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Basis for the remediation of sites polluted by potentially toxic elements in Zimapan, Mexico. Interdisciplinary approach

ARIADNA SCHEHERAZADA SÁNCHEZ LÓPEZ

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PARTIAL REQUIREMENT TO GET THE DEGREE OF:

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BASIS FOR REMEDIATION OF SITES CONTAMINATED BY POTENTIALLY TOXIC ELEMENTS IN ZIMAPAN, HGO., MEXICO: INTERDISCLIPLINARY APPROACH

Ariadna S. Sánchez López, Dr.

2015

ABSTRACT

The present dissertation was performed to gather knowledge about the responses and interaction of plants-endophytic bacteria-potentially toxic elements (PTEs)-soil in a mining area located in a semi-arid environment, Zimapan, Mexico. To address this objective a set of studies were accomplished. 1) Chemical and mineralogical characterization of deposits of mine residues. Results highlight the importance of specific chemical and mineralogical characteristics of mine residues for an adequate phytoremediation strategy. 2) Potential of pioneer plants colonizing mine residues for use in phytoremediation. *Pteridium* sp. showed be suitable for phytostabilization; while *Aster gymnocephalus*, *Gnaphalium* sp. and *Crotalaria pumila* might be a potential phytoextractor. 3) Plants against atmospheric dispersion of particles bearing PTEs from mine tailings (phytobarriers). All plants colonizing mine tailings participate retaining particles on leaf surface. 4) Transgenerational characterization of seed endophytes of a pioneer plant colonizing mine residues. Endophytes improve plant nutrition by solubilization of phosphate, reduce levels of ethylene, and are tolerant to PTEs. These traits may explain the success of certain plants on a multi-PTEs contaminated substrate. Three bacterial isolates were observed in three consecutive seed generations suggesting vertical transmission of selected endophytes. 5) Seed endophytes inoculation on plant growth and PTEs uptake/stabilization by their host plant. Results varied according inoculum and evaluated variable. However, outcomes indicate practical applications of seed endophytic bacteria to promote revegetation and phytostabilization of PTEs-contaminated soil. 6) Endophytic plant colonization of selected bacterial strains using fluorescent protein. Labeled bacterial cells colonized the xylem vessels in root and shoots of plants only in the presence of PTEs. The observations support the theory that seed endophytes can enter root cells, move through the xylem and reach different organs (*e.g.* seeds) and thus they can be transferred to successive plant generations. This new information represents an important step towards a better understanding of PTEs-plant-microorganisms interactions regarding phytoremediation purposes.

Key words: mine residues, heavy metals, phytostabilization, phytoextraction, endophytic bacteria

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GENERAL INTRODUCTION

In recent years potentially toxic elements (PTEs) pollution has become a concern due to the risk of elements mobility in the environment (Bolan *et al.* 2014) and the possible effects on public health (Jaishankar *et al.* 2014; Udeigwe *et al.* 2015). There exist different sources of the mentioned elements; one of the most important is mining. Processing of minerals produces waste which normally contains PTEs that eventually may be spread to the environment.

In Mexico, mining represents an important economic activity which was going on for more than five centuries. This is the case of Zimapan (Mexico). Nowadays, it is possible to observe bare mine tailings produced by different mines along hundreds years. As a consequence, there exist a wide variety of sites that need to be remediated. However, these may reflect a diversity of chemical and mineralogical characteristics; which need to be considered for successful remediation alternatives.

Mine tailings in Zimapan are observed exposed to the environment, under these conditions, PTEs dispersion can occur through wind and water erosion, or by oxidative dissolution of minerals that enhances mobilization and dispersion of PTEs from tailing heaps (Gleisner and Herbert 2002; Jonathan *et al*. 2010). Earlier research has documented that Zimapan mine tailings affect the quality of soil and water in their surrounding areas (González and González-Chávez 2006; Jonathan *et al.* 2010). Additionally, these promote emission and dispersion of PTEs-bearing dust (Duarte-Zaragoza *et al.* 2015), and have negative impacts on public health (Armienta, Rodríguez and Cruz 1997).

Different strategies have emerged in order to solve or diminish PTEs pollution problems. These include physico-chemical and biological methods. Among these latter, phytoremediation was shown to be cost effective and environmental friendly. It involves the use of plants and their

associated microorganisms for the environmental remediation (González-Chávez *et al*. 2005, 2006; Ali, Khan and Sajad 2013; Adki, Jadhav and Bapat 2014; Mani and Kumar 2014). Phytoremediation takes advantage of the plant's ability to remove pollutants from the environment or to immobilize them and make them harmless (Vangronsveld *et al*. 1995, 1996, 2009). Hence, phytoremediation has low installation and maintenance costs compared to physical or chemical remediation options (Van Aken, 2009; Prasad, 2003).

