 2020a) have demonstrated the ecological value and phytostabilization potential of *Agrostis*

Table 6.1: Characteristics of the contaminated soil used in the germination experiment. Data are presented as the mean values and standard deviations of the bulk soil and rhizospheric soil of *A. capillaris* and *F. rubra* growing in association. Concentrations of elements are all given in $mg \cdot kg^{-1}$.

Details	[1]**Bulk Soil**	[2]**Rhizosphere**
Clay	5%	-
Silt	20%	-
Sand	75%	-
pH-H2O	5.95±0.01	5.62±0.27
pH-KCl	5.14±0.03	5.37±0.37
K	6583±293	5534±716
Ca	1778±104	2463±655
S	490±5.6	410±154
Cu	71.9±3.0	84±12
Mn	4276±28	1857±36728
Fe	37408±159	23930±2684
Zn	4002±55	2910±493
Pb	3539±30	2796±425
As	296±5	237±48
Cd	39±0.1	36±6

[1]Data obtained from Vítková et al. (2016). [2] Data obtained from Teodoro et al. (2020a). More details about the site can be found in Ettler et al. (2005).

capillaris L. and *Festuca rubra* L. in soil contaminated mostly with Pb and Zn. The ability of nZVI to successfully stabilize contaminants in the selected soil has also been documented (Michálková et al. 2017; Mitzia et al. 2020; Wu et al. 2018).

6.3 Methods

Sources of Soil and Seeds

For the germination experiment, soil contaminated with Pb and Zn was used (Ettler et al. 2005; Vítková et al. 2016). The soil was collected from the mining and smelting area of Příbram, Czech Republic. The soil was obtained from 12 random sampling sites at two separate times (e.g., March and May, 2017).

After collection, the samples were air dried, homogenized and sieved at 2 mm. Characterization of the soil properties can be found in **Table 6.1**. The nZVI used was an air-stable product (NANOFER STAR) produced by Nano Iron Ltd. (Czech Republic).

Two sources of seeds were tested: (i) "commercial" seeds were purchased from a producer (Planta Naturallis, Czech Republic) and (ii) "native" seeds were obtained directly from the contaminated site. The latter were collected at two different times, namely March and May, 2017 using transect sampling. The seeds were extracted directly from the spikes and were preserved in glass containers at 3°C until their use in the experiment.

Germination Experiment

Prior the experiment, the seeds were disinfected using 30% H_2O_2 and then placed in petri dishes inside a germination chamber at 20°C and 65% relative humidity under a 16:8 h period of light/darkness. In the control dishes, 50 seeds were sown using filter paper as a substrate (10 replicates) while in the study dishes 20 seeds were sown (6 replicates) using the contaminated soil or using contaminated soil treated with 1% nZVI as the substrate.

The total number of germinated seeds was recorded daily starting from the 6th day. After 20 days the seedlings were harvested and root and shoot lengths were measured. The calculations performed include the relative germination rate (RGR, Eq. **6.1**) which is defined as the ratio of the total number of germinated seeds in the control (Gc) and in the treatment soils (Gs):

$$RGR = Gs/Gc \tag{6.1}$$

The elongation inhibition rate (EIR, Eq. **6.2**) is defined as the difference between the root lengths in the control (Lc) and in the treatment soils (Ls), using the formula:

$$EIR = \frac{Lc - Ls}{Lc \cdot 100} \tag{6.2}$$

And the germination index (GI, Eq. **6.3**):

$$GI = \frac{Gs \cdot Ls}{Gc \cdot Lc} \cdot 100 \tag{6.3}$$

Hydroponics Experiment

Native seeds of *A. capillaris* and *F. rubra* were pregerminated in vermiculite : substrate mixture (2:3 w/w), and representative specimens were transferred after 33 days to the hydroponic standard solution (pH 6.5, 2 mM $Ca(NO_3)_2$, 2 mM KNO_3, 1 mM NH_4NO_3, 1 mM KH_2PO_4, 0.5 mM $MgSO_4$, 25 μM H_3BO_3, 2 μM $MnSO_4$, 2 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.1 μM $(NH_4)_6Mo_7O_{24}$, 50 μM $NaCl$ and 20 μM $FeEDDHA$, and 2 mM $MES - KOH$; Eh=300 mV). A total of six plants were placed in a 1.5 L container equipped with an aerator to provide oxygen to the roots and later to maintain the nanoparticles in suspension. During a 29 day period, the solution was renewed regularly and for the final renewal, the solution was modified as follows: the source of Fe was replaced with $FeCl_2$ to avoid interference of the Fe-chelate with the absorption of nZVI (Martínez-Fernández et al. 2015a). From that day forward, four nZVI treatments were used: 0, 25, 50 and 100 mg L^{-1} (T0, T25, T50, T100) with five replicates (containers) per treatment. The optimal culture conditions regarding the availability of nutrients were established in previous experiments using a geochemical/statistical approach model for nanomaterials (Martínez-Fernández et al. 2015a). The growing period under hydroponic conditions took place inside a germination chamber with a regime of 16:8 h light/darkness at 24/18°C and 45% humidity.

One day after the nZVI applications, representative roots of each specimen were marked and their growths were measured after 3, 5 and 8 days. After 40 days in the hydroponic culture, the plants were harvested, separated into roots and shoots, weighed, washed with distilled water and frozen at -18°C for future usage. A portion of 5 g from samples obtained from control (T0) and the highest nZVI concentration (T100) were separated and lyophilized at constant weight. The lyophilized samples were kept under dry conditions until used for further analysis.

Analytical procedures

Five grams of each part/plant were weighed and the exact weight was recorded. These portions were then dried at 60°C for 5 days and the dry weights were measured.

The concentrations of the amino acid proline and of the total amino acids were extracted from 1 g of tissue. The frozen samples were homogenized with a mortar and pestle in liquid N_2 and then mixed with 15 mL of distilled water. The extraction of amino acids was performed by heating the solution (plant/water) at 100°C for 1 h. After filtration, proline determinations were conducted using the method of Bates et al. (1973) and the total amino acid content was determined according to Y. Lee and Takahashi (1966). The absorbances at 520 and 570 nm (proline and total amino acids, respectively) of the product were measured using a spectrophotometer with a calibration curve constructed using L-proline and glycine.

An InVia Raman spectrometer (Renishaw, Wotton-under-Edge, UK) equipped with a Leica confocal microscope was used to measure Raman spectra. The instrument was calibrated to a silicon Raman band at 520.5 cm^{-1}, and a 514.5 nm laser line was used with power 1 mW at the source. We have used an objective with the magnification of 50x (Leica, Wetzlar, Germany), the exposure time was 1s, and at each point, spectra were accumulated 15 times. The Raman spectra of lyophilized plant tissues were measured at 20 °C. The measurement were undertaken in three replicates at distinct positions within each sample. The presented spectra are averages of the three replicates. Raman data were processed using Wire 3.4 software (Renishaw, Wotton-Under-Edge, UK) and Grams AI 9.1 software (Thermo Fisher Scientific, Waltham, USA).

For the elemental concentrations, including Fe, in plant samples, 0.2 g of dried material was digested in 2 mL H_2O_2 and 8 mL of concentrated HNO_3 at 200°C on a hot plate, diluted to 25 mL with deionized water, filtered through a 0.45 μm pore nitro-cellulose syringe filter and analyzed by inductively coupled plasma optical emission spectrometry (ICP OES, 720ES, Varian Inc., CA, USA). For each set of samples, the reference plant material NCS ZC73018 (Bowen's Kale; IUPAC 1979) was also analyzed.

To avoid the dilution effect in plants of different proportions, the concentration of elements was recalculated to obtain the exact amount (in mg) of each element per plant (EP) using Eq. **6.4**:

$$EP = DW \cdot EC \tag{6.4}$$

Where DW is the plant's dry weight (g) and EC is the element concentration in the plant (in mg g^{-1}). The translocation factor (TF) was calculated as the ratio of the element concentration in the shoot to the concentration of the element in the root.

