PHYTOEXTRACTION OF CADMIUM, LEAD AND ZINC BY TWO METAL HYPERACCUMULATOR SPECIES AND METAL BIOAVAILABILITY IN THE RHIZOSPHERE

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Abstract

Contaminated soils pose a potential threat to any ecosystem and to biota. Decontaminating the affected areas is desirable, but often involves elaborate and expensive technical operations. Phytoextraction – the use of metal (hyper-)accumulating plants to extract the contaminant(s) – is being discussed as a low-cost technique for *in-situ* clean-up of metal-polluted soils.

The metal hyperaccumulator species *Arabidopsis halleri* and *Thlaspi caerulescens* were grown in rhizoboxes and rhizobags on two Czech Fluvisols with different levels of metal pollution in order to determine their phytoextraction potential and associated changes in bioavailable cadmium, lead and zinc concentrations in the rhizosphere.

The heavily polluted soil from Litavka contains (total in mg kg^{-1}) 27.4 Cd, 2460 Pb and 3220 Zn, the slightly polluted soil from Píšť any 1.4 Cd, 52.2 Pb and 186 Zn. Both soils have a similar texture (loamy sand) but a different pH (Litavka: 5.8; Píšť any: 7.0). The acetic acid extractable metal fractions reflect the bioavailable pollutant load.

Biomass production of roots and shoots and rosette diameters of both plant species were larger when grown on the heavily polluted soil Litavka in both experiments, indicating the plants' high metal tolerance. The higher pollutant level of soil Litavka was also reflected in larger metal concentrations in roots and shoots. Both plants were able to accumulate >4000 mg Zn kg^{-1} dry matter (d.m.) and >100 mg Cd kg^{-1} d.m. in their shoots, and with few exceptions, bioconcentration and translocation factors were >1, thus confirming the hyperaccumulation trait for these metals. In contrast, accumulation and translocation of lead by both plant species were small.

Independent of the soil trace metal concentration, the bioconcentration factor for cadmium and the cadmium content in biomass of *T. caerulescens* were higher than those of *A. halleri*. The translocation and bioconcentration factors for zinc and the zinc concentration in biomass were highest in *A. halleri* on the highly contaminated soil Litavka, whereas *T. caerulescens* was more efficient in accumulating zinc on the less polluted soil.

T. caerulescens accumulated 42 % and 18 % of the total soil cadmium during the 14 weeks of the experiment when grown on heavily and less polluted soil, respectively. Phytoextraction of zinc amounted to roughly 5 % of total zinc content in the root penetrated soil volume. However, the potential to remove zinc was dependent on the level of soil contamination as *A. halleri* absorbed more zinc on heavily polluted soil and *T. caerulescens* was superior on the soil with low trace metal concentration. The absorption of cadmium and zinc by *T. caerulescens* was reflected in the depletion of the acetic acid extractable metal fractions in the rhizosphere. Distinct rhizosphere activities might account for the observed mobilisation of trace metals on soil Litavka in the rhizosphere of *A. halleri*.

The results of this study confirm the high phytoextraction potential for cadmium and zinc of the investigated metal hyperaccumulator species and reveal the complex interaction with soil pollutant levels that needs to be addressed in the application of phytoextraction technologies.

Kurzfassung

Kontaminierte Böden stellen eine potentielle Gefahr für die Umwelt und für Lebewesen dar. Die Sanierung betroffener Areale ist zwar wünschenswert, gleichzeitig aber aufwändig und kostenintensiv. Phytoextraktion – der Einsatz von metall(hyper)akkumulierenden Pflanzen zur gezielten Extraktion von Kontaminanten – wird gegenwärtig als kostengünstige Maßnahme für die *in-situ* Sanierung verunreinigter Böden diskutiert.

Die spurenmetallhyperakkumulierenden Spezies *Arabidopsis halleri* und *Thlaspi caerulescens* wuchsen in Rhizobox- und Rhizobag-Experimenten auf zwei tschechischen Fluvisolen mit unterschiedlichem Grad der Spurenmetallbelastung, um das Phytoextraktionspotential und damit verbundene Veränderungen der pflanzenverfügbaren Cadmium-, Blei- und Zinkkonzentration in der Rhizosphäre zu ermitteln.

Der stark kontaminierte Boden aus Litavka enthält (Gesamt in mg kg^{-1}) 27,4 Cd, 2460 Pb und 3220 Zn, der Boden mit geringerer Belastung aus Píšťany 1,4 Cd, 52,2 Pb und 186 Zn. Beide Böden haben die gleiche Bodenart (lehmiger Sand), aber verschiedene pH-Werte (Litavka: 5,8; Píšťany: 7,0). Die Extraktion mit Essigsäure spiegelt den pflanzenverfügbaren Anteil der Spurenmetalle wider.

Die Biomasse der Wurzeln und der Sprosse, sowie die Durchmesser der Rosetten beider Arten waren auf dem stark kontaminierten Boden Litavka höher, was die hohe Metalltoleranz der Pflanzen bestätigt. Die höhere Metallbelastung des Bodens Litavka zeigt sich ebenfalls in den höheren Metallkonzentrationen in Wurzeln und Sprossen. Beide Spezies waren in der Lage >4000 mg Zn kg^{-1} Trockenmasse und >100 mg Cd kg^{-1} Trockenmasse zu akkumulieren. Die Biokonzentrations- und Translokationsfaktoren waren, bis auf wenige Ausnahmen, ebenfalls >1, was den Status der Pflanzen als Cd-/Zn-Hyperakkumulatoren untermauert. Demgegenüber waren die Akkumulation und die Translokation von Blei bei beiden Arten gering.

Die Biokonzentrationsfaktoren für Cadmium und die Konzentrationen von Cadmium in *Thlaspi caerulescens* waren, unabhängig von der Metallbelastung des Bodens, höher, als in der verwandten Spezies *Arabidopsis halleri*. Im Gegensatz zu Cadmium ist die Effizienz der Zinkaufnahme vom Grad der Belastung abhängig, da auf dem Boden mit hoher Metalllast *A. halleri* höhere Biokonzentrations- und Translokationsfaktoren sowie höhere Zinkkonzentrationen in der Biomasse aufwies. *T. caerulescens* wiederum war auf dem Boden mit der vergleichsweise geringen Metallbelastung effizienter.

T. caerulescens akkumulierte 42% (hoch kontaminierter Boden) bzw. 18% (gering kontaminierter Boden) des im Boden enthaltenen Cadmiums während des 14-wöchigen Experiments. Die Phytoextraktion von Zink belief sich auf beiden Böden auf etwa 5% des Gesamtgehaltes an

Zink im durchwurzelten Boden, wobei *A. halleri* auf dem stark kontaminierten Boden höhere Werte erzielte und *T. caerulescens* hingegen auf dem weniger belasteten Boden. Die Aufnahme von Cadmium und Zink durch *T. caerulescens* wurde durch die Verringerung der Essigsäure-extrahierbaren Metallfraktion des Bodens reflektiert. Unterschiede in der Wurzelaktivität der beiden Pflanzen könnten die beobachtete Mobilisierung von Metallen in der Rhizosphäre von *A. halleri* erklären.

Die Ergebnisse dieser Studie bekräftigen das große Phytoextraktionspotential für Cadmium und Zink der beiden untersuchten metallhyperakkumulierenden Pflanzenarten. Gleichzeitig verdeutlichen sie die Komplexität der Boden-Pflanze-Interaktionen, welche bei dem Einsatz von Phytoextraktionsmaßnahmen bedacht werden müssen.

Table of Contents

Index of Figures

Index of Tables

1. Introduction

The human impact on soils is manifold. Possibly resulting in soil erosion, soil compaction, sealing, loss of soil organic matter, decline in soil biodiversity, salinisation, floods and landslides and soil contamination.[1] Obvious examples of soil pollution could be waste disposal sites and fertilisation of agricultural areas. Abrasion of rubber tires, exhaustion fumes from cars and industry, pollution from mining and smelter increase the geogenic trace element[2] concentrations, as well.

However, soil is not exclusively a sink for various substances. It serves as a buffer and habitat and is intertwined and integrated in a network with the air, water and biota. Soil is a source for the mineral nutrition of many higher plants. Plants on the other hand, being on the lower end of the food chain, are an integral part of the diet of animals and humans. Potentially toxic substances can thus be transferred from soil to everyone's kitchen table. Recently, a headline in the *Frankfurter Allgemeine Zeitung*, a German supraregional newspaper with high circulation read "Zu viel Cadmium: Schatten über der edlen Kakaokultur" ("Too much cadmium: Shadow over the noble cocoa culture") (Hucklenbroich, 2010).

Reconstituting initial soil conditions is impossible, as soils have been used and altered considerably by man over time. Lowering the acute risk emanating from polluted arable lands by monitoring of and research on contaminated lands and the development of remediation techniques is encouraged by governments and international institutions. Of late, several institutes across Europe intended to collaborate in a biotechnological approach in soil bioremediation termed "UMBRELLA" ("**U**sing **m**icro**b**es for the **re**gulation of heavy meta**l** mobi**l**ity at ecosystem and landscape sc**a**le"), co-financed by the European Union. In addition to the use of microorganisms in on-site remediation projects, specific plants extract and concentrate selected elements from the environment, hence enabling removal of the element(s) in question from contaminated areas (Cunningham et al., 1995; Cunningham and Ow, 1996; Salt et al., 1995).

[1] Eight main threats to soils defined by the EU in its "Thematic Strategy for Soil Protection" (Commission of the European Communities, 2002).

[2] In colloquial language, the term "heavy metal" is widely used, usually referring to toxic elements but not being explicitly defined. Scientific use of the term is not encouraged by the "International Union of Pure and Applied Chemistry" (IUPAC), as meaning depends on the author's definition and could be based on density, atomic mass or atomic number (Duffus, 2002). Due to the different uses and non-existence of a universal definition, the term "heavy metal" will be avoided in this work and "trace metal" or "trace element" will be used instead.

1.1. An overview on plants

Plants are photoautotrophic organisms, meaning that they only rely on sunlight as a source of energy. During photosynthesis, light energy is converted to chemical energy, whereby carbon dioxide and water molecules are rearranged to form molecular oxygen, water and glucose.[3] Energy by itself is not sufficient for the synthesis of new plant material. More than 90% of all plant matter consists of carbon, oxygen and hydrogen molecules. The remaining 10% can be attributed to 16 additional essential chemical elements, which can be sorted by their main functions. They are required for building organic compounds in cells (nitrogen and sulphur), for structural integrity and catabolism (phosphorus, boron and silicon), ionic elements in cells supporting for example enzyme-functioning (potassium, sodium, magnesium, calcium, manganese and chlorine) and for electron transfer (iron, copper, zinc, molybdenum and nickel) (Campbell and Reece, 2002; Taiz and Zeiger, 2000). As these elements have some fundamental physiological function in a plant's life cycle or are required for structural integrity of cellular components, they are considered essential nutrients.[4]

Plants, in contrast to most animals, are immobile and have to cope with their immediate surroundings. Hence, they are obliged to make use of whatever is available to them. As they themselves cannot change the nutrient content in and chemical composition of the soil, roots have to selectively regulate uptake of compounds in order to ensure an optimal mineral nutrition of the whole plant. Plants have evolved diverse mechanisms to strive even under adverse conditions, such as absence and excess of water, extreme temperatures or seasonal changes thereof, limited supply of nutrients and surplus of salts; some species are specialised for specific ecological niches. On soils with elevated trace metal contents, these adaptations may range from the exclusion of toxic or abundant minerals to accumulation of specific trace metals.