Phytoremediation of PTEs is based on two principal principles: phytoextraction and phytoestabilization. The first one involve the uptake of PTEs by plant roots from contaminated soil and translocation of these to shoots (Adki *et al.* 2014; Robinson, Anderson, and Dickinson 2015). In contrast, phytostabilization is the immobilization of pollutants in rhizosphere or roots (Arthur *et al.* 2005; Ali *et al.* 2013). There exist other ways of phytoremediation, e.g. phytovolatilization but this is applicable only for a few elements like Hg and Se (Wang *et al*. 2012; Ali *et al.* 2013).

One of the limitations for phytoremediation is that most of the plant species cannot adapt to the conditions of the sites that need to be remediated (Vangronsveld *et al.* 2009; Mench *et al.* 2010). In the case of Zimapan, besides PTEs pollution, the establishment of plants can be restricted by the semi-arid conditions (extreme variation of temperature, scarce rain, low concentrations of essential elements, etc.). To overcome these limitations and reach a successful establishment of a vegetation cover studies have concentrated on the native flora suitable for phytoremediation purposes (González and Gonzalez-Chavez 2006; Mendez and Maier 2008). Undoubtedly, native plant species found on PTEs contaminated sites possess adaptation mechanisms which allow these species to establish, grow and complete their life cycle in the presence of contaminants (Yoon *et al.* 2006; Barrutia *et al.* 2011). There exists information

reporting candidate species for use in phytoremediation in different environmental conditions. However, taking into account the specific characteristics of mine tailings explained in previous paragraphs, it is necessary continue exploring for plants that are presumably adapted to the mine tailings in Zimapan. In this way, the establishment of a vegetation cover to diminish PTEs dispersion may be ensured. Additionally, a vegetation cover may provide ecosystem services to degraded landscapes and contaminated soils, *e.g*. biomass energy production (Ruíz-Olivares *et al.* 2013), improvement and recover of biodiversity (Wong, 2003; Remon *et al*. 2005), watershed management, soil protection (Vangronsveld *et al*. 1995; Pulford and Watson 2003; Dickinson *et al*. 2009) and carbon sequestration (González-Chávez and Carrillo-González 2012, 2013), and an aesthetic contribution to the landscape.

Generally, phytoremediation studies are mainly focused on phytoextraction followed by these for phytoestabilization of PTEs. Altough the function of vegetation as PTEs sink has been suggested (Tomašević *et al*. 2005), there is no information about the role of plants in retention of particles containing PTEs in mining areas. Particularly in places with semi-arid climate conditions, like Zimapan, where mine tailings are bare and PTEs dispersion by wind erosion is considerably high.

Although the various reports describing native plants suitable for phytoremediation, the benefits that associated microorganisms generate to their host plants were not often taken into account in this context (Gonzalez-Chavez *et al.* 2005; Navarro-Noya *et al*. 2010). The study of these microorganisms in polluted conditions will contribute to better understand how these plants can overcome the adverse environmental factors commonly occurring on polluted sites (Ma *et al.* 2011; Ullah *et al.* 2015). Promising microorganisms to be used in phytoremediation are endophytic bacteria (Weyens *et al*. 2009; Wani *et al.* 2015), due to their close interaction with

their host plants. Endophytes bring to their host benefits similar to those described for plant growth-promoting rhizospheric bacteria (Hardoim *et al*. 2008). They might lower PTEs phytotoxicity and increase tolerance to such elements (Weyens *et al*. 2009). Therefore, the persistence of certain plant species in adverse environments can be explained by the presence and transmission of endophytic bacteria (Johnston-Monje and Raizada 2011; Hardoim *et al*. 2012). Most of research performed concerning endophytic bacteria-plants-PTEs interactions is concentrated on roots. However, plant seeds may act as a vehicle for the transmission of certain endophytes that help their host in the establishment in PTEs contaminated sites (Truyens *et al*. 2013, 2014). Most of this information has been obtained from artificial systems where one or two PTEs were added. Naturally contaminated systems where different PTEs are present have not been studied yet. In consequence, the study of such systems will bring options to reach a successful phytoremediation of PTEs-contaminated sites.

Therefore, the aims of this research were to obtain information about the characteristics of mine residues in Zimapan, as well as to study the native flora adapted to these specific conditions and its possible use for phytoremediation (phytoextraction, phytoestabilization, sink for wind spread contaminated particles). The role of seed endophytic bacteria in the establishment and further growth of plants in PTEs-contaminated sites was also studied. To reach these aims a set of studies were performed, including mine residues characterization and screening for native flora and their potential use in phytoremediation. Seed endophytic bacteria of a plant species colonizing PTEs containing mine residues (*Crotalaria pumila*) were isolated, identified and characterized. Further, selected bacterial strains were tested for their potential to promote establishment and plant growth on PTEs containing soil. Finally, through the use of labeling

techniques and confocal microscopy, the ability of endophytic bacteria to colonize plants in the presence of PTEs was investigated.

OBJECTIVES

Chapter 1. Characterization of tailing heaps from a mine in Zimapan, Mexico.

- To describe the chemical characteristics and mineralogical composition of tailing heaps of a mine in Zimapan, Mexico, in relation to the handle of mine residues.

Chapter 2. Wild flora of mine tailings: perspectives for use in phytoremediation of potentially toxic elements in a semi-arid region in Mexico.