Sections of lyophilized plant roots were analyzed by scanning electron microscopy (SEM, TESCAN VEGA3XMU, Czech Republic) which was equipped with a Bruker QUANTAX200 electron dispersive X-ray spectrometer (EDS) to detect the elemental distribution across the roots with and without nZVI (T100 and T0, respectively). The lyophilized sections were placed on conductive tape and were carbon-coated before analysis.

Statistical Analysis

Values expressed as percentages were normalized by extracting the square root of each value and applying the arcsin function. The remaining variables were analyzed using the Shapiro-Wilk test and were normalized using a logarithmic function when needed. One-way ANOVA was performed to find the differences between treatments and Tukey's Honest Significant Difference method was used with a confidence level of 0.95. All statistical analyses were performed using R 3.6.1 software (The R Foundation for Statistical Computing 2018, under the GNU General Public License).

Table 6.2: Mean values ± standard deviation of the seedling characteristics after the germination experiment. Total germination (%), root and shoot length (cm), relative germination rate (RGR), elongation inhibition rate (EIR) and germination Index (GI). Treatments: control (C), contaminated soil, and contaminated soil + 1% nZVI (nZVI). Source of seeds: native from contaminated site and commercial. Different letters represent significant differences under Tukey's HSD test for $P < 0.05$.

Source	Treatment	Germ %	Root cm	Shoot cm	RGR	EIR	IG
			Agrostis capillaris				
Native	C	^{a}2.8±2.15	^{c}2.5±1.72	^{b}1.6±0.52	1.00	0.00	100.00
	Lit	^{b}19.2±3.76	^{a}0.3±0.17	^{a}1±0.78	6.85	90.73	63.43
	nZVI	^{a}3.3±4.08	^{b}0.5±0.38	^{b}1.8±0.71	1.19	80.14	23.64
Commercial	C	^{c}766.73	^{c}3.71.09	^{b}2±0.49	1.00	0.00	100.00
	Lit	^{d}91.7±7.53	^{b}0.8±0.31	^{c}3.5±0.87	1.21	78.34	26.12
	nZVI	^{d}90±10	^{b}0.8±0.25	^{c}3.2±0.65	1.18	78.97	24.91
			Festuca rubra				
Native	C	^{a}12±2.83	^{bc}3±1.73	^{a}4.2±4.19	1.00	0.00	100.00
	Lit	^{b}34.2±15.3	^{abc}2.4±1.39	^{c}6.9±2.34	2.85	19.37	229.58
	nZVI	^{b}32.5±12.94	^{a}b2±0.91	^{c}7.6±2.31	2.71	32.19	183.65
Commercial	C	^{a}22±8,54	^{c}3.3±1.25	^{a}3.5±1.21	1.00	0.00	100.00
	Lit	^{b}37.5±19.17	^{a}1.7±0.98	^{ab}4.6±2.08	1.70	48.76	87.34
	nZVI	^{b}35±11.4	^{abc}2.4±0.43	^{bc}6.9±1.34	1.59	27.81	114.85

6.4 Results and Discussion

Germination Test

A greater number of seeds germinated in soil (Litavka and Litavka + nZVI) than under the control variant on filter paper. This could simply be due to the humidity factor: well moisturized soil can preserve more water than filter paper. Humidity is a key factor for the germination of seeds as the first step in the germination process is water absorption, which leads to the development of the first organs (Teodoro 2011).

Seeds from different sources presented different responses: those obtained from the contaminated field (native) germinated at considerably lower rates than the commercial seeds (**Table 6.2**, **Figure 6.1**). Native seeds sown in untreated soil had the highest relative germination rate (RGR = 6.85) while for all other treatments, the RGR values were below 1.3; however, values greater

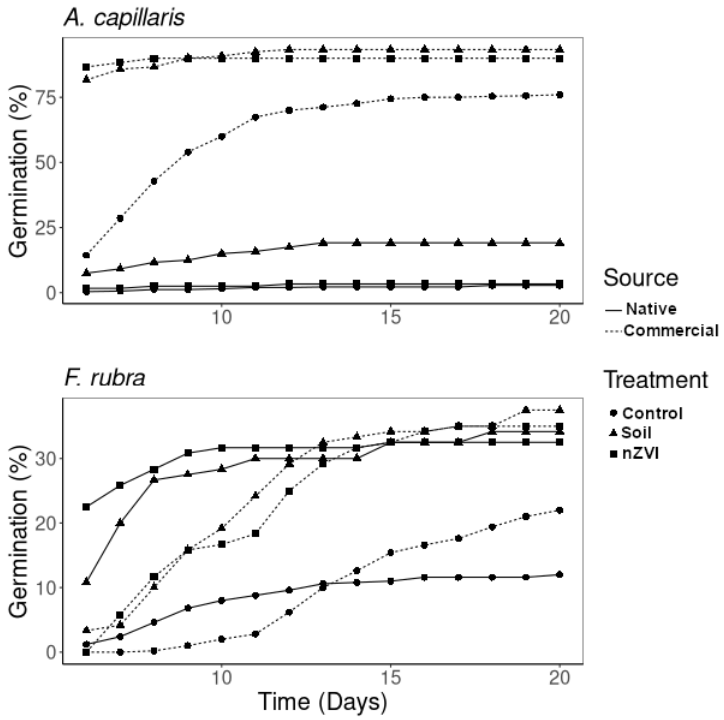

Figure 6.1: Evolution of total seed germination (%) with time (from day 6). Treatments: control, contaminated soil, and contaminated soil + 1% nZVI. Source of the seeds: Native from contaminated site and commercial.

than 1 for all cases reflect the increased total germination in comparison to the controls. Control seeds on filter paper developed longer radicles, as expected, due to the absence of physical obstacles and from the soil itself, which naturally causes mechanical damage to the tissues. In particular, nutrition for the seedlings is provided by the endosperm (Bareke 2018) and thus, nutrient acquisition is not necessary at this stage. The addition of nZVI enhanced the growth of the native seedlings of *A. capillaris* and resulted in lower elongation inhibition rates (EIR) in the nZVI treatment. However, in the commercial seeds, no differences were found between treated and untreated substrates (**Table 6.2**).

Application of nZVI did not significantly influence the germination of *F. rubra* seeds. Most of the native seeds of this species germinated during the first 8 days while the commercial seeds needed 12 days (**Figure 6.1**). Although no significant differences were found between these seed types, soil treatments and seed sources (**Table 6.2**), some differences in germination times were found

(**Figure 6.1**), as commercial seeds needed longer times to germinate while native seeds treated with nZVI reached maximum germination faster. The RGR was higher for native than for commercial seedlings (**Table 6.2**). Radicle lengths in the native seedlings showed no significant differences between soil treatments but for the commercial seeds, there was an increase in the lengths under nZVI treatment when compared to the untreated soil (**Table 6.2**). The effects of nZVI on *F. rubra* seedlings were more visible for the shoot lengths as the longest shoots were found on the seedlings treated with nZVI (**Table 6.2**). In addition to germination, root elongation is one of the first visible symptoms of toxicity: root tips are sensitive to toxicity due to the influence of metal(loid)s in cell division and cell elongation (Visioli et al. 2014). This effect is visible in the EIR of *F. rubra*, which was higher for the commercial seedlings growing in contaminated soil (48.8) and lower for the native seedlings in the same treatment (19.4), thus showing the adaptation of the native seeds for growth under metal(loid) contamination. Our results reflect that the application of nZVI not only presents no signs of toxicity in the early plant stages but also that for some seeds, application of the amendment improved their development.