1.1.1. Rhizosphere

A prerequisite for nutrient absorption is the bioavailability of the element in question. Sorption processes, weathering and biological degradation continuously influence the chemical equilibrium between the solid, gaseous and liquid phases of the soil system. Salts and their ions, colloids and organic solutes are thus passed to the interstitial water. Dissolved minerals are transported towards the plant via transpiration-driven mass flow and diffusion. At the root

[3] The process of photosynthesis can be summarized in the chemical equation:
$6\ CO_2 + 12\ H_2O + \text{photons} \rightarrow C_6H_{12}O_6 + 6\ O_2 + 6\ H_2O$

[4] Following Arnon and Stout (1939), an element is considered essential, if a deficiency in this nutrient hinders the plant to accomplish its life cycle and to produce a new generation of offspring. The element in question must be directly involved in the nutrition of a plant and characteristics of a deficiency would be specific for this one element, apart from possible amendments to better an unfavourable growth environment.

surface, inorganic ions in the soil solution[5] are absorbed and passed across the membrane, usually in exchange for other ions with the same charge.[6]

For obvious reasons, plant-induced changes in the soil body are most pronounced closest to the root surface. Additionally, various microorganisms[7] densely populate this area due to favourable chemical and physical changes in the soil. The influence of plant roots on the adjacent soil has already been described by Hiltner a century ago, who introduced the term "rhizosphere"[8] for the "zone of soil surrounding the root which is affected by it" (Hiltner, 1904).

By emitting exudates[9] from roots, plants can also alter "(...) the soil microbial community in their immediate vicinity, influence resistance to pests, support beneficial symbioses, alter the chemical and physical properties of the soil, and inhibit the growth of competing plant species" (Bertin et al., 2003).

Despite the introduction of protons to the soil, total soil pH is usually unaltered, as it is buffered by exchangeable base cations (Ca^{2+}, Na^{+}, Mg^{2+}, K^{+}). Nevertheless, a local lowering of soil pH either by direct release of protons or organic acids from the root or by dissolution of carbon dioxide in water and its conversion to carbonic acid and subsequent deprotonation to carbonates[10] increases the solubility of nutrients, among others phosphate, copper and zinc (Jones, 1998). Next to acidification, mobilisation of nutrients or metallic elements varies even with the type of acid secreted by plants (Schwab et al., 2008). Nevertheless, enhancing the mobilisation of macronutrients (e.g. zinc) might not necessarily correlate with an increased rate of zinc uptake (McGrath et al., 1997). Generally, excretion of protons and the resulting acidification and release of organic carbon compounds from roots are thought to enhance the solubility of essential nutrients and bioavailable elements in rhizosphere soils. Zhao et al. (2001) found no effect of carbonate exudates excreted by *Thlaspi caerulescens* on mobilisation of cadmium and zinc. But the excretion of low-molecular weight organic acids by rice (*Oryza sativa* (L.)) was found to increase

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5 Soil solution is here defined as the "dilute solution of electrolytes at equilibrium with definable solid phase and gas phase components of the soil" (Adams, 1974).

6 Protons (H^{+}) and hydroxides (OH^{-}) are dispensed by the root surface when taking up cations and anions, respectively (Haynes, 1990).

7 E.g. bacteria, fungi, algae, lichens, unicellular protozoa or nematodes.

8 In the original German version of the essay, the definition reads: "Der unmittelbar an eine Wurzel angrenzende Raum, der von ihr beeinflußt wird und sich durch eine besondere Flora und Fauna auszeichnet.". See Darrah (1993) for explanation and history of this term. Rhizosphere processes include physical (e.g. pressure exhibited by roots), chemical (e.g. change in redox potential, removal of water or dynamics of nutrients) and biological (e.g. accumulation of microorganisms, root exudation or oxygen release from the roots) effects.

9 Predominant exudates are sugars and amino acids (Campbell and Greaves, 1990), but all soluble chemical compounds occurring in plant cells could be lost to soil (Whipps, 1990).

10 Carbon dioxide results from the decomposition of organic matter and might react with the soil pore water to form carbonic acid, which might dissociate in two reactions to carbonate and protons:
$CO_2 + H_2O \rightleftarrows H_2CO_3 \rightleftarrows H^+ + HCO_3^- \rightleftarrows 2H^+ + CO_3^{2-}$ (Robbins, 1985).

bioavailable soil zinc concentrations (Hoffland et al., 2006) and cadmium was solubilised by exudates of *Nicotiana* spp. (Mench and Martin, 1991). Although these data were retrieved under uncontaminated or even deficient nutrient status, they show that plant-induced cadmium and zinc solubilisation is theoretically possible. Concerning trace metal mobilisation by *Arabidopsis halleri*, the effects of exudates have not been documented yet.

Changes in soil pH are often observed in rhizosphere soil, as plants counterbalance the charges resulting from the uptake of ionic nutrients by secreting protons or hydroxides (Haynes, 1990). The released ions may enhance the availability of a series of nutritive substances (Lucas and Davis, 1961) by altering the pH of the soil (if soil buffer capacity is exhausted). If acidification is the dominant mechanism by which *T. caerulescens* accumulates zinc and cadmium is being discussed controversially (Knight et al., 1997; Luo et al., 2000; McGrath et al., 1997; Wang et al., 2006).

1.1.2. Hyperaccumulators

The first who introduced the term "hyperaccumulator" for plant samples with an unusually high metal concentration in their tissues were Brooks et al. (1977). They described herbarium species containing more than 1000 mg nickel kg^{-1} dry matter in foliar matter. As different plants can accumulate a variety of different metals, the term was more generalised; accumulation of trace metals and translocation to any above-ground plant tissue of plants grown in their natural habitat, resulting in approximately 100 times higher concentrations than in normal species is pivotal (Baker and Brooks, 1989). In terms of zinc, lead and cadmium, the hyperaccumulation criterion applies, if metal concentration in leaves is greater than 10000 mg kg^{-1} (1%), 1000 mg kg^{-1} (0.1%) and 100 mg kg^{-1} plant dry matter (0.01%), respectively (Baker and Brooks, 1989; Shah and Nongkynrih, 2007).

Hyperaccumulating plants have been found in a variety of plant families, each assimilating one or more specific trace metals. *Arabidopsis halleri* and *Thlaspi caerulescens* are both members of the family *Brassicaceae* and both known for their ability to hyperaccumulate cadmium and zinc. Even though they produce rather little biomass, their advantage is a relatively short life-cycle, poor colonisation with mycorrhiza[11], their small size, which makes them ideal for pot experiments, and the widely-accepted implementation as model organisms (e.g. the related *Arabidopsis thaliana* in genetics and molecular biology and *Thlaspi caerulescens* or *Thlaspi goesingense* as hyper-

[11] Symbiosis with mycorrhiza in the family Brassicaceae (equivalent to "Cruciferae" (McNeill et al., 2006)) is not widespread (Newman and Reddell, 1987; Harley and Harley, 1987). According to Regvar et al. (2003), roots of *Thlaspi* spp. show signs of fungal colonisation. The authors believe that this symbiosis does not affect the plant's tolerance to and uptake of trace metals, as colonisation is faint.

accumulators) provides a multitude of background research. As *Thlaspi caerulescens* naturally accumulates cadmium and zinc in high concentrations, and has been known for hyper-accumulation for over a century (Sachs, 1865), this species has been investigated most (Reeves and Baker, 2000, cited in Küpper et al., 2000; Lasat, 2002). Measured trace metal concentrations of 43710 mg Zn kg^{-1}, 2740 mg Pb kg^{-1} (Reeves and Baker, 2000) and 12000 mg Cd kg^{-1} (Mádico et al., 1992) dry matter in the shoot tissue of *T. caerulescens* have been reported. Cadmium concentrations in *A. halleri* shoot tissue were found to exceed 1000 mg kg^{-1} dry matter (Zhao et al., 2006), lead concentrations 70 mg kg^{-1} dry matter (Bert et al., 2002) and zinc concentrations 32000 mg kg^{-1} dry matter (Zhao et al., 2000).

Adaptations at the physiological level are required to achieve these trace metal concentrations and to avoid phytotoxicity. In contrast to non-accumulating species, zinc transporters in the plasma membrane of *Thlaspi caerulescens* are much more abundant (Pence et al., 2000), metal sequestration in the root vacuole is reduced, enabling transport of zinc back across the tonoplast, the xylem loading rate is increased and transfer of zinc across the leaf cell plasma membrane is enhanced (Küpper et al., 1999; Lasat et al., 1998; Milner and Kochian, 2008; Papoyan et al., 2007).

The mitigation of hazardous environmental pollution mediated by natural hyperaccumulators or genetically modified plants, bacteria, fungi or algae in soils or water is termed bioremediation. In recent times, their potential to immobilise or degrade organic pollutants or to accumulate certain trace metals in harvestable material has been discussed as a possible *in-situ* decontamination method (Salt et al., 1995). The verification of *T. caerulescens* and *A. halleri* as cadmium and zinc hyperaccumulators makes them suitable candidates for further research in the field of phytoextraction.

1.2. Trace metals and their bioavailability in soils

Organic toxicants can potentially be alleviated by decomposition and degradation processes (Meagher, 2000). In contrast, metallic elements and ions cannot be eliminated. As chemical elements, mitigation of trace metals in soils occurs solely by adsorption to soil particles, complexation, speciation and precipitation or uptake into and metabolisation by biota (Eklund, 1995; Ernst, 1996; Yong et al., 1992).

Of the metal fractions associated with soil, free ionic metals and soluble complexes containing metallic elements are mobile and can diffuse towards living organisms in the soil (Lasat, 2000). Additionally, metals "adsorbed to inorganic soil constituents at ion exchange sites […] are readily available for plant uptake" (Lasat, 2000). Trace metals bound electrostatically to charged

soil surfaces are buffering trace metal concentration in soil solution (Whiting et al., 2003). Soils and trace metals are affected by a range of biotic and abiotic processes. As exchangeable trace metals are replenishing element concentrations in soil solution, they eventually become part of the fraction of soil, which is available for plant uptake (Marschner, 1995).

Besides, bioavailability requires the chemical element to become involved in an organism's metabolism (Adriano, 2001). Soil solution trace metal concentration is impacted by ionic strength, pH, competing ions, loading rate and counter ions in soil (Harter and Naidu, 2001). Trace metal mobility is also based on soil type and characteristics, such as amount of Fe- and Mn-oxides, cation exchange capacity (CEC), the soil's redox potential and organic matter content (Kookana et al., 1999; Römkens and Salomons, 1998). However, some fractions of an element might not be bioavailable at all.

Apart from soil parameters, plant species and genotype, the stage of the plant's life-cycle and exudation of organic acids or carbohydrates all interfere with the absorption of nutrients and non-essential elements. To assess the transfer of contaminants or chemicals from the environment into living tissue, the bioconcentration factor was introduced as the ratio of the concentration of the chemical in the plant or its organs and the contaminant concentration in the soil (see Equation 1 on page 15). In a general ranking of accumulated quantitative transfer of elements from the soil to the plant, zinc has been established in the front rank. Following are in decreasing order: copper, chromium, nickel, manganese, lead, cadmium, arsenic and mercury (Chojnacka et al., 2005).