- To identify wild plant species suitable for phytoextraction in mine residues containing PTEs at Zimapan, Mexico.
- To identify plant species adequate for phytoestabilization of PTEs on bare mine tailings at Zimapan, Mexico.

Chapter 3. Phytobarriers: plants reduce atmospheric dispersion of particles containing potentially toxic elements originating from mine tailings in semiarid regions.

- To document the retention of solid particles containing PTEs by different plant species spontaneously colonizing mine tailings.
- To investigate which leaf structure(s) of studied plants contribute(s) (and to what extent) to retention of particles.
- To identify plant species with highest potential to retain particles containing potentially toxic elements arising from mine residues.

Chapter 4. Seed endophytic bacteria of a pioneer plant species colonizing mine residues in a semi-arid region: trans-generational characterization.

- To compare the metabolic fingerprints of the endophytic communities of three consecutive generations of *C. pumila* seeds from plants growing on multi-PTE contaminated mine residues.
- To study composition and functions of the cultivable endophytic communities of three consecutive generations of *C. pumila* seeds from plants growing on mine residues containing PTEs. Then, compare these against composition and functions of endophytic community of seeds originating from a non-contaminated site.

Chapter 5. Seed endophytic bacteria from *Crotalaria pumila* and their potential to promote remediation of soils contaminated by potentially toxic elements.

- To investigate the possible beneficial effects that inoculation of endophytic bacteria isolated from *C. pumila* seeds bring to their host in the presence of PTEs.
- To verify whether seed endophytic bacteria keep their plant growth promotion traits on different batches of *C. pumila* seeds (one batch collected from mine residues and another one from a non-contaminated site).

Chapter 6. Endophytic colonization of *Crotalaria pumila* by seed endophytic mCherry-tagged *Methylobacterium* sp.

- To study the ability of seed endophytes to colonize *C. pumila* plants in the presence of PTEs.

HYPOTHESES

Chapter 1. Characterization of tailing heaps from a mine in Zimapan, Mexico.

- The percentage of extractable PTEs, regarded as a result of chemical characteristics and mineralogical composition of mine residues is affected by the management of the mine tailings.

Chapter 2. Wild flora of mine tailings: perspectives for use in phytoremediation of potentially toxic elements in a semi-arid region in Mexico

- Among native flora colonizing mine residues there are plant species appropriate for phytoextraction or phytoestabilization of PTEs.

Chapter 3. Phytobarriers: plants reduce atmospheric dispersion of particles containing potentially toxic elements originating from mine tailings in semiarid regions.

- Plant species colonizing mine residues can retain PTEs containing particles on their leaf surfaces.
- The amounts of retained particles on leaves differ among plant species.

Chapter 4. Seed endophytic bacteria of a pioneer plant species colonizing mine residues in a semi-arid region: trans-generational characterization

- Seeds of *C. pumila* growing on mine tailings contain cultivable endophytic bacteria that are transferred transgenerationally.
- Seed endophytic bacteria present functional characteristics that contribute to the establishment and growth of *C. pumila* plants on mine tailings containing PTEs.
- The composition of seed endophytic bacterial community of *C. pumila* growing on mine tailings is different from that of plants of the same species growing in non-contaminated site.

- Functional traits of endophytic bacteria of *C. pumila* are different between seeds collected on mine tailings and seeds from non-contaminated site.

Chapter 5. Seed endophytic bacteria from *Crotalaria pumila* and their potential to promote remediation of soils contaminated by potentially toxic elements.

- Selected seed endophytic bacteria stimulate biomass production of *C. pumila* arising from two different seed batches (collected from mine residues or non-contaminated site) when the plants are growing on PTEs polluted soil.
- Studied seed endophytic bacteria affect translocation of PTEs of *C. pumila* arising from two different seed batches (collected from mine residues or non-contaminated site) when the plants are growing on PTEs polluted soil.
- Inoculation with seed endophytic bacteria promotes activity of antioxidative enzymes of *C. pumila* arising from two different seed batches (collected from mine residues or non-contaminated site) in the presence of PTEs.

Chapter 6. Endophytic colonization of *Crotalaria pumila* by seed endophytic mCherry-tagged *Methylobacterium* sp.

- Seed borne *Methylobacterium* sp. colonizes the inner root tissue of *C. pumila* in presence of PTEs.
- Seed endophyte *Methylobacterium* colonizes the transport system of its host plant when growing in the presence of PTEs.
- Labeled *Methylobacterium* migrates from roots to shoots of *C. pumila* in the presence of PTEs.

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CHAPTER 1. CHARACTERIZATION OF TAILING HEAPS FROM A MINE IN ZIMAPAN, MEXICO.