Germination tests are commonly used to evaluate toxicity in plants. Our results show that not only different species (*F. rubra* seedlings grew longer than *A. capillaris* ones; **Table 6.2**) but also different seed sources (probably indicating different genotypes) responded differently: native seedlings of *A. capillaris* from the contaminated site exhibited higher EIR. For *F. rubra*, the addition of nZVI to the soil clearly improved the growth of the commercial seedlings but had showed no significant effect on the native seedlings. Germination experiments are a quick and effective way to test for toxicity in plants; it has been proven that application of elements such as Ni, Hg, Cd, Co, Cu and Pb result in reductions of germination rates and seedling growth (Visioli et al. 2014). However, different plant species react differently to the same components; Munzuroglu and Geckil (2002) found that applications of the same elements with the same concentrations had different effects on seed germination of wheat (*Triticum aestivum*) and cucumber (*Cucumis sativus*): wheat seeds are more sensitive to the presence of metal(loid)s whereas cucumber seeds can cope better with their presence. Thus, testing ENPs with the potential for metal(loid) stabilization is necessary and germination tests are useful tools for evaluating this potential.

Other ENPs have also been tested in plants with phytostabilization potential and have exhibited no risk of toxicity: silver ENPs had no effect on seed germination and plant growth of *Ricinus communis* L. (Yasur and Rani 2013); four kinds of Zn ENPs had no impact on the seed germination of Chinese cabbage (Xiang et al. 2015). In some cases, application of ENPs improved seedling development. A demonstration of this was found by Savithramma et al. (2012) by using silver ENPs on the seeds of *Boswellia ovalifoliolata*, which caused increased seed germination, seedling growth and hastened the germination period from 17 days in control to 8 days in treated seeds. The authors concluded that this effect could be due to the ability of the ENP to penetrate the seed coat, which could either lead to generation of new pores which allowed better nutrient flux or that ENPs may act as nutrient carriers. Research aimed at investigating the toxic effects of specific amendments and for specific plant species is an important factor for phytostabilization processes. To date, there is still very little information about the effects of nZVI on seeds and seedlings.

Hydroponics Experiment

Root growth after nZVI application to the hydroponic solution exhibited small variations during the first 5 days; the main findings were for *F. rubra* development under T50 and T100, which grew less than 13 mm, while all of the roots under the other treatments grew between 11 and 59 mm (**Figure 6.2**). After 8 days with nZVI in the solution, the effects were more evident.

The biomass production of *A. capillaris* was lower when it was not treated with nZVI (**Table 6.3**) but the roots grew longer (up to 108 mm in some replicates) than when nZVI was added to the growing medium (**Figure 6.2**). Plants treated with nZVI showed similar root biomass production and the root lengths were longer for T50, followed by T100 and T25; however, the differences were minimal (5 mm between treatment types). Regarding shoot biomass, fresh weight production followed the order: T100>T25>T50>T0 while no significant differences were found for the dry weights.

F. rubra did not show significant differences in root biomass production (**Table 6.3**) although differences between treatments were evident in the root

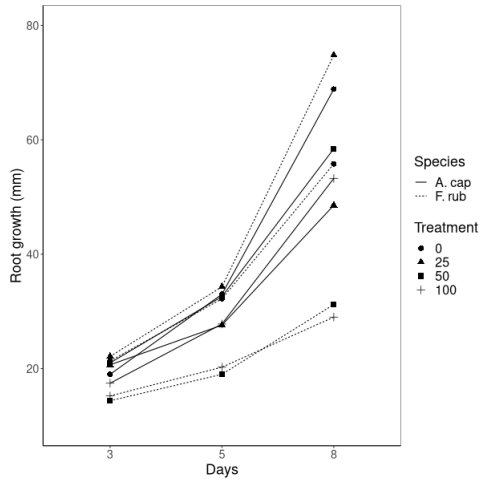

Figure 6.2: Root growth of *A. capilaris* and *F. rubra* after the application of 0, 25, 50 and 100 mg nZVI L^{-1} to the hydroponic solution.

lengths (**Figure 6.2**). Plants treated with 25 mg L^{-1} nZVI grew the longest roots (75 mm average at final time and a maximum value of 106 mm), followed by T0 (55 mm average) while the highest concentrations of nZVI (50 and 100 mg L^{-1}) developed the smallest roots.

Microscopic root Observations

Roots of plants growing in the hydroponic solution treated with 0 and 100 mg L^{-1} nZVI (T0 and T100, respectively) were investigated using SEM/EDS (**Figure 6.3**) and nZVI particles were observed adhering to the roots of plants under T100 (**Figure 6.3**-B4 and -D8). In agreement with this finding, nZVI particles adhering to plant roots (*Helianthus annuus* L. and *Lolium perenne* L.) have previously been identified using SEM/EDS by Vítková et al. (2018). nZVI particles usually form aggregates which limit their functionality for sorption and cause lower mobility of the product (Mitzia et al. 2020). On the root surfaces, they create a black coating that is visible without effort (Ma et al. 2013; Martínez-Fernández et al. 2015a), however such coating was not found in our samples. Nevertheless, nZVI was detected under electron microscope. Observations under bright-field optical microscopy in previous studies (Libralato et al. 2016) showed that the nZVI particles aggregated outside of the cell walls.

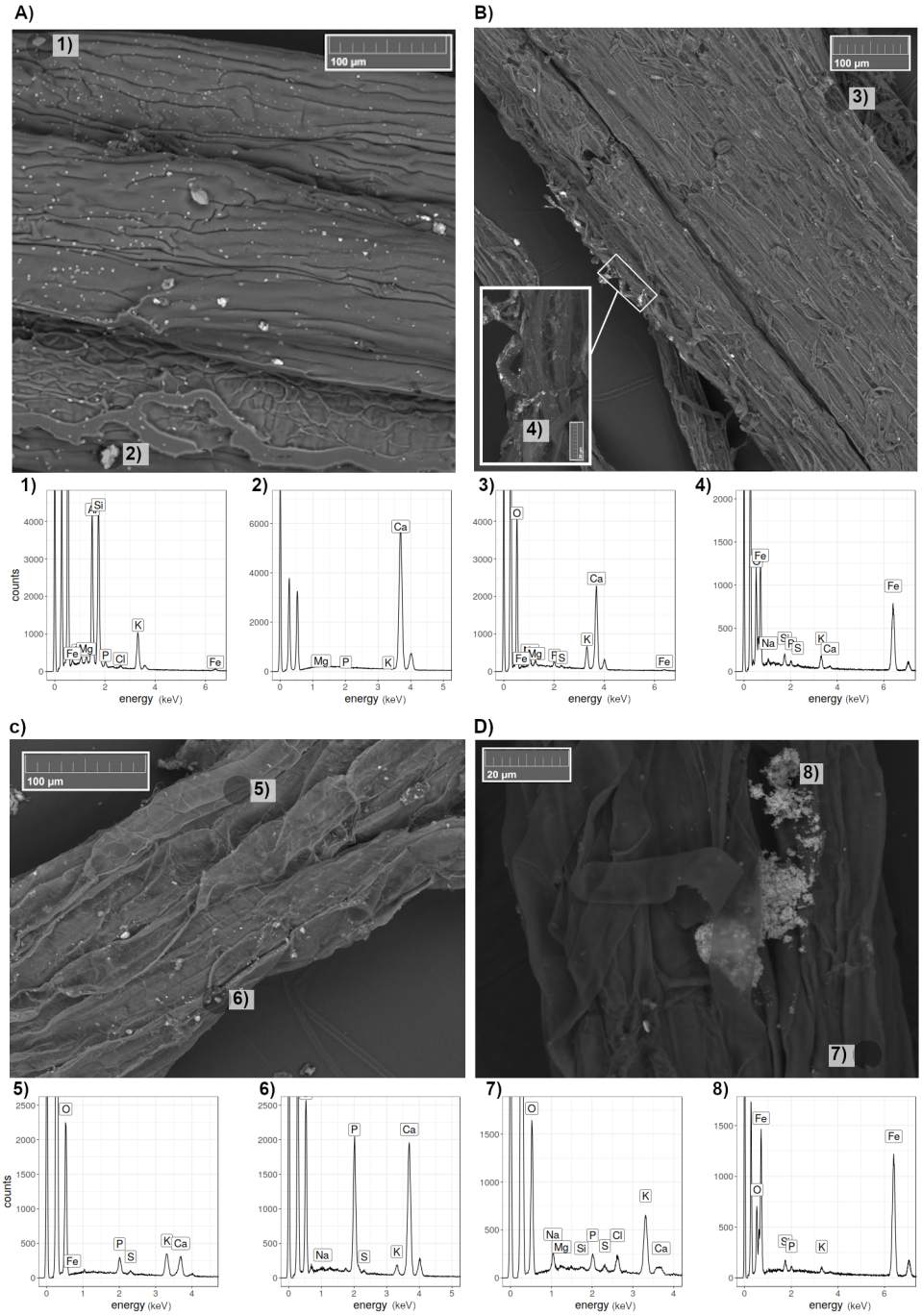

Figure 6.3: Roots of *A. capillaris* (above, A, and B) and *F. rubra* (below, C, and D) treated with (T100)/without (T0) nZVI (right and left, respectively) under a scanning electron microscope (SEM) with their corresponding EDS spectra (1-8).