Table 1: Statutory limits for cadmium, lead and zinc in arable lands (in mg kg^{-1} dry soil) of Germany, the Czech Republic and Austria. The Federal Republic of Germany decreed precaution values for trace metals in arable lands extracted with aqua regia (Bundesbodenschutz- und Altlastenverordnung). Regulations in the Czech Republic allow for the listed values in soil trace metal concentration in "aqua regia" extracts (Anonymous, 2001). In Austria, maximum levels of trace metal contamination are defined by the federal states themselves. The values presented here refer to the ÖNORM L 1075, which depicts admissible maximum levels of harmful substances.

	Germany	**Czech Republic**	**Austria**
Cadmium	0.4; 1.0; 1.5 [a]	0.4; 1.0 [b]	1.0 [c]; 0.5 [d]
Lead	40; 70; 100 [a]	100; 140 [b]	100 [c]
Zinc	60; 150; 200 [a]	130; 200 [b]	300 [c]

[a] For sandy soils; loamy soils; clays.
[b] For light soils; other soils.
[c] Benchmark (so-called "Richtwert") in ÖNORM L 1075.
[d] For light or slightly acidic soils.

The mobile pool of elements is equivalent to the sum of the soluble and the exchangeable element fractions in soil, specifying the bioavailable forms. In practice, the mobile pool of trace elements is related to their concentration in soil solution (Kabata-Pendias, 1995) or could be approximated from dried soils after elution with extracting agents. Essential nutrients can potentially hold a detrimental feature, as toxicity of trace metals is concentration dependent. The uptake into plants poses a potential threat of the trace metal being transferred to the food chain, hence governments have set critical values for a range of chemicals to be present in soils. Table 1 shows admissible levels of selected trace metal in arable lands in selected European countries.

2. Research Objective

Many hyperaccumulating plants have been examined for their potential of remediating trace element contaminated soils. Among the cadmium- and zinc-accumulating perennial plants, *Thlaspi caerulescens* and *Arabidopsis halleri* are the most explored species. However, information on the interaction of their phytoextraction potential with soil properties, in particular pollutant level, is still limited but needed for the improvement of phytoextraction applications. Moreover, little is still known about changes of metal bioavailability in the rhizosphere of phytoextraction crops.

Therefore the main objectives of this study were:

- To investigate the potential for phytoextraction of cadmium, lead and zinc of the two related hyperaccumulators *Arabidopsis halleri* and *Thlaspi caerulescens* at differing levels of soil pollution.
- To examine the impact of the plants on an extractable metal fraction in soil to assess possible effects of rhizosphere activities on metal bioavailability.

3. Material and Methods

3.1. Soil Pre-treatment

This study was conducted with two Fluvisols from the Czech Republic. Both feature trace metal contamination from anthropogenic sources, especially with cadmium, lead and zinc. Soils were sampled from the heavily polluted alluvium of the river Litavka and from the alluvium of the river Labe (called "Elbe" in German), near the village of Píšt'any. In this study, soils will be labelled "Litavka" and "Píšt'any", according to their points of origin. Concentrations of cadmium and zinc (see Table 2) on both sampling sites are above Czech statutory limits for arable lands of 0.4 mg kg^{-1} and 130 mg kg^{-1}, respectively (Anonymous, 2001). Table 2 depicts some soil characteristics and total cadmium, lead and zinc concentrations of the experimental soils.

Table 2: Important soil characteristics of those soils used in this study. Oxidisable carbon content ($Carbon_{ox}$) and pH were determined after extraction with 1M $K_2Cr_2O_7$ and 0.01M $CaCl_2$. Soil was sieved in order to determine the sand (particle size: 0.05 mm – 2 mm), silt (0.002 mm – 0.05mm) and clay (≤ 2 μm) fractions. Total trace metals concentrations were determined after digestion with aqua regia as described by Száková et al. (2009).

Location	$Carbon_{ox}$ (mg kg^{-1})	pH	Sand (g kg^{-1})	Silt (g kg^{-1})	Clay (g kg^{-1})	Cadmium (mg kg^{-1})	Lead (mg kg^{-1})	Zinc (mg kg^{-1})
Litavka	23.1	5.8	659	256	85	27.4	2460	3220
Píšt'any	37.5	7.0	654	229	116	1.4	52.2	186

Soil samples were dried and mixed thoroughly to ensure a homogeneous character in all conducted experiments. Soil was stored in plastic bags at outside temperature.

3.2. Preparation of Plants

Arabidopsis halleri (L.) O'KANE & AL-SHEHBAZ (ecotype: Litavka, Czech Republic) and *Thlaspi caerulescens* J. & C. PRESL (ecotype: Ganges, France) were sown into commercially available potting soil. At the two-leaf stage, plants were individually transferred to growth trays with separate cells filled with potting soil. Seedlings were watered twice daily with demineralised water.[12]

[12] For these experiments, only demineralised water was used for irrigation. On August 14th, 2008, pH of the water was 6.12 (pH-metre: regom instruments, WTW, Typ: pH 340i) and conductivity was measured to be 0.001 mS cm^{-1}.

3.3. Rhizobags

Six kilograms of soil were fertilised with 0.5 g nitrogen, 0.154 g phosphorus and 0.45 g potassium, supplied as NH_4NO_3 and K_2HPO_3, each dissolved in demineralised water. Fertiliser was well incorporated into the soil to prevent nutrient hot spots. Soil was sieved to ≤ 2 mm. One kilogram of the prepared soil was filled into a nylon bag with a mesh size of 43 µm, restricting growth of roots to the outer compartment, but not of root hairs.[13] These rhizobags were placed in the centre of commercially available five litre plastic buckets and surrounded by the remaining five kilograms of soil so that soil surfaces inside and outside the bags were on equal level. Drill holes in the bottom plate of buckets allowed drainage of excess water.

One sprout was planted centrally into the nylon bag of each bucket. Each soil-plant combination was prepared in quadruples.

Plants were watered twice a day and kept at semi-covered, rain-proof outdoor stands. Fungicide (0.15% Topsin®) was applied after two weeks of growth. The experiment was terminated after 14 weeks by harvesting plants.

3.4. Rhizoboxes

The rhizobox-system developed by Wenzel et al. (2001) was used in this experiment. Soil was sieved to ≤ 2 mm. Small quantities were filled to upper rhizobox compartments (50 x 64 x 130, height x depth x width (in mm)), sprayed with demineralised water and slightly compacted. Repeating this procedure ensured an even soil density within each compartment and between compartments. Three wicks for water supply were inserted into each box via lateral sockets as soon as the compacted soil was on the same level as the opening and covered with further soil layers. On average, 461 g ± 8 g[14] dry soil from locality Litavka and 567 g ± 6 g dry soil from locality Píšť'any were processed per box.

As much potting soil as possible was removed from roots of pre-grown seedlings. Three prepared plants were planted in a row into each box, when the soil level was approximately 15 mm

[13] Assuming a mean root hair diameter of 5-17 µm for Angiosperms in general (Jungk, 2001), and more specifically 6-7 µm for plants of genus *Arabidopsis* (Ketelaar et al., 2008) and 10 µm for *Thlaspi goesingense* (Himmelbauer et al., 2005). Diameter of roots themselves amounts to 80-100 µm in genus *Arabidopsis* (Bowman, 1994) and in family *Brassicaceae*, root diameter of main roots is specified with up to 1.2 mm (*Cardamine pratensis*), in rare cases even exceeding 1 cm (*Lepidium crassifolium*). Secondary roots commonly range from 0.7 mm to 0.9 mm in diameter in *Brassicaceae* (Kutschera et al., 1992).

[14] Representing ± one standard error of the mean.

below its upper rim. Boxes were then filled to the top with soil. In total, eight boxes were prepared per soil. Four compartments of each soil were cropped with *A. halleri*, whereas the second half was planted with *T. caerulescens*.

All upper compartments were kept in an air-conditioned room (21 °C) in the greenhouse. The incorporated wicks were inserted into an elevated water reservoir for continuous passive watering. Plants were additionally watered twice a day from above.

Some plants did not cope with the transfer to contaminated soil or were slightly damaged in the process, as they wilted within one week. These were replaced by new ones of the same age.

Fungicide (Topsin®) in a concentration of 0.15% was sprayed on all plants in the upper parts of the rhizobox-system 20 days after planting.

Lower compartments were prepared another three weeks later. Four wicks were inserted and placed to the rear boundary of each compartment at differing heights for an even water supply. Bottom plates of upper parts were removed and upper and lower compartments containing soil from the same sampling site were assembled, hence enabling roots to grow through the slit into the free space between the acrylic window and the membrane. The membrane's mesh size was 43 µm and restricted growth of roots, but not of root hairs, to the soil-only compartment.[15] To protect roots from sunlight, the lower part of the system was wrapped with aluminium foil. All wicks were inserted into elevated plastic water-reservoirs containing demineralised water for irrigation.

The speed of root growth into the free space in the lower compartment varied considerable between plant species and soil type. The clamp device for fixing the acrylic window was installed 33 days after assembly. Plants were harvested ten weeks after having assembled the upper and lower compartment.

[15] See footnote on page 10 on diameter of root hairs and roots.

3.5. Analysis of plant material

3.5.1. Plant harvest

Foliar plant material was sampled by cutting plants right above the substrate's surface. Above-ground biomass was cleaned by washing with demineralised water and blotted dry. Fresh weight was recorded and plant tissue was dried in aluminium trays at 40°C. Roots were separated from soil particles as good as possible, rinsed with demineralised water and blotted dry. Fresh weight was taken before roots were dried at elevated room temperature (~ 25°C). Dry weight was measured prior to homogenisation of plant tissue in an ordinary kitchen blender. Root and shoot samples were stored separately in paper bags.

3.5.2. Trace metals in plant material

Approximately one gram of plant material was transferred to a glass digestion flask. The exact weight of the sample was recorded to three positions after decimal for later determination of trace metal concentration. If possible, two aliquots of each plant sample were analysed. Biomass was dry-ashed and its residues were dissolved in 20 mL 1.5 % HNO_3. Individual test tubes were sealed with Parafilm® and kept in the fridge (4°C) until analysis. Blanks without plant matter amounted to ten per cent of total sample number. Trace metal concentration was determined by inductively coupled plasma optical emission spectroscopy with axial plasma configuration (ICP-OES; VARIAN, VistaPro, equipped with auto-sampler SPS-5, Australia) at the Department of Agrochemistry and Plant Nutrition at the Czech University of Life Sciences (CZU - CULS), Prague, Czech Republic.

3.6. Analysis of soil material

Different extracting agents were used in order to determine initial total trace metal concentration in both soils and the exchangeable fractions of selected trace metals of experimental soils.

3.6.1. Rhizobag soil sampling

Batches of soil from inside and from outside the rhizobag were taken, dried at outside temperatures, ground and sieved to ≤ 2 mm. Individual samples were stored in plastic bags in the dark at room temperature.

3.6.2. Rhizobox soil sampling

At point of harvest, different soil fractions from rhizoboxes were sampled for analysis. From the upper compartment, a single soil sample devoid of plant material (especially roots) was withdrawn. Soil in the lower compartment of rhizoboxes was chipped into sections measuring 2 mm in thickness using a slicing device developed by Fitz et al. (2003a). Starting at the membrane-side of the lower compartment, four slices were sampled.[16] A fifth soil sample was collected towards the rear of the box, referred to as "bulk soil" in this study. Weight of all samples was recorded before and after soil samples were dried in petri dishes at room temperature. Dry soil samples were transferred to small plastic bags for storage.