Abstract

In order to describe the chemical characteristics and mineralogical composition of mine residues under field conditions, waste samples were taken from tailing deposits aged for different periods of time, ranging from less than one to 40 years. The total, DTPA- and water-extractable concentrations of Ca, Cd, Cu, Fe, Mn, Pb and Zn were determined. These results together with mineralogical composition were useful to assess the present stage of oxidation and the risk of potentially toxic elements (PTEs) release into the environment. The results varied among tailing deposits, and in some cases between the plateau and sloped zones of a given tailing heap. The proportion of soluble and DTPA-extractable PTEs were the result of the chemical characteristics and mineralogical composition of mine residues. The handling of mine residues and possibly, the method and the site of tailing construction, affected the chemical and mineralogical features of studied tailing heaps. The results highlight the importance of the specific characteristics of tailing heaps in future mitigation and remediation efforts.

Keywords: sulfide oxidation, heavy metals, environmental mineralogy

Introduction

Mining has impacted local culture, economy and ecology in Mexico for more than five centuries, from ancient villages to the modern municipalities. From the beginning of industrial mining until the present time great quantities of mine residue have been generated. Zimapan Village, which is located near the current mine site, is situated in the mine exploitation area of central Mexico, where intensive mining was started during the 17th century. The principal products have been Mn, Zn, Cu, Ag and Au (SGM, 2011).

There are two principal operations in mineral processing: liberation and concentration. The first one consists in crushing and grinding to obtain clean separated particles of valuable minerals and gangue or waste minerals (Napier-Munn, 2006). Sulfide ore minerals are tipically concentrated by flotation after milling. The resulting fine particles (clay to silt size) are mixed with water to form a slurry, which is treated with surfactants to turn the sulfide mineral surfaces hydrophobic. The surfactants used are variable, but those most commonly used for flotation include xanthates and dithiophosphates (Fuerstenau, Chander and Woods 2007). Subsequenlt, the fine-grained slurry is poured into flotation vessels, where a frothing agent is added. The hydrophobic sulfide minerals will then attach by surface tension to the bubble interface formed during frothing and are transported to the surface of the flotation slurry. The minerals adhering to the froth are collected (Lindsay *et al*. 2015). After the ore minerals have been processed the resulting waste is commonly deposited in tailing heaps or piles (Blowes, Ptacek and Jurjovec 2003) up to 15 m high that remain exposed to the environment.

The processing of sulfide ore minerals produces tailings containing gangue minerals and residual sulfides. Minerals reported as components of residues include sulfides (pyrite, sphalerite, galena, pyrrothyte, arsenopyrite, chalcopyrite, bornite), carbonates such as calcite, and silicates

such as quartz (Moreno-Tovar, Barbason and Coreño-Alonso 2009; Moreno-Tovar, Téllez-Hernández, and Monroy-Fernández 2012). Sulfide minerals are abundant and often contain potentially toxic elements (PTEs) as a component of the mineral structures.

Since mine residues are exposed to the atmosphere (oxidizing conditions) oxidative weathering processes begin. Under these conditions, PTEs dispersion can occur through wind and water erosion, or after the alteration or dissolution of the original mineral components. Oxidative dissolution of sulfide minerals results in acidification (Edwards *et al.* 2000; Alakangas, Lundberg, and Nason 2012), which leads to the enhanced mobilization and dispersion of PTEs from tailing deposits (Gleisner and Herbert 2002; Jonathan *et al*. 2010). Once in solution, there are several mechanisms affecting the fate of PTEs (Lapakko, 2002). Generally, during mine operation the oxidation is often relatively slow but is enhanced after the residue accumulation (Romero *et al*. 2008). Mineral reactivity in the tailing heaps differs from site to site, as a function of the mineral composition (Jennings, Dollhopfa, and Inskeep 2000; Hiller *et al*. 2013) and chemical characteristics (Cappuyns and Swennen 2008; Hofmann and Schuwirth 2008). An understanding of the mechanisms and factors affecting the oxidation process and thus the release of PTEs into the environment is important for prediction, remediation and prevention of PTEs contamination. The detailed characterization of changes in sulfide mine residues provides information about the probable future PTEs release from the tailings. Therefore, a comparative study among tailing heaps originating from the same mine was performed. The aim of this work was to describe the mineralogical composition and chemical characteristics of nine tailings heaps and to evaluate the probable impacts of handle of residues on the PTEs release from the tailing heaps.

Materials and methods

Site description

This research was conducted with residues of a mine located next to San Francisco village (20°49’32.5” N and 99°22’20.1”W) in Zimapan municipality, Hidalgo State in central Mexico. The weather is temperate semidry, annual average temperature varies between 15 and 22 °C, and the average precipitation is 500 mm (CNA, 2002). Geology of Zimapan is basically sedimentary outcrops formed by calcareous shales and limestone (Alcayde and Cserna 1982). The studied mine has been in operation since the 1970’s, during this time 9 tailing heaps have been constructed, making the mine an interesting case study for evaluating chemical and mineralogical changes as a result of sulfide oxidation under natural conditions.

Sampling

Composite samples, formed by mixing 10 subsamples, were obtained from each tailing. The depth of sampling varied from 0.3 m (Tailings 4, 5, 6, 7, 8 and 9) to 1 m (Tailings 1, 2 and 3). In case it was possible samples were taken from both, plateau and slope of the tailing heap. At Tailings 1, 2, 3, 4, 5 and 6 sampling was done at the plateau. While, at piles 7 and 9 samples were obtained from the plateau and the slope of the pile.