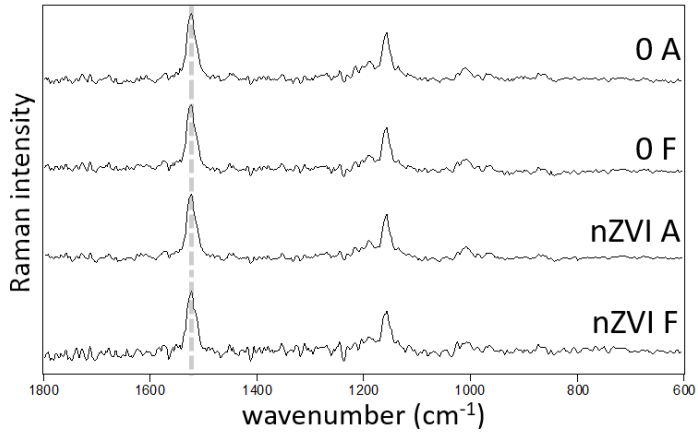

Figure 6.4: Raman spectra of *A. capillaris* (A) and *F. rubra* (F) untreated (0) and treated with 100 mg L^{-1} nZVI, presented as an average from three measurements at distinct zones of the leaf. The spectral features correspond to carotenoids; the v1(C=C) band position (dashed line) that may reflect changes in the carotenoid stucture remains the same (1523 cm^{-1}) among the measurements.

It has been hypothesized that such aggregation could be the reason for the reduction in water movement to the plant and thus, nutrient uptake, however this has not been clearly proved yet (Ma et al. 2013; Martínez-Fernández et al. 2015a).

Our observations of *F. rubra* roots under T0 also showed several particles of calcium phosphate (**Figure 6.3**-C6) adhering to the roots. These particles could possibly be aggregated due to the presence of $Ca(NO_3)_2$ in the hydroponic solution, which is known to control P release in solutions due to its oxidative effect on reduced substrates (Y. Zhan et al. 2019).

Physiological Parameters

For the stress markers analyzed in the plants at the end of the hydroponic experiment, no significant differences were found between treatments (**Table 6.3**, **Figure 6.4**). Proline is an amino acid that plays several roles in plants such as biosynthesis of proteins, scavenging of reactive oxygen species, protection of photosystems, and regulating cellular pH (Sharmila et al. 2017). Proline accumulation often occurs in the presence of elevated metal(loid)s concentra-

tions in plants (F. Wang et al. 2008), e.g., Ullah et al. (2019) found a strong correlation between Pb, Cr and proline concentrations in plant tissues. Proline concentrations can also be indicators of nutrient deficiency and of salt and/or drought stress (Kaur and Asthir 2015; Trovato et al. 2008). Our results showed no differences in the levels of proline and total amino acids (**Table 6.3**) in plant tissues with increasing nZVI concentrations, which can indicate no toxicity. The carotenoid composition and structure also show no differences between treatments (**Figure 6.4**). Carotenoids, non-enzymatic antioxidant pigments, are involved in the protection of chlorophyll production during oxidative stress; other studies have found differences in the carotenoid activity in plant species of the *Poaceae* family, in the presence of soil contamination, compared with no contamination or amended soils (Banerjee et al. 2019; Gajić et al. 2020; Teodoro et al. 2020b). Our results correlate well with other research which has found no or little effects of nZVI on the regular physiological functions of different plant species: concentrations of 0, 250 and 1,000 mg Kg^{-1} had no impact in seed germination, biomass production or root and shoot lengths of *Oryza sativa* (Wang et al. 2016a); likewise 5 and 50 g L^{-1} had no significant effects on the germination index, seedling elongation or dry weight of *Lepidium sativum, Sorghum saccharatum* and *Sinapis alba* (Libralato et al. 2016).

A. capillaris plants showed differences in the Fe concentrations in the roots, which were very low in the plants exposed to the lowest concentration of nZVI (25 mg L^{-1}) and were similar to those observed when no nZVI was applied (**Figure 6.5**). Significantly higher Fe concentrations were observed at higher nZVI doses. Consequently, a decrease in the translocation factor (TF) with an increase in nZVI in the growing solution was observed. Iron is required for various cellular processes in plants, including respiration, chlorophyll biosynthesis and photosynthesis and serves as a cofactor for enzymes involved in electron or oxygen transfer (Kobayashi et al. 2019). However, the mechanisms of uptake and accumulation of nZVI into the plant tissues is still uncertain; its movement within the plant is unknown. Copper usually binds well with Fe and thus its distribution in plants is similar with lower levels in plants with the lowest nZVI concentrations and increasing levels with higher doses of the amendment; this results in a decrease of TF with increases in nZVI concentrations in the hydroponic/growing solution.

Table 6.3: Plant characteristics at the end of the hydroponic experiment. Treatment (T) expresses mg of nZVI per L of hydroponic solution. ANOVA was performed for shoot and root separately. Different letters represent significant differences when using Tukey's HSD test $P < 0.05$.

T	Part	Fresh weight (g)	Dry weight (g)	Aminoacids μMg^{-1}	Proline μMg^{-1}
			Agrostis capillaris		
0	Shoot	^{a}8.72±1.68	^{a}2.17±1.56	^{a}0.15±0.04	^{a}5.21±2.48
25		^{a}21.56±4.39	^{a}4.13±0.85	^{a}0.1±0.06	^{a}4.02±2.36
50		^{ab}12.89±5	^{a}3.48±2.12	^{a}0.17±0.06	^{a}5.42±1.57
100		^{c}26.29±9.41	^{a}5.08±1.58	^{a}0.16±0.04	^{a}3.4±0.88
0	Root	^{a}5.11±1.08	^{a}0.43±0.09	^{a}0.07±0.02	^{a}2.71±2.68
25		^{b}16.76±3.43	^{ab}1.42±0.2	^{a}0.08±0.01	^{a}4.53±2.11
50		^{ab}15.87±10.15	^{ab}1.33±1	^{a}0.07±0.01	^{a}4.44±2.94
100		^{b}19.42±6.45	^{b}1.67±0.48	^{a}0.08±0.02	^{a}3.86±2.53
			Festuca rubra		
0	Shoot	^{a}12.21±5.11	^{a}2.16±0.76	^{a}0.16±0.04	^{a}5.93±2.77
25		^{a}9.63±4.68	^{a}1.87±0.8	^{a}0.16±0.03	^{a}4.31±1.47
50		^{a}8.89±3.29	^{a}1.78±0.61	^{a}0.16±0.06	^{a}3.21±1.08
100		^{a}10.05±5.07	^{a}2±1.04	^{a}0.14±0.03	^{a}4.42±0.85
0	Root	^{a}9.81±3.67	^{a}0.75±0.26	^{a}0.05±0.01	^{a}3.48±2.73
25		^{a}8.43±4.57	^{a}0.7±0.32	^{a}0.08±0.05	^{a}3.46±2.62
50		^{a}8.41±2.99	^{a}0.72±0.27	^{a}0.05±0.01	^{a}2.77±2.86
100		^{a}8.71±5.6	^{a}0.77±0.48	^{a}0.07±0.03	^{a}3.63±2.8