3.6.3. Aqua regia digestion

Total trace metal concentration in soil was determined with aqua regia as described by Száková et al. (2009) before beginning the experiments.

3.6.4. Organic carbon content of soil

Organic carbon content of soil samples was determined spectrophotometrically after oxidation of the organic fraction of soil with $K_2Cr_2O_7$ following the procedure of Sims and Haby (1971). 1g of soil was agitated well with 10 mL 1M potassium dichromate and 20 mL sulphuric acid (conc.) and left to stand for 20 minutes. Distilled water was used to bring the volume to 100 mL. The suspension was filtered and absorbance of the filtrate was measured at 600 nm.

3.6.5. Determination of soil pH

Soil pH was determined in an aqueous 0.01 M $CaCl_2$ extract. A soil sample of 1 g was mixed with 20 mL of $CaCl_2$, left for equilibration and filtered. The pH of the filtrate was determined (pH-metre: regom instruments, WTW, Typ: pH 340i). The room temperature was kept constant at 20 ± 1 °C during the procedure.

[16] Due to the permeability of the membrane by root hairs, soil in the first slice (0-2 mm) was likely directly influenced by the plants, as root hair length of *Thlaspi caerulescens* varies from 1.9 mm to 2.1 mm on zinc-rich soil (Whiting et al., 2000; Whiting et al., 2001). Similar values were assumed for *Arabidopsis halleri*.

3.6.6. Extraction with acetic acid

Extraction with 0.11 M acetic acid is supposed to liberate bioavailable and exchangeable trace metals and carbonate salts (Chen et al., 2001) and partially trace metals bound to oxides (Száková et al, 2000), therefore possibly overestimating trace metal concentrations potentially available for plant uptake.

Aliquots of soil samples weighing one gram were agitated with 20 mL 0.11 M acetic acid overnight (16 h). The liquid phase was poured to test tubes after centrifugation for 10 minutes at 20 °C, 2000 *g*. Vials were sealed with Parafilm®, stored in the fridge (4°C) and analysed as soon as possible.

3.6.7. Analysis of soil extracts

If not specified differently, cadmium and lead concentrations in the extracts from soil Píšť any were measured by graphite furnace atomic absorption spectroscopy (GF-AAS) at the Department of Chemistry at CZU, Prague. The higher concentration of these elements in soil Litavka and the zinc concentration from both soils permitted determination by ICP-OES (VARIAN, VistaPro, equipped with auto-sampler SPS-5, Australia) at the Department of Agrochemistry and Plant Nutrition at CZU, Prague.

3.7. Calculation of hyperaccumulation criteria

As hyperaccumulation is characterised by a high uptake of contaminants from the soil and a high translocation to aerial plant parts, the bioconcentration factor and the translocation factor were used to verify the extent of the hyperaccumulation potential.

3.7.1. Bioconcentration Factor

The bioconcentration factor was calculated according to Equation 1.

$$Bioconcentration\ Factor = \frac{Concentration_{Plant}}{Concentration_{Soil}} \quad \text{(Equation 1)}$$

3.7.2. Translocation Factor

The translocation factor (see Equation 2) assesses the ratio of trace metal concentration in shoots to the concentration in roots.

$$Translocation\ Factor = \frac{Concentration_{Shoots}}{Concentration_{Roots}} \quad \text{(Equation 2)}$$

3.8. Verification of accuracy of measurements

At least ten per cent of the sample number was assigned for pure extractants, which were carried along in the process of trace metal extraction.

Tobacco leaves CRM CTA-OTL-1 with certified trace metal concentrations were additionally analysed in duplicates for monitoring data quality. Attested cadmium, lead and zinc concentrations are 1.12 ± 0.12 mg kg^{-1}, 4.91 ± 0.80 mg kg^{-1} and 49.9 ± 2.4 mg kg^{-1}, respectively. Determined trace metal concentrations in tobacco leaves averaged at 1.13 ± 0.10 mg kg^{-1}, 4.84 ± 0.41 mg kg^{-1} and 51.5 ± 3.4 mg kg^{-1} for cadmium, lead and zinc, respectively.

3.9. Statistical analysis

The arithmetic mean ($\bar{x}$; Equation 3) and standard error of the mean (SEM; Equation 4) were determined. Sample size (n) equalled 4, if not stated differently.

Analysis of variance (one-way ANOVA) was performed using PASW®Statistics, Version 18.0.0, for determination of differences in trace metal concentrations and contents in plant biomass and soil resulting from growth of either species. Significance for statistical tests was accepted at $\alpha = 0.05$. ANOVA-presuppositions of homoscedasticity and normal distribution were checked using Levene's Test for homogeneity of variance and Shapiro-Wilk-Test. Posthoc Tests were attempted but not valid for the given data structure.

$$\bar{x} = \frac{1}{n-1}\sum_{i=1}^{n} x_i \qquad \text{(Equation 3)}$$

$$SE = \frac{\sqrt{\frac{\sum (x-\bar{x})^2}{(n-1)}}}{\sqrt{n}} \qquad \text{(Equation 4)}$$

4. Results

The successive sections will present the results of the rhizobag- and the rhizobox-experiment separately.

4.1. Rhizobags

Two *T. caerulescens* plants growing on soil Píšť any died until the end of the experiment. The first died after installation of micro-suction cups and the second wilted after the first sampling of soil solution. Retrievable biomass of these plants was analysed, but does not contribute to determined average values presented later.

One of the *A. halleri* plants growing on soil Píšť any was affected by caterpillars during the growth period, possibly resulting in its small growth and deformed leaves.

Table 3: Mean values (± SEM) of plant growth characteristics (biomass is given in g) in the rhizobag experiment at harvest (n=4; *: n=2).

Soil	Species	Diameter of rosette (cm)	Fresh weight of shoots	Dry weight of shoots	Fresh weight of roots	Dry weight of roots
Litavka	*Arabidopsis halleri*	27.4 (± 0.8)	166 (± 10)	27.9 (± 2.0)	29.0 (± 2.3)	4.4 (± 0.6)
	Thlaspi caerulescens	24.9 (± 1.1)	164 (± 10)	24.7 (± 2.3)	22.6 (± 1.2)	4.1 (± 0.4)
Píšť any	*Arabidopsis halleri*	18.2 (± 2.9)	47 (± 14)	10.0 (± 3.0)	4.0 (± 1.3)	1.4 (± 0.6)
	*Thlaspi caerulescens**	20.7 (± 3.1)	85 (± 10)	9.8 (± 0.9)	10.0 (± 0.5)	2.4 (±0.3)

At harvest, plants grown on soil Litavka outperformed plants from soil Píšť any in diameter of the rosette and mass of shoots and roots. *A. halleri* produced on average more biomass than *T. caerulescens* when both were grown on soil Litavka. *A. halleri* plants from the less polluted soil Píšť any have grown to a lesser extent than *T. caerulescens* (Table 3).

4.1.1. Cadmium in plant biomass

As pictured in Figure 1, both plant species accumulated considerably more cadmium in shoot material than in roots when grown on soil Litavka. *T. caerulescens* absorbed significantly more cadmium than *A. halleri* per kilogram dry matter in shoots and roots on both soils.

Cadmium content (Table 4) in individual specimen of *A. halleri* ranged from 2.6 mg to 4.1 mg on soil Litavka and from 40 µg to 230 µg on soil Píšť'any. Examples of *T. caerulescens* grown in rhizobags incorporated between 9.9 mg and 15.1 mg cadmium on soil Litavka and between 233 µg and 579 µg cadmium on soil Píšť'any. The differences in cadmium content between both plant species grown on either soil were significant.

The cadmium concentration in the growth substrate was reflected in the plant material: plants grown on the highly contaminated soil Litavka had larger cadmium concentrations than those grown on soil Píšť'any. According to the bioconcentration factors, plants from soil Píšť'any accumulated relatively more cadmium (Table 4).

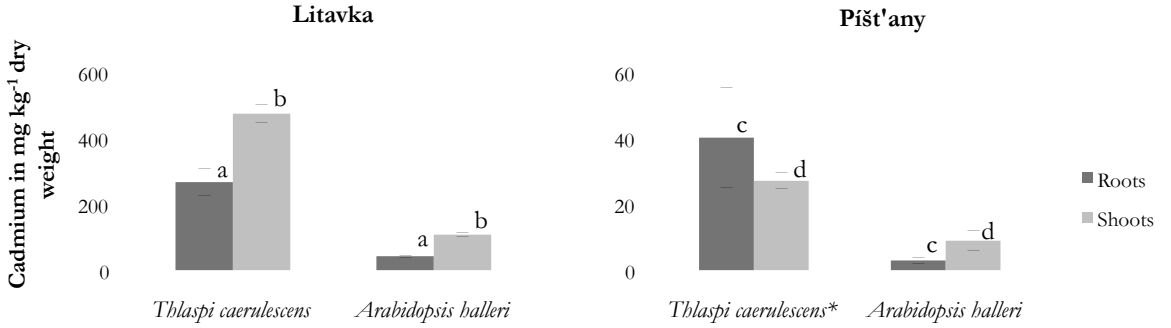

Figure 1: Cadmium concentrations in shoot and root material of *A. halleri* and *T. caerulescens* in mg kg^{-1} grown on soils Litavka and Píšť'any (n=4; *:n=2). Error bars indicate standard error of the mean. Data marked by the same letter are statistically different with $\alpha = 0.05$.[17,18]

Table 4: Cadmium content (in µg) in biomass and bioconcentration and translocation factors (± SEM) of cadmium for *Arabidopsis halleri* and *Thlaspi caerulescens* grown on experimental soils (n=4; *:n=2).

Soil	**Litavka**		**Píšť'any**	
Species	*Arabidopsis halleri*	*Thlaspi caerulescens*	*Arabidopsis halleri*	*Thlaspi caerulescens**
Cadmium content	3200 (± 320)	12800 (± 1120)	97 (± 47)	360 (± 39)
Bioconcentration factor	3.6 (± 0.2)	16.2 (±0.6)	6.0 (± 2.1)	21.2 (± 0.4)
Translocation factor	2.6 (± 0.2)	1.9 (± 0.3)	3.1 (± 0.4)	0.8 (± 0.3)

[17] Homoscedasticity (homogeneity of variance) was not observed for data on cadmium concentration of plants grown on soil Píšť'any. However, the F-test is supposed to be robust to violations of this assumption (Lindeman, 1991).

[18] Normal distribution of data was not identifiable for *T. caerulescens* grown on soil Píšť'any, as n=2.

4.1.2. Lead in plant biomass

The lead concentration in both species was higher in the root material, regardless of the extent of the initial soil contamination (Figure 2). The shoot lead concentration of plants grown on soil Litavka amounted to less than 5% of plant root concentration. The difference in the level of contamination between root and shoot material was less pronounced on the less contaminated soil Píšť any. The lead concentration in shoots of *A. halleri* grown on soil Litavka was significantly higher than in shoots of *T. caerulescens*. On soil Píšť any, differences between species in lead concentration of shoots and of roots and lead content in plant material were not significant with $\alpha = 0.05$.