Mineralogical analysis

A MMA X-ray diffractometer utilizing Cu K-alpha radiation from a Cu source operated at 35 kV and 25 mA was used for mineralogical analysis. The 2θ range was 2-75° with 0.03° steps. Prior to analysis the samples were ground using an agate mortar. Approximately 2 g of ground sample were set on the analysis plate. Mineral identifications from the X-ray patterns were obtained by taking into account the interplanar distances and relative peak intensities. Mineral identification by powder X-ray diffraction was done using online free access mineralogical

databases. The databases used were the mineralogical database Webmineral and the American Mineralogist Crystal Structure Database. Due to the difficulty in identifying sulfide and oxyhydroxides minerals by X-ray diffraction alone, mineral optical identification was also performed. Slides were prepared for petrographic microscopic analysis. Mine residue grain samples were fixed and imbedded in an epoxy resin on a microscope slide and observed under plain and cross-polarized light using a petrographic microscope. Prepared slides and grain samples were also observed under incidental light (Lynn, Thomas, and Moody 2008).

Chemical characterization

Prior to chemical analysis mine residues samples were sieved (2 mm). All procedures were performed at room temperature (20-25 °C). Redox potential was measured with a platinum electrode. pH and electrical conductivity (EC) were determined using a potentiometer and a conductivity bridge, respectively. These three variables were analyzed in the supernatant of a 2.5:1 (v:w) deionized water:residue slurry. To measure pH and redox potential the slurry was shaken for 15 min in a reciprocating shaker. The pH meter was calibrated with the 4 and 7 buffer solution for pH and with the electrolytic solution for redox. The same slurry was subsequently shaken during 16 h and then EC reading was obtained. Afterwards, the mixture was centrifuged (700 rcf for 5 min) and filtered (Whatman No. 42). The filtrate was used to determine sulfate concentration and to analyze the water-extractable PTEs concentrations (Ca, Cd, Cu, Fe, Mn, Pb and Zn). For determination of available sulfate concentration, the $BaCl_2$ turbidimetric method was used (Chesnin and Yien 1951). The resulting turbidity was measured using a spectrophotometer (UV-Visible, Cary 50Varian) and compared with a standard curve.

To prevent PTEs precipitation in the filtrate, after the aliquot for sulfate analysis was taken, a drop of HNO_3 was added to the filtrate (~20 mL). Samples were stored at 4°C until analysis. PTEs

concentrations were determined by flame atomic absorption spectrometry (FAAS) (Perkin Elmer 3100).

Mine residues samples for diethylenetriaminepentaacetic acid-triethanolamine-calcium chloride (DTPA)-extractable and total PTEs concentrations were air dried and sieved (2 mm) before analysis. DTPA-extractable PTEs concentrations were determined according to Lindsay and Norvell (1978). The extraction ratio was 1:4 (0.5 g of sample plus 20 mL of extractant). Total concentrations of PTEs were determined following acid digestion of the tailings samples (0.5 g) in HNO_3 (EPA 3050) at a digestion ratio of 1:100 (w:v). Afterwards the digests were readjusted to 50 mL total volume and filtered (Whatman 42). From these concentrations were calculated the soluble and extractable percentage of each element:

$$\%Soluble = \frac{[water - soluble]}{[total]} \text{ x } 100$$

$$\%DTPA = \frac{[DTPA - extractable]}{[total]} \text{ x } 100$$

The total carbonate content in the solid phase was determined by the volumetric calcimeter procedure (Loeppert and Suarez 1996). The total free iron oxides (Fe-ox) were extracted by the dithionite-citrate buffered method (Loeppert and Inskeep 1996) and the concentrations were determined by FAAS.

Certified standards were used to prepare the calibration curves.

Statistical analysis

Principal components analysis was applied by including all chemical variables determined. Free software R version 3.1.0 was used for statistical analysis (R Core Team, 2013).

Results

Field observations

The Zimapan mining area is located in the Mexican West mountain range; thus most of the region has rough terrain. In the area there exist three main watercourses: the Tula, Amajac and Metztitlan Rivers (INEGI, 2009). Due to the terrain roughness, there are several small basins. San Francisco mine is located in one of those, but water discharged is affluent of the main river. Tailings are distributed along a hydrologic basin composed of connected "stair-step" impoundments, with the tailing deposits having different heights and topographic positions (Figure 1). In order to diminish the runoff received by the tailings from the surrounding elevations, a reception and redirection canal is constructed along the basin. However, during the rainy season, this design might be not sufficient to divert the flow of incoming drainage water causing slope collapse.

Handling of residues

Tailing heaps 1, 2 and 3 are the oldest deposits and are located at higher elevations along the basin (Figure 1). At the deeper side the deposits are 25 m thick. Tailings 1 and 2 were covered with a soil layer approximately 0.4 m thick. A gravel layer was put on the top of Tailing 1, in order to use the area as a parking lot and for the administrative facilities of the mine. The plateau of Tailing 3 has been used as an open-space to store equipment and heavy machinery that is not used any longer. Mine tailing 4 was constructed during the 1980's, but 3 years ago it was covered with fresh residues. In some parts, the layer of fresh residues reaches 3 m thickness.