The application of increasing concentrations of nZVI also affected the uptake by *A. capillaris* of some major nutrients (**Figure 6.5** and **Figure 6.6**). The Ca, Mg and B concentrations in the shoots of plants treated with 25 and 100 mg L^{-1} were significantly different than those in plants with no nZVI application. A different effect was observed in the roots of this species, which showed great variability and only the Mg concentrations at the highest nZVI dose showed significant differences compared to those in the control (no nZVI) solution. The TF of B was highest in plants treated with 25 mg nZVI L^{-1} and means that, with this particular treatment, an increase in B transport to the upper plant parts took place. Other nutrients including K, Mo, Mn, Na and Zn were found in the lowest concentrations in the shoots of *A. capillaris* treated without nZVI and no other differences were found between treatments

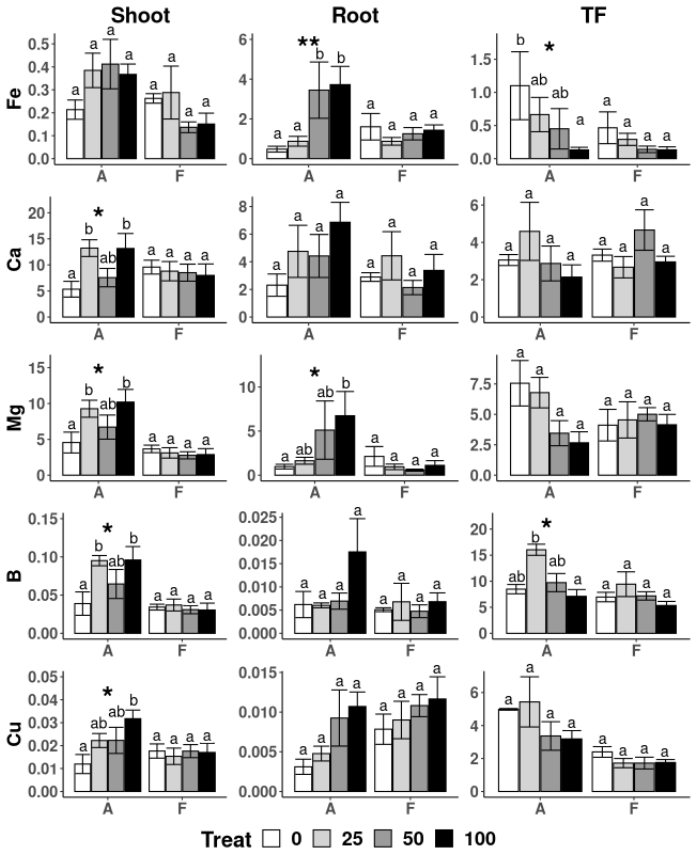

Figure 6.5: Translocation Factor (TF) and uptake of elements (g) in shoot and root biomass of *A. capillaris* (A) and *F. rubra* (F) at the end of the hydroponic experiment for the different treatments (e.g., 0, 25, 50 and 100 mg nZVI L^{-1}). For the one-way ANOVA *P $<$ 0.05, **P $<$ 0.01, no asterisks for P $>$ 0.05. Different letters above the bars represent significant differences according to Tukey's test at P $<$ 0.05.

(**Figure 6.6**).

Regarding *F. rubra* plants, no effects of the nZVI applications were found in the physical, physiological or chemical parameters measured (**Table 6.3**, **Figure 6.4**). Consistent with these findings, Martínez-Fernández and Komárek (2016) reported no significant effects on the growth of *Solanum lycopersicum* under hydroponic conditions with nZVI addition. Nevertheless, in the present experiment there was evidence of aggregation of nZVI particles onto the root surfaces of *F. rubra* (**Figure 6.3**-D). This suggests that *A. capillaris* possesses mechanisms which are able to assimilate nZVI more so than *F. rubra*. One

possibility could be the formation of iron plaque at the root levels. Research shows that the formation of iron plaque on the roots induces variations in the movement of nutrients and trace elements within the plant, e.g. increases of P, S and Mg uptake (Zandi et al. 2020; Zhang et al. 1999) which could explain the increase of Mg and Fe concentrations in the roots of *A. capillaris* at higher nZVI concentrations (**Figure 6.5**). Most of the information available on iron plaque formation comes from experiments performed on rice and other well-known halophytes or flood-resistant species. Our seeds were collected from a grassland dominated by *A. capillaris* that is regularly flooded, which suggests that this species and, in particular, this genotype could potentially be adapted to such conditions. Furthermore, other species of the family *Poaceae* also have been shown to develop iron plaque, such as *Paspalum urvillei* and *Setaria parviflora*, which grow in sand with excesses of iron and developed layers of Fe oxyhydroxide (ferrihydrite) in the cell walls and vacuoles; these were detected only by synchrotron μXANES analysis (Araujo et al. 2020). The evidence suggests that one of the mechanisms of *A. capillaris* to cope with nZVI could be the formation of iron plaque, however further investigation is necessary to evaluate this concept.

The two plant species have been found to grow in close association in multi-contaminated soil, and our previous studies have shown evidence of their potential as phytostabilizing plants (Teodoro et al. 2020a). The differences in their responses to the application of nZVI highlights the importance of biodiversity in phytoremediation projects and supports the possibility of nZVI applications as amendments for assisted phytostabilization using *A. capillaris* and *F. rubra* associations.

6.5 Conclusions

Two species of the family *Poaceae*, which grow in close association in a contaminated site, responded differently to the application of nZVI. In particular, the two species exhibited no symptoms of toxicity under nZVI application but rather showed support for the development of certain characteristics, especially for *A. capillaris*. The diversity in the behavior and response of each plant

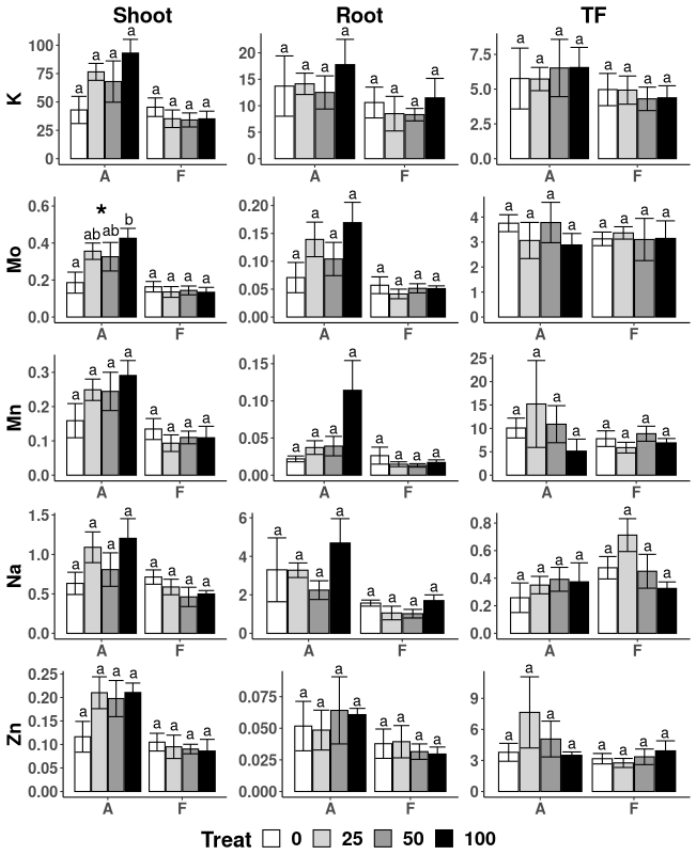

Figure 6.6: Translocation Factor (TF) and uptake of elements (g) in shoot and root biomass of *A. capillaris* (A) and *F. rubra* (F) at the end of the hydroponic experiment for the different treatments (e.g., 0, 25, 50 and 100 mg nZVI L^{-1}). For the one-way ANOVA *P $<$ 0.05, **P $<$ 0.01, no asterisks for P $>$ 0.05. Different letters above the bars represent significant differences according to Tukey's test at P $<$ 0.05.

species suggests that the use of *A. capillaris* and *F. rubra* in association could be the best strategy for successful stabilization of contaminants in soil when assisted by nZVI.