Lead content (Table 5) in whole plants of species *Thlaspi caerulescens* grown on soil Litavka varied from 0.9 mg to 2.6 mg and from 19 µg to 51 µg when grown on soil Píšť any. *A. halleri* incorporated from 2.2 mg to 4.2 mg and from 8 µg to 29 µg on soil Litavka and on soil Píšť any, respectively.

The bioconcentration factor for lead in total plant biomass was low in all four cases. The highest value was calculated for *Arabidopsis halleri* grown on soil Litavka (0.11) and was lowest for *T. caerulescens* (0.06) on soil Litavka. All values are shown in Table 5.

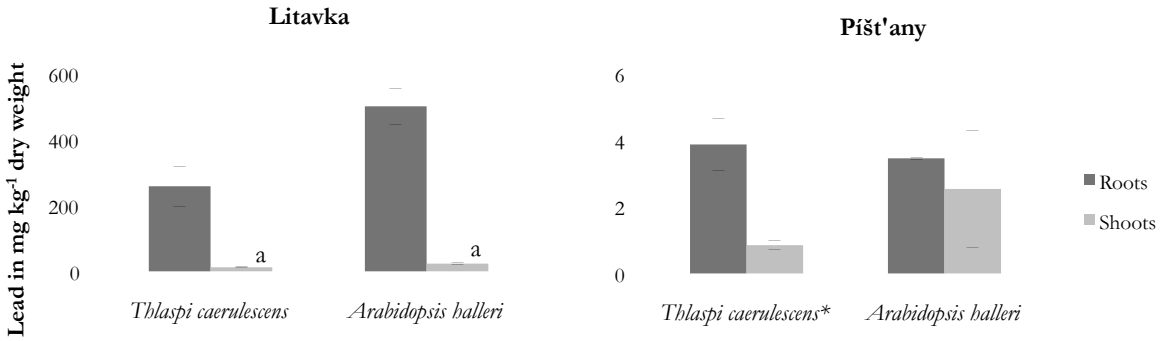

Figure 2: Lead concentrations in shoot and root material of *A. halleri* and *T. caerulescens* in mg kg^{-1} dry matter. Sample size amounts to four (n=4), except *: *T. caerulescens* grown on soil Píšť any (*:n=2). Error bars indicate standard error of the mean. Data marked by the same letter are statistically different with $\alpha = 0.05$.

Table 5: Lead content (in µg) in biomass and bioconcentration and translocation factors (± SEM) of lead for *Arabidopsis halleri* and *Thlaspi caerulescens* grown on experimental soils (n=4; *:n=2).

Soil	**Litavka**		**Píšť any**	
Species	*Arabidopsis halleri*	*Thlaspi caerulescens*	*Arabidopsis halleri*	*Thlaspi caerulescens**
Lead content	2950 (± 440)	1420 (± 390)	19 (± 5)	17 (± 3)
Bioconcentration factor	0.04 (± 0.01)	0.02 (± 0.01)	0.05 (± 0.03)	0.03 (± 0.00)
Translocation factor	0.05 (± 0.01)	0.05 (± 0.02)	0.75 (± 0.47)	0.22 (± 0.06)

4.1.3. Zinc in plant biomass

A. halleri was more effective in accumulating zinc on soil Litavka, whereas it was *T. caerulescens* on soil Píšť any, as shown by the zinc concentrations of shoots and roots in Figure 3. The averaged shoot concentration always exceeded the root zinc concentration in the dry plant matter. The root and shoot concentrations of Píšť any-grown *A. halleri* plants did not differ to a great extent and the root concentration of single plants was higher than the shoot zinc concentration. Differences in zinc concentration in roots and shoots between *A. halleri* and *T. caerulescens* were significant when plants were grown on soil Litavka.

The zinc content of plants grown on soil Litavka was highest in *A. halleri* (Table 6). When *A. halleri* was grown on soil Píšť any, its zinc content averaged at 8 mg, whereas the zinc content in *T. caerulescens* averaged slightly higher at 10 mg.

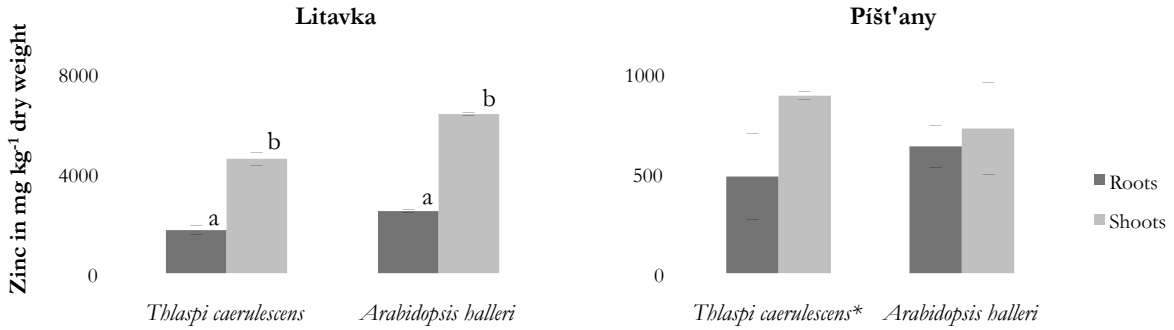

Figure 3: Zinc concentration in mg kg^{-1} dry plant material of the hyperaccumulating species *A. halleri* and *T. caerulescens* (n=4, *:n=2). Error bars indicate standard error of the mean. Data marked by the same letter are statistically different with $\alpha = 0.05$.[19]

[19] Homoscedasticity was not observed for data on zinc concentration in shoots of plants grown on soil Litavka.

Table 6: Zinc content (in mg) in biomass and bioconcentration and translocation factors (± SEM) of zinc for *Arabidopsis halleri* and *Thlaspi caerulescens* grown on experimental soils (n=4; *:n=2).

Soil	**Litavka**		**Píšt'any**	
Species	*Arabidopsis halleri*	*Thlaspi caerulescens*	*Arabidopsis halleri*	*Thlaspi caerulescens**
Zinc content	188 (± 10)	120 (± 11)	8 (± 3)	10 (± 1)
Bioconcentration factor	1.8 (± 0.0)	1.3 (± 0.0)	3.9 (± 1.1)	4.4 (± 0.2)
Translocation factor	2.6 (± 0.0)	2.7 (± 0.3)	1.2 (± 0.3)	2.3 (± 1.0)

The bioconcentration factor for zinc was for both species higher on soil Píšt'any (Table 6). Within the Píšt'any-grown plants, bioconcentration factors for *T. caerulescens* were higher than those for *A. halleri*. On soil Litavka, translocation of zinc to aerial biomass was almost similar for both species, whereas it was higher in *T. caerulescens* when grown on soil Píšt'any.

4.1.4. Trace metal concentrations in acetic acid extracts

Whenever *A. halleri* was grown, the element concentration in soil within the rhizobag was larger in comparison to that outside the bag, except for the level of extractable cadmium and zinc in soil Píšt'any (Figure 4). Here, the concentration of cadmium differed by only 11 μg kg^{-1} dry soil and zinc concentration was reduced by 1.78 mg kg^{-1} soil

The concentration of soil trace metals outside the rhizobag was higher than inside when *T. caerulescens* was grown on it. As pictured in Figure 4, only the zinc concentration of soil Litavka was higher inside the rhizobag than outside.

Soil samples derived from buckets planted with *A. halleri* had a higher concentration of elements than those planted with *T. caerulescens*, independent of whether they originated inside or outside the rhizobag. This finding was only reversed by the level of lead in the "outside"-fraction of soil Píšt'any.

Differences in soil trace metal concentration after growth of either species were statistically significant for the cadmium concentration inside and outside the rhizobag on soil Litavka and inside the rhizobag on soil Píšt'any.

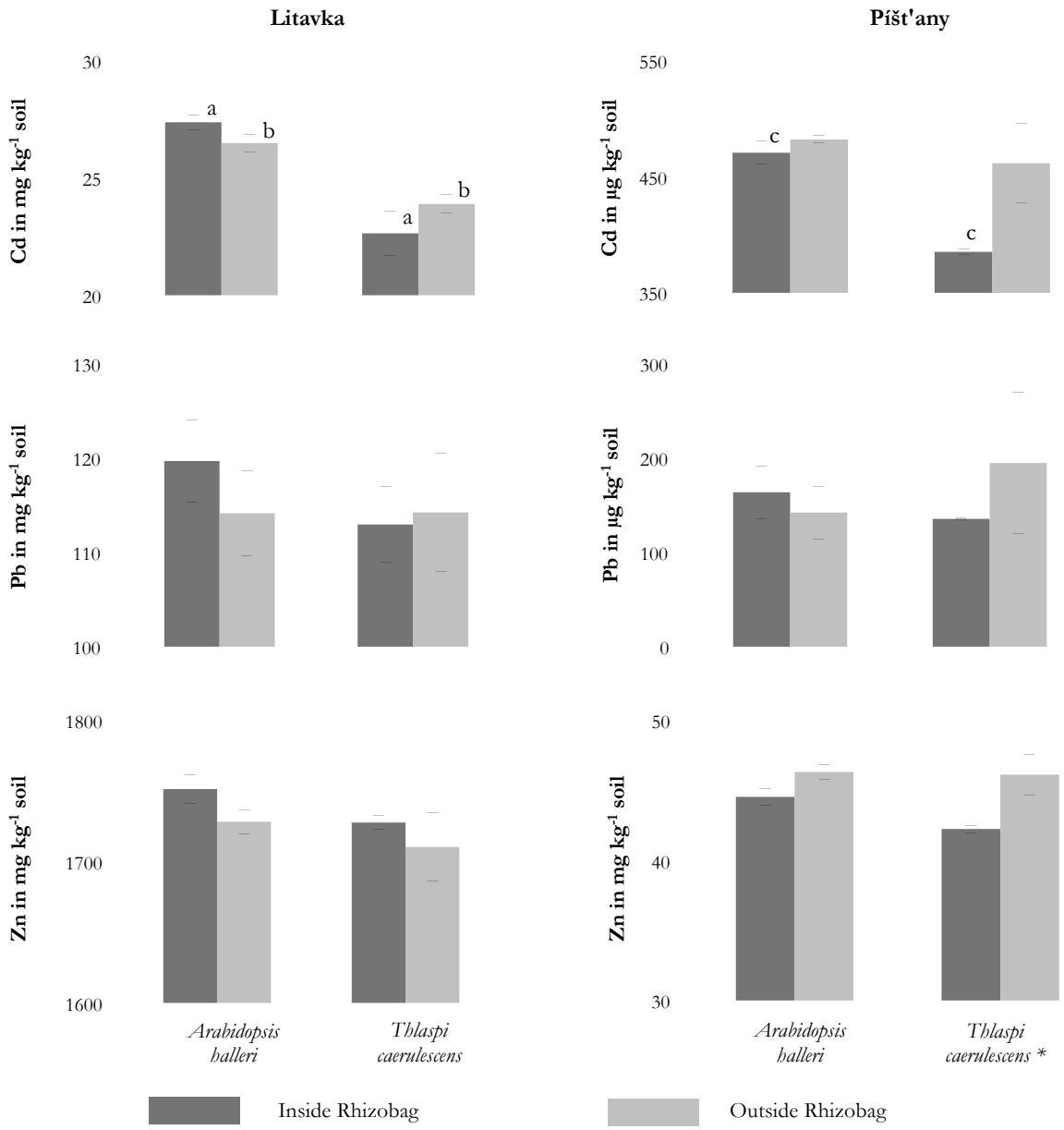

Figure 4: Cadmium, lead and zinc concentration in soil samples from inside and outside the rhizobags. Note that cadmium and lead concentration of soil Píšť any are plotted in µg kg^{-1} dry soil and data in remaining diagrams is presented in mg kg^{-1} dry soil. Sample size totals four (n=4) and two (*: n=2) if marked with asterisk. Error bars indicate standard error of the mean. Data marked by the same letter (a, b, c) was statistically different with $\alpha = 0.05$.