Yellow, red or orange colours, observed at Tailings 3, 5, 8 and the slope of tailing 7, are an indication of oxidation and thus potential risk to the environment. For this reason when was evident, as indicated by the colour, some tailing heaps were covered with fresh grey (unoxidized)

mine residues. Mine Tailing 7 is approximately 9 years old, with grey colour residues on the plateau, compared to red-orange colour residues on the slope. This difference is explained by the addition of a layer of fresh residues; however, during a period of heavy rain the layer of fresh residues from the slope collapsed. As a result, the oxidized residues were exposed and the fresh and old residues were mixed.

Additionally, not all tailing heaps were constructed following the same method. The first six deposits were constructed following the downstream procedure of depositing the slurry as layers. In contrast Tailing dam 9 is being constructed following the upstream method of separating coarse and fine particles by cycloning. The coarse particles (250-2000 μm) form the heap slope, while the fine particles (<250 μm) are deposited on the plateau. Tailing 7 was constructed by the same method. Tailing deposit 9 is approximately 65 m deep.

Mineralogical composition

Mineralogical composition is presented in Table 1. Quartz, calcite and pyrite were minerals identified in all tailing heaps. Among silicate minerals, besides quartz were diopside and hedenbergite. The most common carbonate mineral was calcite, followed by dolomite and ankerite. Dolomite was present at Tailings1, 2, 4 and the plateu of Tailing 7, while ankerite was identified in Tailing 2, 3, 5, 6, at the slope of Tailing 7 and the plateau of Deposit 9.

Some characteristic peaks corresponding to sulfide minerals were identified by X-ray analysis. Their presence in mine residues was corroborated by incidental light identification under the microscope. According to Klein and Hulburt (1997), sulfide minerals can be distinguished by their colour and luster. In all tailing heaps sulfide minerals were identified, among which were: arsenopyrite, pyrrhotite, galena and sphalerite.

The iron oxides and oxyhydroxides identified were goethite, hematite and ferrihydrite. The identification of this last mineral was checked by X-ray diffraction and complemented by observations at the microscope. Ferrihydrite shows a pattern with maxima at 2.54, 2.35, 1.97, 1.72, 1.51, 1,47; however, these peaks are generally weak (Wilson, 1987). Observations under the microscope were useful to verify the presence of ferrihydrite by recognizing its characteristic reddish-brown colour, with hues in the 5YR to 7.5YR range (Bigham, Fitzpatrick and Schulze 2002).

Goethite was more frequently observed in the mine tailings than were hematite and ferrihydrite; it was observed in all heaps except Tailing 5 and the slope of Tailing 9. The three minerals, goethite, hematite and ferrihydrite, were observed in Tailing 3 and the slope of Tailing 7. Among secondary sulfate minerals, gypsum, jarosite, plumbojarosite and anglesite were identified; the three minerals were presented in Tailing 3, 5, 8 and the slope of Tailing 7. Gypsum was identified in all tailing heaps excepting Tailing 2 and the plateau of Tailing 9. Jarosite was most frequent in Tailing 8. Lead sulfosalts identified were boulangerite, jamesonite and freibergite; the first two minerals were observed in Tailings 3 and 8.

Chemical characteristics

The chemical analyses are summarized in Table 2. The values of redox potential were variable; the lowest value was observed for the slope of Tailing 9, which represent the newest and most highly reduced tailing, dominated by sulfide minerals. The highest redox values were observed in the Tailing 5 and the slope of Tailing 7, where there was also evidence of enhanced oxidation. The pH values of the mine tailings ranged from 1.7 to 8.4. Tailing heaps 3, 5, 8 and the slope of Tailing 7 had acidic pH (<5). The highest pH was 8.4, in the slope of Tailing 9; which

was under construction at the time of sampling. The EC varied from $5.14x10^4$ and $1.63x10^6$ µS m^{-1} in the slopes of Tailings 9 and 8, respectively.

The plateau and slope of Tailing heap 9 contained the highest concentrations of total carbonates, followed by Tailing 6 and the plateau of Tailing 7. Mine tailing 3 had the lowest total carbonates concentration; as well as Tailings 5, 8 and the slope of Tailing 7. The slope of Deposit 7 had the highest concentrations of Fe-ox and dissolved sulfate. Iron oxides were less abundant in the slope of Tailing 9 (Table 2). The colour of the residues was in the YR hue. Dry and wet residues from Tailings 4, 6, plateau of 7 and plateau and slope of 9 varied from light to dark grey. Residues from Tailing 5 were reddish yellow and those from Tailing 8 were yellow (Table 1). Tailings 1, 2 and 3 were from brown to strong brown.