Additionally, our findings suggest that nZVI could be assimilated by the plants, although in different proportions depending on the species. *A. capillaris* develops longer roots and more biomass in the presence of nZVI. Hence we hypothesize that *A. capillaris* has mechanisms that can better assimilate nZVI than *F. rubra*. However, such mechanisms are still unknown and require investigation. Further research to study nZVI availability and mobility within

plants and iron plaque formation in the roots can provide valuable insights to understanding nZVI-plant interactions.

CHAPTER

SEVEN

SUMMARY

In order to guarantee the sustainability of a phytostabilization project, all the elements of the ecosystem and its modifications have to be taken into account. The ecosystem includes the community of living organisms in conjunction with the non-living components of their environment, their interactions and energy flows (Scheiner and Willig 2007). When stabilizing amendments are used in the ecosystem, studies on their interactions are crucial for its recovery. Therefore, particular cases have to be studied, regarding the specific contaminants in the soil, main plant species colonizing it, usage of the land, other living organisms interacting and the meteorological and edaphic characteristics. At the same time, common knowledge about different amendments, contaminants and plant species resistant to contamination provide us valuable tools as starting point to assess a particular problem of soil contamination. It is important to consider that once the amendments enter into the soil system, they become part of the ecosystem and their negative effects can be as difficult to correct as the contaminants themselves. This thesis proposes the process shown in **Figure 7.1** as a system that can help to make informed decisions, considering the appropriate elements and steps necessary to be evaluated before the application of amendments at field scale.

A good description of the site is important to understand the problem, propose solutions and project results. Therefore, the exploratory research is the first step in the process, and it should consider as many elements of the ecosystem as possible. Knowledge of the soil chemistry, crystallography, plant biology, agricultural practices and ecotoxicology provide a broad background for the development of phytostabilization strategies, e.g., information about the pH provides knowledge about the metal(oid)s mobility, information about the nutrients tells us what kind of fertilizers are needed, information about the local plants provides us with options for the restoration process, presence of slopes or strong winds require special techniques such as mulching (Mench et al. 2005). The research project presented here takes as a starting point a general knowledge of the studied site: previous research has been conducted there concerning different organisms living on the site, including the vegetation established there (Brizova 2008; Mayerova et al. 2017), metal(loid)s accumulation in edible mushrooms (Komárek et al. 2007), microbial activity (Donkova et al. 2009) arbuscular mycorrhiza fungi species adapted to the site (Malcová

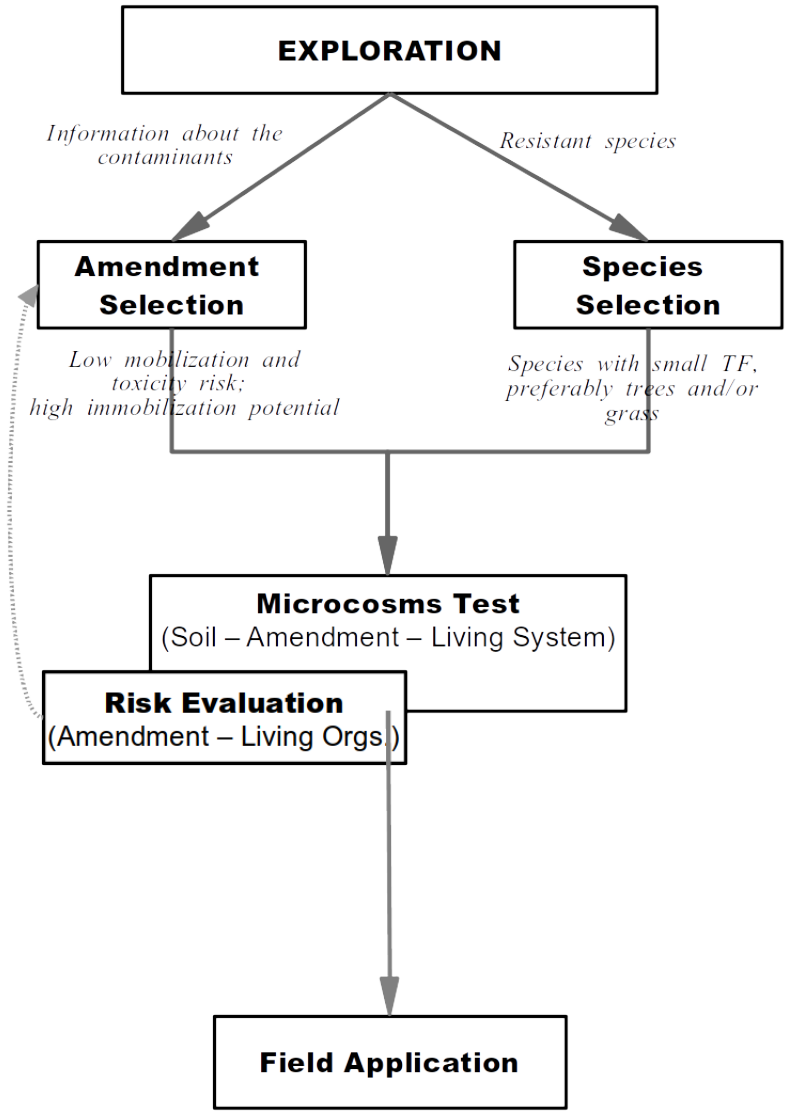

Figure 7.1: The process for the phytostabilization should begin with a good knowledge of the site (Exploration): weather, soil characteristics, geology, contaminants and ecological community. Based on this, plant species and suitable amendments can be chosen. The evaluation of the risk can be performed as part of the microcosms test, or separately, prior to the application at field scale. If a threat is found, a new amendment should be chosen. The present dissertation thesis takes as a target of study a soil contaminated by Pb, Zn, Cd, Cu and As. The site of study is located at the alluvium of the river Litavka, in the mining and smelting district of Příbram, Czech Republic. All our experiments originate from the same area.

et al. 2003) and a description of the adaptation of the moss *Bryum argenteum* (Hejcman et al. 2014), as well as the chemical and geological characteristics of the contaminants (Zak et al. 2009), including the speciation and distribution of Pb (Chrastny et al. 2014; Ettler et al. 2005; Ettler et al. 2004) and soil hydraulic properties (Sipek et al. 2019).

Several plant species resistant to this particular soil conditions have been studied in the past, however the research focused mostly on phytoextractors (Mayerova et al. 2017) such as *Salix* and *Populus* species (Komárek et al. 2008; Kubatova et al. 2016; Syc et al. 2012; Vondrackova et al. 2015) or relevant crops like corn (Komárek et al. 2007; Neugschwandtner et al. 2008) and sunflower (Martínez-Fernández et al. 2015a). To our knowledge, the only studies oriented to species with phytostabilizing potential growing on the studied site are for *Rumex obtusifolius* (Hejcman et al. 2012; Vondrackova et al. 2014). Hence in order to find more species with phytostabilization potential, our research begins with an exploratory evaluation of the community of grasses growing in the contaminated area.