4.2. Rhizoboxes

Growing the same plant species in rhizoboxes filled with either soil Píšť any or Litavka altered results on soil and plant trace metal concentrations and on trace metal content in biomass.
All plants grown in rhizoboxes did not produce as much biomass as those from the rhizobag experiment. Table 7 reflects mean fresh weight and mean dry weight of plants at harvest.

Table 7: Biomass (in g) of roots and shoots (± SEM) of *A. halleri* and *T. caerulescens* when grown in rhizoboxes filled with soil from Litavka and Píšť any (n=4).

Soil	Species	Fresh weight of shoots	Dry weight of shoots	Fresh weight of roots	Dry weight of roots
Litavka	*Arabidopsis halleri*	10.7 (± 0.4)	2.0 (± 0.0)	2.7 (± 0.7)	0.3 (± 0.0)
	Thlaspi caerulescens	15.8 (± 0.4)	3.7 (± 0.5)	3.4 (± 0.8)	0.6 (± 0.1)
Píšť any	*Arabidopsis halleri*	2.4 (± 1.2)	0.5 (± 0.1)	0.4 (± 0.2)	< 0.1 (± 0.0)
	Thlaspi caerulescens	4.5 (± 0.4)	1.1 (± 0.1)	1.1 (± 0.2)	0.3 (± 0.0)

Overall, plants from Litavka-boxes spread more and grew more uniformly. In contrast to plants grown on the less polluted soil Píšť any, where individual roots in front of the rhizobox membrane were distant and distinguishable, roots of plants grown on soil Litavka were more abundant and generally covered the complete membrane.

4.2.1. Cadmium in plant biomass

The concentration of cadmium in shoot material of *T. caerulescens* on both soils and from *A. halleri* when grown on soil Píšť any was higher than in the associated root material (Figure 5). Cadmium content in *T. caerulescens* was higher than in plant tissue of *A. halleri* when grown under the same conditions (Table 8). Differences between species are significant for the cadmium content in biomass, as well as for root and shoot concentration individually.[20]

[20] Even though homoscedasticity (homogeneity of variance) was not observed, the F-test is supposed to be robust to violations of this assumption (Lindeman, 1991).

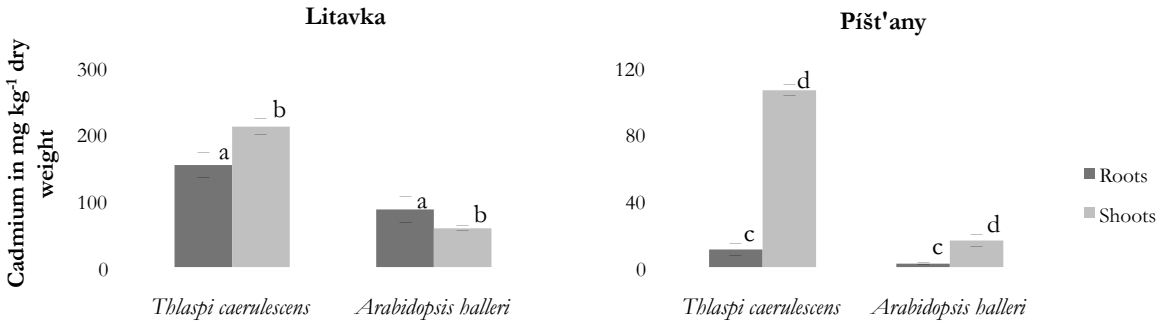

Figure 5: Cadmium concentration (± SEM) in samples of *A. halleri* and *T. caerulescens* when grown on contaminated soils from the Czech Republic. Data marked with the same letter are statistically significantly different with $\alpha = 0.05$.

The cadmium concentration in *T. caerulescens* was roughly seven times higher than in soil Litavka, whereas cadmium concentration in *A. halleri* was twice the concentration of the growth substrate (see bioconcentration factors in Table 8). Even though cadmium content in plants grown on soil Píšť any was lower, bioconcentration factors of plants grown on soil Píšť any exceeded those of plants grown on soil Litavka.

Translocation of cadmium to shoots of either species was better accomplished on soil Píšť any than on soil Litavka (Table 8). On soil Píšť any, the difference in shoot and root concentration was highest in *A. halleri*, whereas it was higher in *T. caerulescens* on soil Litavka

Table 8: Cadmium content (in µg) in biomass and bioconcentration and translocation factors (± SEM) of cadmium for *Arabidopsis halleri* and *Thlaspi caerulescens* grown on experimental soils (n=4).

Soil	**Litavka**		**Píšť any**	
Species	*Arabidopsis halleri*	*Thlaspi caerulescens*	*Arabidopsis halleri*	*Thlaspi caerulescens*
Cadmium content	141 (±12)	886 (±128)	8.8 (±3.9)	123 (±11)
Bioconcentration factor	2.2 (± 0.2)	7.4 (± 0.3)	9.1 (± 1.9)	60.3 (± 3.3)
Translocation factor	0.8 (± 0.1)	1.5 (± 0.3)	23.3 (± 17.0)	12.8 (± 3.2)

4.2.2. Lead in plant biomass

Especially when soil from locality Litavka was the growth substrate, lead concentration in plant roots was up to 36 times higher than in shoot material. Even though lead concentration in dry root biomass of *A. halleri* was higher than in root biomass of *T. caerulecens* (Figure 6), the lead content of *T. caerulescens* was slightly higher than the lead content of *A. halleri* when grown on either soil (Table 9).

Uptake of lead was rather poor and accumulation non-existing, as all bioconcentration factors were below 0.1 (Table 9). Data in Table 9 and the illustration in Figure 6 suggest that *A. halleri* was more successful in taking up this element than *T. caerulescens*, resulting in higher lead concentrations and higher bioconcentration factors. Lead was stored in root tissue by all plants as all translocation factors (Table 9) are lower than 0.5.

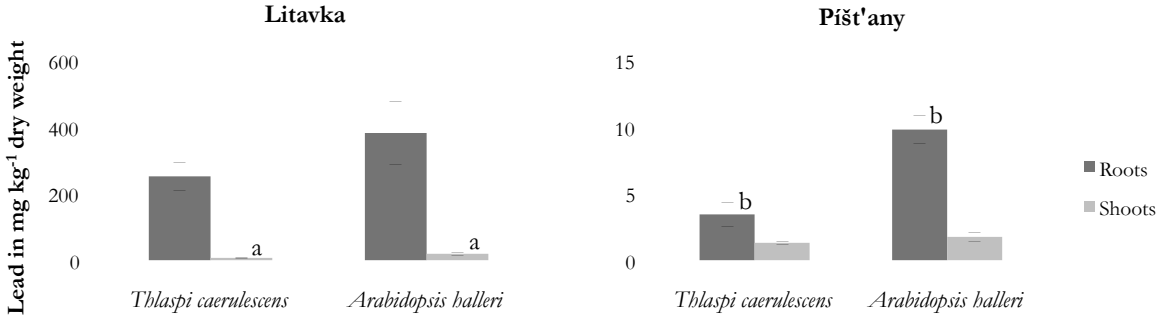

Figure 6: Lead concentration (± SEM) in mg kg^{-1} dry biomass of the hyperaccumulating plants *A. halleri* and *T. caerulescens* grown in two contaminated soils differing in level of pollution. Sample size was four (n=4). Statistically significant differences between species with $\alpha = 0.05$ were detected if data are marked by the same letter.[21]

Table 9: Lead content (in µg) in biomass and bioconcentration and translocation factors (± SEM) of lead for *Arabidopsis halleri* and *Thlaspi caerulescens* grown on experimental soils (n=4).

Soil	**Litavka**		**Píšť'any**	
Species	*Arabidopsis halleri*	*Thlaspi caerulescens*	*Arabidopsis halleri*	*Thlaspi caerulescens*
Lead content	142 (± 12)	161 (± 20)	2.0 (± 0.2)	2.4 (±0.2)
Bioconcentration Factor	0.03 (± 0.00)	0.02 (± 0.00)	0.07 (± 0.01)	0.03 (± 0.00)
Translocation Factor	0.06 (± 0.02)	0.03 (± 0.01)	0.18 (± 0.02)	0.47 (± 0.12)

4.2.3. Zinc in plant biomass

Concentration of zinc in dry biomass of *T. caerulescens* (roots and shoots) was significantly higher than the zinc concentration of *A. halleri* when both species were grown on soil Píšť'any (Figure 7). On soil Litavka, zinc concentration in shoots of *A. halleri* was significanty higher than in *T. caerulescens*.

Zinc content in plants on either soil was higher in *T. caerulescens* (Table 10).

[21] Homoscedasticity does not apply for data on lead concentration in shoots of plants grown on soil Litavka.

Regarding the bioconcentration factors for zinc (Table 10), plants grown on soil Píšť any absorbed a greater portion of total soil zinc than plants grown on the heavily polluted soil Litavka (bioconcentration factors ≤ 1.0). Even though *Thlaspi caerulescens* was more effective in using the available zinc sources when grown on soil Píšť any, it was performing worse than *A. halleri* when grown on soil Litavka.

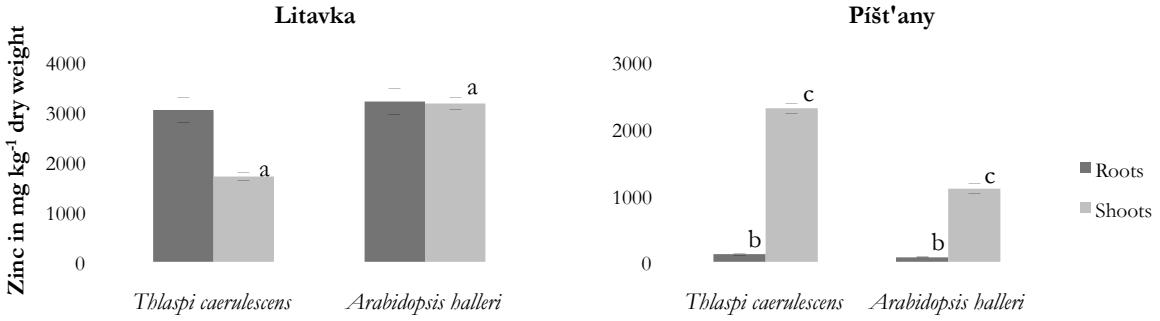

Figure 7: Zinc concentration (± SEM) in the plant species *A. halleri* and *T. caerulescens* after growth on two contaminated soils in mg kg^{-1} dry plant material. Sample size was four (n=4). Data marked with the same letter are statistically significantly different with $\alpha = 0.05$.

Table 10: Zinc content (in µg) in biomass and bioconcentration and translocation factors (± SEM) of zinc for *Arabidopsis halleri* and *Thlaspi caerulescens* grown on experimental soils (n=4).