PTEs concentrations

Dissolved PTEs concentrations. The total concentrations of PTEs obtained by the acid digestion procedure are shown in Table S1. The relative PTEs abundance was Ca > Fe > Pb > Zn > Cd = Cu > Mn; however, concentrations of each PTEs varied among tailings. Calcium concentrations ranged from $3.25x10^4$ and $1.54x10^5$ mg kg^{-1}, corresponding to Tailings 5 and 6, respectively. The range for Fe was $2.20x10^4$ to $1.45x10^5$ mg kg^{-1}. The highest concentrations of Fe and Cu were registered in the slope of Tailing 9. The lowest concentrations of Cu, Fe and Mn were detected in Tailing 5. Lead was most abundant in Tailing 3 ($1.02x10^4$ mg kg^{-1}); Cd varied between 533 mg kg^{-1} in Tailing 1 and 1,190 mg kg^{-1} in the slope of Tailing 7.

Extractable-PTEs concentrations. The water- and DTPA-extractable concentrations are presented in Tables S2 and S3, respectively. The percentages of DTPA-extractable and water-soluble PTEs compared to the total concentrations varied among tailing heaps (Figure 2). Since it

was initially assumed that pH is the main factor controlling PTEs fate, the percentages are arranged according the pH value registered in each tailing heap.

Percentage of water soluble PTEs with respect to total concentrations. The water-soluble percentages of Ca, Cd, Cu, Mn and Zn were higher in tailing deposits with pH values lower than 6.6 (Tailings 3, 5, 8 and the slope of Tailing 7). Except for Ca, the water-soluble percentages diminished to values close to zero in tailing deposits of pH 6.6 or higher (Tailings 1, 2, 4, 6, and the plateau of Tailing 9). Tailing 8, pH 1.9, showed the highest percentages of water soluble Mn (42%), Fe (32%) and Cu (15%). The highest values for Zn (29%) and Cd (2%) were determined in the slope of deposit 7, which had pH 1.7. Lead exhibited different behavior from the other elements; the soluble fraction of Pb was close to zero in all tailing dams.

DTPA-extractable concentrations. DTPA extractable iron and Mn represented the highest percentages with respect the total concentration, around 7% in tailings with pH lower than 3.6 (Tailing deposits 5, 8 and the slope of Tailing 7). For Pb, the trend was different, with the DTPA-extractable percentages increasing as the pH values increased. The highest value (12%) was observed in the slope of Tailing 9 (pH 8.4). For Cd and Zn, two peaks of high DTPA-extractable percentage were observed. The first peak was at pH < 3.6 (Tailings 5, 8 and the slope of Tailing 7); the second peak was around neutral pH, mainly in Tailing 6. Copper values were variable; despite of the highest percentage detected in an acidic tailing (pH 3.6), there were values lower than 1% in the other acidic tailings (Tailing 5 and the slope of Tailing 7). In deposits with neutral pH, DTPA-extractable Cu also varied. In Tailing 1 (pH 7) and Tailing 6 (pH 7.1) the DTPA-extractable Cu was around 6%; whilst at pH 7.2 (plateau of Tailing 7) the percentage was lower than 1. For Ca, the highest percentages were observed at pH 2.4 (14%) and 3.6 (11%), in Tailings 5 and 3, respectively, but the values were lower in the most acidic tailings.

Oxidation advance of mine tailings

A principal component analysis was performed (Figure 3) by taking into account all chemical variables. It was possible to distinguish three groups; the first one is composed by Tailings 1, 2, 4, 6, slope and plateau of Tailing 9 and plateau of Tailing 7. This group is characterized by neutral to alkaline pH, low redox potentials and EC, relatively low concentrations of Fe-ox, abundant carbonates (Table 2) and low water-soluble and DTPA-extractable PTEs concentrations (Figure 2, Table S2).

A second group was formed by Tailings 3, 5 and the slope of Tailing 7; which are characterized by acidic pH, high EC, low carbonate content (Table 2) and high water-soluble and DTPA-extractable PTEs concentrations except Pb and Mn (Figure 2). Additionally, in these tailings secondary minerals such as oxides, oxyhydroxides and sulfates were identified (Table 1). Tailing 8 shared the aforementioned characteristics; however, this tailing had the highest values for EC (Table 2) and water-soluble Fe concentration (Figure 2).

Discussion

PTEs concentrations

PTEs concentrations (total, DTPA-extractable and water-soluble) varied widely between residues (Figure 2; Table S1, S2, S3). Regarding total concentrations, the most abundant PTEs were Ca, Fe and Pb, followed by Zn. Cu, Cd and Mn were less abundant (Table S1). However total concentration does not reflect the risk of PTEs mobility. Chelating agents, in this case DTPA, could release a relatively large proportion of PTEs by displacing them from insoluble organic or organometallic complexes in addition to those sorbed on inorganic soil components (Ure and Davidson 2002). Hence, DTPA might give an estimation of easily or potentially

leachable PTEs concentration (Rao, Sahuquillo and Lopez-Sanchez 2008). DTPA-extractable PTEs concentrations varied with each element and tailing heap. Acidic residues in Tailing 8 and in the slope of Tailing 7 had high concentrations of Cu, Fe, Zn and Cd (Figure 2). The slope of Tailing 9, with alkaline pH, represents the highest risk for Pb leaching (Figure 2).