Our focus of research was set on a grassland established naturally on alluvial soils. Species of the family *Poaceae* produce an extensive root system, creating a net that immobilize the contaminants at the root level; moreover, grasses present a rapid growth and establishment, quick adaptation, high longevity and easy maintenance (Doubkova and Sudova 2016; Kucharski et al. 2005; Mench et al. 2010; Mendez and Maier 2008). We evaluated concentrations of target contaminants (Pb, Zn, As and Cd) in the rhizosphere of three communities of grasses naturally established in the area of the Litavka alluvium: *Agrostis capillaris* with *Festuca rubra*, *Arrhenaterus elatius* with *Molinia caerulea* and *Calamagrostis epigeios*. Plant tissues were analysed, separated into below and above ground parts in order to obtain detailed information about the movement of contaminants into the above-ground biomass. Plants of *A. elatius* and *M. caerulea* translocated high amounts of Pb, As and Cd into the above-ground tissues. On the other hand, the association of *A. capillaris* and *F. rubra* proved to be the most suitable option for phytostabilization: our data showed that it correlates well with the concentrations of metal(oid)s in its below-ground biomass, while translocating relatively low amounts to the aboveground biomass, showing potential for reducing the mobility of the contaminants in soil. Hence

further research was performed using this particular species.

Once that adequate plant species have been chosen, the selection of a suitable amendment is another step that has to be carefully studied prior its application in the field. Some amendments can produce negative side effects under certain edaphic and/or meteorological conditions, such as toxicity or higher mobility of the contaminants. Therefore, the application of amendments to a particular soil has to be tested first in the laboratory. Due to the high levels of Pb, Zn, Cd and As in our studied soil, several studies have been conducted in order to find suitable amendments. Materials with good sorbent qualities like layered double hydroxides (LDHs) and mixed oxides (thermally treated LDHs; CLDHs) proved to be efficient for As, Pb and Zn sorption in aqueous solutions obtained from our soils (Hudcová et al. 2019). Application of engineered nanoparticles (ENP) of Fe and Mn oxides showed to be successful stabilizing Cd, Cu and Pb in batch and column experiments (Michálková et al. 2014), particularly a novel synthetic amorphous Mn oxide (AMO) was the most efficient due to its good adsorption capacity.

Application of ENP at the field scale still comes with several challenges that have to be considered. One of the main aspects is the cost/benefit relation, the price of applying ENP is dependent on the source and type of material, the amount produced, costs of transportation and, in some cases, also costs of laboratory and field experiments in the target area (Stefaniuk et al. 2016). Another aspect is its low mobility, ENP have the potential to aggregate, which reduces bioavailability, toxicity and mobility, resulting into a reduction of its potential ability to reach its target (Martínez-Fernández and Komárek 2016; Stefaniuk et al. 2016). On the other hand, the benefits of its application have to be balanced with its potential risks. Although toxicity for plants is rather scarce, the risk exists and it has to be researched prior application (Ghorbanpour et al. 2017). Meanwhile, the potential toxic effects of ENP to the soil micro biota is higher, and its implication for plant health have to be taken into account (Lefevre et al. 2016).

Application of biochar (BC) proved to be not only efficient reducing the mobility of contaminants in the studied soil (Mitzia et al. 2020) but also in improving soil hydraulic conductivity (Jacka et al. 2018; Sipek et al. 2019). The

mechanisms of metal(oid)s immobilization by BC have been widely investigated and described. These include chemical and physical sorption, precipitation, pH changes, increase concentrations of organic matter, P and C, and changes of the soil porosity and structure (Beesley et al. 2015). The co-composting of organic materials with activated BC has shown to increase the porosity size and specific surface area of the BC, as well as increase its ion exchange capacity which enhance the adsorption capacity of contaminants (Ye et al. 2019). Application of BC in soils with low fertility is an efficient strategy that can be successfully combined with other additives such as fertilizers (both, organic or inorganic) or further amendments. However, the source of the BC has to be carefully considered before its application to a contaminated soil. Some kinds of BC have proved to increase the availability of potential toxic elements and/or decrease soil micronutrient availability (Agegnehu et al. 2017; El-Naggar et al. 2019). Commercial BC with well-known characteristics usually has high cost; hence its application so far has been limited to small-scale proportions (El-Naggar et al. 2019). The combination of BC with a low-cost compost has the potential of balancing the cost/efficiency ratio.

Two materials were used in this work, nZVI and BC. One of the aims was to evaluate the efficiency of these additives as stabilizing agent of contaminants in our Litavka soil in the presence of plants, and at the same time, to evaluate its influence on plant development. Therefore, we performed an experiment using BC, in addition or not of compost, and in different proportions. The application of the amendments had no effect in the variables measured in the plants (chlorophyll production, total proteins and activity of enzymes related to oxidative stress) proving to have low or no risk of toxicity. Addition of compost successfully reduced the available fractions of contaminants in the soil (pore water and $CaCl_2$ extractable) and its movement to the plant, independently of the BC additions. Addition of BC improved the water retention capacity of the soil, and fastened the maturing of the compost. The different proportions of the mixtures had no effect in the parameters related to plant – rizosphere relations.

Application of compost clearly increases the availability of nutrients in the soil and reduces the mobility of metal(oid)s contaminants (pore water and $CaCl_2$ extractable and plant uptake) probably due to 1) the stabilization of

pH, 2) the ability of some contaminants to form stable metal chelates with humic substances and 3) the increase of the biological activity at the rhizosphere level (root exudates, microorganisms, etc.) thanks to the rise of nutrient availability (Forjan et al. 2018; Novak et al. 2019; Padmavathiamma and L. Y. Li 2012; Z. Yang et al. 2018). The addition of BC seems to have little or no influence in these processes: several studies have found, similar to the present one, no differences in the availability of contaminants and/or nutrients between different treatments (compost/BC ratio), but only between control and treated soils (Beesley et al. 2015; Novak et al. 2019; Oustriere et al. 2017). However, application of BC increased easily available water content (EAWC) in soil due, in all likelyhood, to the high porosity of BC and the loading of H_2O molecules on the BC surface through the hydrogen bonds (Jacka et al. 2018). The increase of the EAWC in combination with the effects obtained by addition of compost could have a positive impact for the plants in the long term, increasing the water disponibility and nutrient abailability. Hence, long term experiments are necessary to observe the full effect that BC could represent as amendment.

The addition of nZVI in previous batch experiments using Litavka soil caused a decrease of the contaminants in the $CaCl_2$ and H_2O extractable fractions of the soil, as well as a reduction of its mobility into both, the soil water solution and the plant system due to mechanisms such as (i) sorption onto both existing and newly formed Fe (hydr)oxides, (ii) formation of secondary Fe-As phases, and/or (iii) sorption onto Mn (hydr)oxides. (Michálková et al. 2017; Mitzia et al. 2020; Vítková et al. 2018, 2016). Not only the efficiency of nZVI as stabilizing amendment for the Litavka soil has been proved, but also its low toxic potential for plants (Martínez-Fernández et al. 2015a; Michálková et al. 2017), making it good candidate as amendment. However, little is know about its effects to microorganisms. Thus, in order to evaluate the nZVI-mediated metal(oid) behavior in the arbuscular mycorrhizal fungal (AMF)-maize association, we conducted an experiment where plants were grown in Litavka soil, with or without AMF inoculation (extracted from the same site), and with application or not of nZVI. The application of nZVI had little toxic effect on the plants by reducing root hydraulic conductivity and curling of green leaves, however it also reduced the mobility of the contaminants to the plant biomass. In any case its application inhibited AMF colonization to the roots. The inoculation

of AMF alleviated the physiological stress caused under nZVI application by improving the plant-water relations, which at the same time resulted in a higher uptake of both, nutrients and contaminants by the plant. The independent application of either nZVI or AMF had relatively similar effects in the movement of contaminants to the plant system. Our results provide information for making decisions in the selection of either AMF or nZVI, or in combination, as a support for the growth of the plants. However further research is needed using specific plants with phytostabilization potential. Moreover, a long-term experiment at field scale will provide valuable insight about indigenous mycorrhizal species, together with other microorganisms.