Soil	**Litavka**		**Píšť any**	
Species	*Arabidopsis halleri*	*Thlaspi caerulescens*	*Arabidopsis halleri*	*Thlaspi caerulescens*
Zinc content	7210 (± 190)	8030 (± 940)	565 (± 168)	2630 (± 220)
Bioconcentration factor	1.0 (± 0.0)	0.6 (± 0.0)	4.7 (± 0.3)	9.7 (± 0.5)
Translocation factor	1.0 (± 0.1)	0.6 (± 0.0)	21.1 (± 3.2)	17.8 (± 3.5)

The translocation of zinc to shoots was low by both plant species when grown on soil Litavka (Table 10) in comparison to plants grown on the less contaminated soil Píšť any. As a result, zinc concentration in shoots of *T. caerulescens* grown on soil Litavka was even lower than in *T. caerulescens* grown on the less contaminated soil Píšť any (Figure 7).

4.2.4. Trace metal concentrations in acetic acid extracts

The concentrations of all three chemical elements were lower in the lower compartments (soil slice "0-2 mm" and more distance to the membrane) of rhizoboxes filled with soil Píšť any when planted with *A. halleri* than when planted with *T. caerulescens* (Figure 8). Only soil in the upper

compartment, in direct contact to the roots of *T. caerulescens*, contained less acetic acid-extractable elements than those planted with *A. halleri*.

As shown in Figure 8, lowest cadmium concentrations in acetic acid extracts of samples of soil Píšt'any were found in the root-penetrated upper compartments. Here, difference in cadmium concentration in soil influenced by either plant species was significant with $\alpha = 0{,}05$.

Data on cadmium concentration display that it declined with decreasing distance to the root surface. Maximum concentration was found with 4-6 mm distance to the membrane after growth of *T. caerulescens* (703 μg kg^{-1} dry soil) and in the 6-8 mm soil slice after growth of *A. halleri* (669 μg kg^{-1} dry soil). In all measurements of the lower compartment, cadmium concentration after growth of *T. caerulescens* was greater than in corresponding soil sections after growth of *A. halleri*. Significant differences in soil cadmium concentration in soil Píšt'any due to growth of the two hyperaccumulators were noticed in soil with 2-4 mm and 4-6 mm[22] distance to the membrane.

A similar picture was found for the lead and zinc concentrations of soil Píšt'any (Figure 8). The lead concentration in the upper compartment was almost the same for both hyperaccumulators (*T. caerulescens*: 221 μg kg^{-1}; *A. halleri*: 231 μg kg^{-1}). Highest lead concentration (839 μg kg^{-1} dry soil) was again found with four to six millimetres distance to the membrane, when boxes were planted with *Thlaspi caerulescens*. For boxes influenced by *A. halleri*, the highest lead concentration with 702 μg kg^{-1} dry soil was found six to eight millimetres away from the root mat. Significant differences in soil lead concentration were noticed with 2-4 mm and 4-6 mm distance to the membrane on soil Píšt'any.

On soil Píšt'any, the zinc concentration in soil intensively influenced by plant roots (upper compartment and soil fractions with less than two millimetres distance to the membrane) was lowest, regardless of plant species grown thereon. Further data on zinc concentration were higher and also differed significantly with plant species grown on the soil.[23] When soil Píšt'any was influenced by *T. caerulescens*, it contained up to 10 mg more of acetic acid extractable zinc per kilogram dry soil than after growth of *A. halleri*.

Soil Litavka obtained from the rhizobox-experiment planted with *T. caerulescens* contained generally less exchangeable cadmium, lead and zinc than after growth of *A. halleri*. The only exception

[22] Data on the soil fraction with 4-6 mm distance to the membrane do not exhibit homogeneity of variance.
[23] The soil fraction with 4-6 mm distance to the membrane does not exhibit homoscedasticity.

was the cadmium content in the bulk soil (Figure 8). The cadmium and zinc concentrations in rhizoboxes after growth of either species were lowest in the upper compartments.

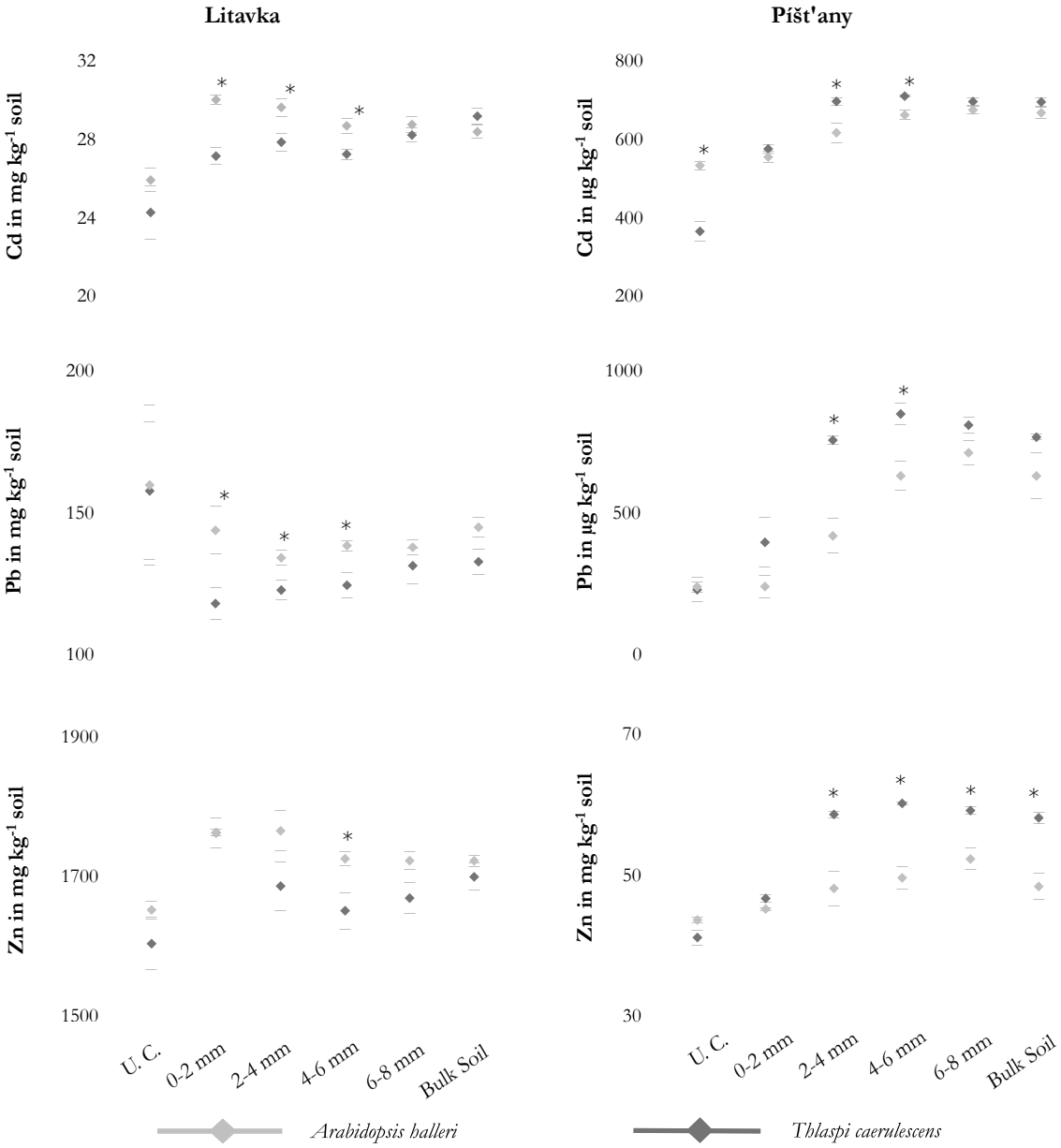

Figure 8: Cadmium, lead and zinc concentrations in acetic acid extracts of dried samples (n=4) of soils Litavka and Píšť'any after growth of the hyperaccumulators *A. halleri* and *T. caerulescens*. Soil fractions of rhizoboxes consist of upper compartment (U.C.), four soil slices with 2 mm width each and soil from the bulk soil. Error bars indicate the standard error of the mean. Trace metal concentrations are given in mg kg^{-1}, except concentrations of cadmium and lead of soil Píšť'any (in µg kg^{-1}). The difference in soil trace metal concentration induced by growth of either of the two species is significant with $\alpha = 0.05$ for individual soil slices, if marked with asterisk (*).

In the lower box, extractable cadmium concentration decreased from 29.9 mg kg^{-1} dry soil to 28.2 mg kg^{-1} dry soil with increasing distance to the membrane after growth of *A. halleri*. Besides, the cadmium concentrations in the first two soil fractions of the lower compartment (0-2 mm and 2-4 mm) were significantly higher when planted with *A. halleri* than when rhizoboxes were planted with *T. caerulescens*.

The lead concentration in rhizoboxes after growth of both plant species was highest in the upper compartment (Figure 8). When *A. halleri* was the plant grown, soil lead concentration decreased until four millimetres distance to the root and increased slightly towards the rear of the rhizobox. After growth of *T. caerulescens*, the lowest lead concentration was in the immediate root vicinity and increased with increasing distance to the membrane. Differences in soil lead concentration between the two species were significant with 0-2 mm, 2-4 mm and 4-6 mm distance to the membrane.

The acetic acid extractable zinc concentrations in the lower compartment of rhizoboxes peaked at roughly 1760 mg zinc kg^{-1} dry soil after growth of either species. After growth of *A. halleri*, the adjacent soil slice (with 2-4 mm distance to the root surface) had an equally high zinc concentration, whereas the zinc concentration in soil with this distance to the membrane decreased to 1680 mg kg^{-1} dry soil after growth of *T. caerulescens*.

5. Discussion

This evaluation aims at examining the differences in cadmium, lead and zinc concentration in plant matter of two hyperaccumulator plants and the resulting changes in trace metal concentration in two soils differing in level of pollution. Some considerations on the experimental procedure and general considerations on trace metal behaviour in soil will preface the discussion.

5.1. Experimental problems and considerations

The influence of mycorrhiza on the hyperaccumulation of trace metals was neglectible, as both plant species do not accommodate symbiotic fungi in pivotal amounts (Regvar et al, 2003). No measures were taken to limit the existence of bacteria and other unicellular microorganisms. Photoautotropic organisms were definitely present in the lower compartments of the rhizoboxes, predominantly at the more lucid rear panel, despite the tinfoil wrapping. Biofilms also developed in the shade of plants in rhizobags, but their influence on soil was expected to be less pronounced, due to a greater soil volume. Other microscopical life-forms were probably present in all samples, possibly influencing trace metal solubility in soil (Gadd and Sayer, 2000; Hughes and Poole, 1989; Klein and Thayer, 1990; Schinner and Sonnleitner, 1996).

The concentrations of cadmium, lead and zinc in biomass of each plant were determined in the same filtrate. Differences in metal concentrations between shoots and roots in two of the determined trace metals were usually large enough to make a substantial bias by inaccurate metal concentrations in roots for the third trace metal (e.g. the lead concentrations in Figure 2 and Figure 6) due to contamination with soil particles unlikely.

Hyperaccumulation and translocation of lead were not expected in both species. However, lead is strongly bound to the root surface and in the waterfree space of roots (Broyer et al., 1972), resulting in potential overestimation of lead concentrations in roots.