Some ENP made from different sources can have opposite effect in AMF, as described in a review by Varma et al. (2017), nano particles of Zn and Ag increased AMF growth and colonization in *Zea mays* and *Trifolium repens*, respectively. Nevertheless, according to Prasad (2010), this are some of the few exceptions, as most of the research done so far reflect rather negative effects of nano particles in microorganisms. The negative effects of ENP are usually related to its adherence into the root surface, due to their great surface activity, which causes a reduction in the water and nutrients uptake (Martínez-Fernández and Komárek 2016). Colonization by AMF can balance this effect by increasing root activity, where both, intra and extraradical mycelia help the plant to acquire nutrients at distances that plant roots cannot reach (Wang et al. 2016a). These effects are evident in our experiment as inoculation by AMF increased leaf water potential, which at the same time has been inhibited by the presence of nZVI. The improvement of the root activity benefits not only the plant, but also the microbial activity in the rhizosphere (Cao et al. 2016). As a result we have a system that is directly dependent on each other: increase of AMF colonization stimulates root activity and thus microbial activity; on the contrary, increase on the concentrations of ENP reduces root activity, hence reducing microbial activity (AMF included). Despite of the fact that research shows a clear reduction in the AMF colonization with the increase of ENP proportion in the soil, AMF colonization occurs even at the highest doses, indicating its tolerance to stress (Wang et al. 2016a). Our results show that application of AMF together with nZVI can help the plant to cope with side effects caused by the additives at the cost of reducing AMF colonization and

growth, but at the same time reducing the mobility of contaminants in the soil.

Other microcosms experiments have been conducted in the past using Litavka soil, with the goal of evaluating the interactions between plant – soil – amendment(s), although very few using potential phytostabilizers. *Rumex obtusifolius*, a weed widely spread in temperate grasslands, was found growing naturally in our studied site, proving to be resistant to contamination (Hejcman et al. 2012). Among the additives tested are superphosphate, quick lime (Vondrackova et al. 2014), nano-maghemite and nZVI (Martínez-Fernández et al. 2015a; Michálková et al. 2017).

Based on results from previous research we concluded that nZVI could be one of the best candidates for the stabilization of our target soil, but its interaction with other plant species like phytostabilizers is still unknown. Hence in order to assess the toxicological risk of nZVI in our selected species (*A. capillaris* and *F. rubra*), our final experiment was divided into two tests: germination of the seeds and plants growing under hydroponic conditions. Germination tests are typically used to evaluate potential toxicity in plants, resulting in a quick and simple method. Hydroponic experiments are a good strategy to evaluate the direct interaction between a particular additive and mature plants. Our results showed no negative effect of the application of nZVI in the physiological responses of the plants. However, *A. capillaris* developed longer roots and more biomass in the presence of nZVI, and it took up more extensively Mg and Ca. The differences in the responses of two plant species that grow in close association in a contaminated site is an indicator of the importance of the biodiversity in the process of phytostabilization. Therefore, we propose the association of *A. capillaris* and *F. rubra* as adequate species for a phytostabilization project of the alluvium of Litavka river, assisted by nZVI.

All our experiments used individual plant species for the tests, with the exception of the exploratory evaluation of the Litavka vegetation, which shows the importance of plants associations. Moreover, our results reflect that different plant species respond differently to similar edaphic conditions. However, little is known about the dynamics of this association: their interactions competing for space and nutrients, their influence in the rhizosphere, and thus, the response they might have, as an association of plants, when a new additive is applied

to their rhizosphere. According to Mench et al. (2006), the evaluation of the overall effectiveness of amendments for chemical immobilization should combine physicochemical and biological methods; it is important to survey several endpoints such as biodiversity, bioaccumulation in living organisms, changes in metabolite and protein patterns, as well as toxicity at different trophic levels. Further investigation is necessary using more elements of the ecological community, not only of plant associations, but their interactions with microorganisms and other living components, and at different levels of detail. Our results show that application of nZVI has minimal negative impact for our studied system, but it represents an important contribution for the stabilization of contaminants and the growth of the plants. Therefore, a long-term experiment on field conditions could be the best technique to directly evaluate the effects of phytostabilization with *A. capillaris* and *F. rubra* assisted by nZVI on the studied site.

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CURRICULUM VITAE AND LIST OF PUBLICATIONS

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EDUCATION

1016 - Present PhD. Applied and Landscape Ecology

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Thesis: "Interactions of soil microorganisms and plants with stabilising amendments in contaminated soils"

2012-2014 M.Sc. Natural Resources Conservation

University: Instituto Politecnico Nacional (IPN)
Faculty: Interdisciplinary Centre for Rural Investigations (CIIDIR, Oaxaca)
Thesis: "Study of plant community growing in tailing with silver mine wastes and its phytoremediation capacity"

2004-2008 Bsc. Forest Restoration

University: Chapingo University (UACH)
Faculty: Forestal Sciences Department (DICIFO)
Thesis: "Dormancy break of *Mimossa lacerata* seeds."

Publications

2020 - Manuel Teodoro, Lukáš Trakal, Brett N. Gallagher, Pavel Šimek, Peter Soudek, Micheal Pohořelý, Luke Beesley, Lukáš Jačka, Martin Kovář, Samar Seydsadr and Dinesh Mohan *Application of co-composted biochar significantly improved plant-growth relevant physical/chemical properties of a metal contaminated soil.* Chemosphere, 242: 125255.
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2019 - Ariana Latini, Giovanni Bacci, **Manuel Teodoro**, Daniele Mirabile Gattia, Annamaria Bevivino and Lukáš Trakal. *The impact of soil-applied biochars from different vegetal feedstocks on Durum Wheat plant performance and rhizospheric bacterial microbiota in low-metal contaminated soil.* Frontiers in Microbiology, 10: 2694.
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DOI: 10.1021/acs.est.7b05516

Participation in Projects

- GACR-17-25536Y "Phytoremediation of contaminated soils using nanoparticles: implications for the rhizosphere"
- CIGA-20174205 "Interactions of arbuscular mycorrhiza with nano zero-valent iron and their influence on metal(oid) behaviour in plant-soil system"

- IGA-4240013123156 “Effects of nZVI on the symbiosis AMF - plant: The use of species tolerant to contamination for assisted phyto-stabilization”
- QK1910056 Ministry of Agriculture. “Long-term test of the biochar application produced from waste biomass to solve drought in intensively farmed areas of the Czech Republic”

Participation in Conferences

August 2019 — Goldschmidt Conference. Barcelona, Spain

Poster "Seasonal development of Zn, Pb, As and Cd contents in the biomass of selected grass species growing on contaminated soils: Implications for phytostabilization".

October 2018 — XV International Phytotechnologies Conference. Novi Sad, Serbia

Presentation "Aided Phytostabilization: Effect of biochar and nano zero-valen iron on seed germination".

September 2017 — XIV International Phytotechnologies Conference. Montreal, Canada

Poster "Use of nano-iron for soil remediation: metal/metalloid behaviour within the rhizosphere".

July 2017 — IX International Conference on Mycorrhiza. Prague, Czech Republic.

Poster "Presence of arbuscular mycorrhizal fungi in indigenous grasses growing in contaminated soils".

April 2014 — Local symposium Jornadas Politecnicas. Oaxaca, Mexico.

Presentation "Phytoremediation as alternative for polluted soil restoration".

April 2014 — XVI National Congress of Agronomic Sciences. Chapingo, Mexico.

Presentation "Structural description of vegetation established in silver mine tailing in San Jeronimo Taviche, Oaxaca".

November 2013 — VII International Congress Red Latinoamericana de Ciencias Ambientales. San Carlos, Costa Rica

Main author of the presentation "Species richness of indigenous plants in tailing from silver mine wastes in Oaxaca, Mex."

Experience

Feb 2012 - Feb 2013 — Consultant. ETRADETER, S.C.

Environmental supervision in civil engineering and social projects

Feb 2011 - Jan 2012 — Supervisor. PROBOSQUE

Technical supervision for forest management programs to be in accordance with federal laws.

Oct 2008 - May 2009 — Technical support. CONAZA

Design and implementation of projects for Conservation and sustainable use of water and soil (program COUSSA).