5.2. Biomass production

Both plant species grew on the contaminated soils and accumulated cadmium and zinc. In both experiments, more biomass was generally produced on the highly polluted soil Litavka. Differences in biomass production between species were also observed. In the rhizobox experiment, *T. caerulescens* produced more biomass than *A. halleri* on both soils (Table 7).

However in the rhizobag experiment, which resembled field conditions more closely, *A. halleri* grown on soil Litavka produced more biomass than *T. caerulescens* (Table 3).

Especially if plants will be used for phytoremediation, site-specific screening for high-biomass species (or even ecotypes) is advisable for high trace metal contents in plants (content [mg] = concentration [mg kg^{-1}] x biomass [kg]) (Schmidt, 2003; Shah and Nongkynrih, 2007).

5.3. Hyperaccumulation traits and phytoextraction potential

Thlaspi caerulescens and *Arabidopsis halleri* are both confirmed cadmium and zinc hyperaccumulator plants (Reeves and Brooks, 1983; Baker et al., 1994; Bert, et al., 2000; Escarré et al., 2000; Lombi et al., 2000; Zhao et al., 2000), but show no hyperaccumulation trait for lead. Therefore it comes to no surprise that neither the threshold value for hyperaccumulation of 1000 mg lead kg^{-1} dry matter in above-ground biomass is met (Figure 2 and Figure 6), nor that factors of bioconcentration and translocation are below one (Table 5 and Table 9). Still, both species were tolerant to the soil lead levels in the experimental soils (up to 2460 mg kg^{-1}).

The hyperaccumulation threshold for metal concentration in shoots, as defined by Baker and Brooks (1989), was met by the cadmium concentrations in biomass of both species grown in rhizobags on soil Litavka (Figure 1) and in rhizoboxes by *T. caerulescens* only, though on both soils (Figure 5). However, the definition considers single plants growing under natural conditions. Criteria for hyperaccumulation have since been amended by physiological adaptations to hyperaccumulation by the plants (Reeves, 1992), such as the translocation factor and the bioconcentration factor. Here, cadmium and zinc accumulation were detected in both plant species. Bioconcentration (rhizobox experiment only) and translocation of cadmium and zinc were in both plant species higher when grown on soil Píšťany, possibly indicating, similar to the remark by Robinson et al. (1998), an inversely related effectiveness of plant physiological adaptations of accumulation to the degree of soil contamination.

For the purpose of phytoextraction however, the trace metal content in harvestable biomass is pivotal. Only the shoots of *T. caerulescens* and *A. halleri* were considered harvestable for remediative purposes due to the delicate and branched structure of plant roots.

Removal of cadmium by *T. caerulescens* amounted to 42.8% and 18.7% of total soil cadmium on soils Litavka and Píšťany, respectively[24]. Removal rates of up to 40% of total soil cadmium were also observed by Wang et al. (2006) on a soil with a cadmium contamination level of 5 mg kg^{-1} soil and up to 36% cadmium removal were determined for *T. caerulescens* on soil with higher cadmium contamination (25.4 mg kg^{-1} soil). In this study, the capacity to extract cadmium from either of the the two soils differed even within the same species. However, the bioavailability of this trace metal on soil Píšťany was lower than on soil Litavka, as cadmium concentration in acetic acid extracts of unplanted aliquots of soil Píšťany contained about a third of total soil cadmium but nearly the total of soil cadmium of soil Litavka. The high potential to phytoextract cadmium is also attributable to the fact that cadmium is generally quite mobile in soil (Harter and Naidu, 2001), resulting in a relatively easily accessible cadmium fraction in soils and facilitating absorption by plants (Schwartz et al., 2003).

The potentially possible rates of zinc phytoextraction are lower than those for cadmium, reaching a removal rate of total soil zinc of approximately 7%, achieved in 391 days by *T. caerulescens* (ecotype Ganges) grown on a soil contaminated with 2920 mg zinc kg^{-1} soil (Lombi et al., 2001). In this study, phytoextraction of zinc represented at most 5.5% of total soil zinc on soil Litavka (average of the four replicates of *A. halleri*) and 4.7% on soil Píšťany (average of the two replicates of *Thlapsi caerulescens*). On soil Litavka, the zinc concentration in *A. halleri* was higher than for *T. caerulescens* (Figure 3), resulting in higher zinc content in biomass and a higher phytoextraction potential. Whereas the zinc concentrations in biomass of both species grown on soil Píšťany were alike (Figure 3) and the higher amount of biomass of *T. caerulescens* was pivotal for a greater capacity to extract zinc.

Mitigating the risk of contaminated soils is preferable and could possibly be achieved by phytoremediation. From the data gathered in this study, removal of total cadmium and zinc of soil Píšťany would take roughly 21 croppings when using *T. caerulescens*. On soil Litavka, removing the soil cadmium content with *T. caerulescens* would be achieved within 3 years. Yet, extracting soil zinc on soil Litavka would be more tedious, lasting at least 18 years when cropping *A. halleri* and even longer (29 croppings) when *T. caerulescens* would be grown.

However, removing total soil trace metal content might not be possible and necessary. The total contaminant concentrations in soil are typically not a good indicator for bioavailability.

[24] Determined for the root-influenced compartment of the rhizobag experiment, assuming complete penetration by roots of each rhizobag.

Decreasing (stripping) the bioavailable trace metal fractions to within a remedial target, as proposed by Fitz et al. (2003b), would hence allow for a faster return to use of polluted soils.

Lombi et al. (2002) presented evidence that the cadmium uptake of *Thlaspi caerulescens* depends on the plants' ecotype and is ecpecially high in the ecotype Ganges. Besides, the cadmium accumulation in this ecotype is not dependent on the zinc uptake system (Zhao et al., 2002), as was proposed for the accumulation of several trace metals in samples of *T. caerulescens* from the UK (Baker et al., 1994). However, in a metallicolous population of *Arabidopsis halleri* from Blankenrode, Germany, and in backcross progenies of *A. halleri* from Northern France and *Arabidopsis lyrata* ssp. *petraea* from the Czech Republic, cadmium uptake is at least partly dependent on the zinc system (Zhao et al., 2006; Bert et al., 2003). Albeit possible differences in the pathway of cadmium uptake between ecotypes in *A. halleri*, specific cadmium carriers in the root membrane of *T. caerulescens* could explain the higher cadmium concentration in biomass of *Thlaspi caerulescens* in all experiments. Especially if the transporter for the shared cadmium/zinc uptake pathway has a high affinity for zinc and thus supresses the cadmium uptake (Pence et al, 2000).

In hydroponic experiments, accumulation of zinc by *A. halleri* seemed to be greater than by *T. caerulescens* (Shen et al., 1997; Zhao et al., 2000). This finding is in line with the data on zinc concentration of plants grown on soil Litavka only (Figure 3 and Figure 7). As has been shown by Huitson and Macnair (2003), at low soil zinc concentrations, *Arabidopsis halleri* reaches a plateau in zinc concentration much earlier than at higher soil zinc concentrations. This circumstance might be reflected in the reversed ranking of plant species with highest zinc concentration on soils Litavka and Píšťany.

5.4. Changes of metal extractability in the rhizosphere

The trace metal extractability by acetic acid was changed in the central root compartment of the rhizobags as compared to that measured in bulk soil (Figure 4). In the less contaminated soil Píšťany, the concentrations of cadmium and zinc inside the rhizobag after growth of either species were lower than outside. This difference is likely to result from uptake of bioavailable trace metal fractions by the plants. However, cadmium and zinc contents in biomass of both species were higher than the assessed depletion of trace metal content in rhizobags as determined by the soil trace metal concentration in acetic acid extracts (soil mass in rhizobags x

trace metal concentration). Replenishment of soil trace metals from less exchangeable fractions might account for the observed difference.

On the heavily polluted soil Litavka, the trace metal concentrations inside the rhizobag planted with *Arabidopsis halleri* were increased in comparison to the concentration outside the rhizobag. Hyperaccumulator plants depend on a sufficient supply of trace metals from the soil (Shen et al., 1997; Assunção and Schat, unpublished, cited in Assunção et al., 2003). However, on the highly polluted soil Litavka, the demand for trace metals by both hyperaccumulators was likely satisfied or even lower than the re-supply from less accessible fractions. This may explain that no depletion of acetic acid extractable trace elements was found in the rhizosphere of *A. halleri* (Figure 4). However, the buffer power of the polluted soil cannot explain increases of the extractable trace element fractions in soil. It is therefore asssumed that trace metals were mobilised in the rhizopshere of *A. halleri* by root activities.

The exudation of low molecular organic compounds by plants (e.g. phytometallophores) triggers the formation of metal complexes, improving the metal nutrition of plants, especially in the case of metal deficiency (Fan et al., 1997). Possible impacts of such exudates on an enhanced trace metal mobilisation by hyperaccumulator plants have been studied. However, studies on *A. halleri* on this aspect are apparently lacking and results on other plants are being discussed controversially. Solubilisation of cadmium by organic acids and, to a lesser extent, by amino acids was observed in the rhizosphere of tobacco (*Nicotiana* ssp.) by Mench and Martin (1991), but not in an isotope dilution assay conducted on soil planted with *T. caerulescens* (Hutchinson et al., 2000). Zhao et al. (2001) found no effect of exudated organic carbon compounds on the mobilisation of zinc, cadmium, copper and iron in the rhizosphere of *T. caerulescens*. Whereas dissolved organic carbon forms complexes with nickel in the rhizosphere of the nickel hyperaccumulator *Thlaspi goesingense* Hálácsy (Wenzel et al., 2003), especially oxalate exudated by *T. goesingense* or associated rhizobacteria plays a role in the "ligand promoted dissolution of nickel in forsterite-type minerals" (Puschenreiter et al., 2005). Hence, differences in trace metal solubilisation between species are likely and require further studies for an in-depth examination.

At low trace metal concentrations in soils (Píšťany), it appears that the rate of trace metal uptake by *A. halleri* exceeded the re-supply from less acessible fractions and the action of soil buffer power and root activities were not able to compensate for depletion.

6. Conclusion

Within the scope of this work it was possible to determine differences in the accumulation ability of two hyperaccumulating herbaceous plant species. *Thlaspi caerulescens*, as well as *Arabidopsis halleri*, accumulated cadmium and zinc in high amounts and complied with plant physiological criteria for hyperaccumulation. Hyperaccumulation of lead was not observed, as absorption and translocation of lead were marginal.

The potential to extract cadmium from both experimental soils was highly pronounced in both plant species. Yet, *T. caerulescens* is considered more efficient in remediating soil cadmium. The phytoextraction potential for zinc differed between plant species with the level of soil contamination, so that *A. halleri* should be the plant of choice in remedial assignments on highly polluted soil.

The concentration of bioavailable trace metals increased in the rhizosphere of *A. halleri* when grown on soil Litavka, indicating metal mobilisation by root/microbial activities. Metal mobilisation in the rhizosphere could not be observed for the other plant-soil combinations.

This study shows that plants, soil properties, in particular pollutant levels, as well as their interactions need to be considered in the design of phytoextraction technologies.

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I confirm that the thesis at hand is my own work; and that all published or other sources of material consulted have been acknowledged in notes to the text or the bibliography. The thesis in this form or in any other form has not been submitted to an examination body and has not been published.

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