The water-soluble phase contains free ions and ions complexed with soluble organic matter and other soluble components; this phase represents the most mobile and potentially the most bio-available PTEs species (Rao *et al*. 2008). The water-soluble concentration of each PTE varied between tailings. Tailings 3, 5, 8 and the slope of Tailing 7, which are acidic tailings, showed the highest water-soluble concentrations of PTEs, except of Pb (Figure 2).

Sulfide minerals

Different sulfide minerals were present in studied tailing heaps (Table 1). It has been mentioned that pyrite is one of the principal acid-producing sulfide minerals (Jennings *et al*. 2000); this sulfide mineral was detected in all studied tailing heaps. Another, acid-producing mineral identified was pyrrhotite. Non acid-producing sulfide minerals, e.g. sphalerite and galena (Dold, 2005), were also detected but not in all tailing heaps (Table 1).

Neutralizing minerals

One of the factors affecting the rate of oxidation of sulfides is the presence of acid-neutralizing minerals, like silicates and carbonates. These minerals can consume H^+ as a result of surface adsorption and dissolution reactions (Al, Martin, and Blowes 2000; Jurjovec, Ptacek, and Blowes 2002). Silicate dissolution reactions are usually slower than those of the carbonate minerals (Dold 2005). Moreover, silicates are sometimes considered to be active in acid neutralization only at very low pH values (Gusinger *et al.* 2006). In consequence, carbonate minerals are especially important for their reasonably high neutralization capacity and rate of dissolution. Because of its

abundance and relative reactivity, calcite is likely the main carbonate mineral with respect to its neutralizing capability, followed by dolomite and ankerite (Al *et al*. 2000). Both types of neutralizing minerals, i.e., carbonates and silicates, were identified in the current study (Table 1). Due to the presence of calcite in all tailing heaps (Table 1) it is reasonable to consider that this mineral might slow down the oxidation process. Nevertheless, according to the current results, the calcite-neutralizing capability might be not have been sufficient to neutralize all of the acidity production. It is also possible that secondary mineral phases coated calcite surface, hence reducing its effectiveness in acid neutralization. It is possible that precipitation of secondary minerals form coatings on the surface of primary minerals (Jurjovec *et al*. 2002; Moreno-Tovar *et al*. 2009). In this way, the secondary layers might inhibit or slow calcite dissolution (Edwards *et al*. 2000; Farkas *et al*. 2009) and thus diminish its neutralizing capability.

Secondary minerals and PTEs mobility

Different processes affect PTEs mobility from tailing heaps, among them is the formation of secondary minerals that could contribute to the immobilization and subsequent concentrations of soluble PTEs in mine tailings (Lapakko, 2002; Piantone, Bodénan and Chatelet-Snidaro 2004). The reported mechanisms are precipitation as oxides, hydroxides or carbonates, inclusion in amorphous oxides, co-precipitation and adsorption on other solid phases (Gieré, Sidenkob and Lazarevab 2003; Hita, Torrent and Bigham 2006; Alakangas *et al*. 2012; Hiller *et al*. 2013). However, tailings where secondary minerals were detected (Tailings 8, 5, 3 and the slope of Tailing 7) also showed high water-soluble and DTPA-extractable Cd, Cu, Fe, Mn and Zn concentrations (Figure 2). This result indicates that the secondary minerals are not permanently trapping PTEs from solution.

pH and PTEs mobility

Results showed that the lowest pH values generally corresponded with the highest water-soluble and DTPA-extractable PTEs concentrations, except Pb (Figure 2). This result suggests that acidic pH favored mineral dissolution and hence the release and mobility of PTEs from the mine tailings, which is in agreement with previous studies (Jurjovec *et al*. 2002; Jonathan *et al*. 2010; Tabelin *et al*. 2014).

Formation of secondary minerals might result in reduced concentrations of soluble PTEs in mine residues. However, the results did not support this trend. In mine tailings with secondary minerals, like Tailings 3, 5, 8 and the slope of Tailing 7 (Table 1), PTEs immobilization was limited and high dissolved PTEs concentrations were observed. This result can be explained by the influence of pH. The aforementioned tailing heaps had acidic pH values (Table 2). Precipitation of PTEs-bearing secondary minerals is often associated with pH increase (Bigham *et al*. 2002; Gieré *et al*. 2003). As the acidity advances in the tailings the secondary minerals become unstable and dissolution proceeds; as a result the PTEs previously sequestered are released (Weisener, Smart, and Gerson 2004; Gusinger *et al*. 2006).

The restricted Pb mobility observed at low pH (Figure 2) may be a result of precipitation as anglesite (Gusinger *et al*. 2006; Sima *et al*. 2011), that along with plumbojarosite are stable under acidic conditions (Farkaz, Weiszburg and Pekker 2009). Both, anglesite and plumbojarosite were identified in mine residues as well as boulangerite and jamesonite (Table 1).

Actual oxidation stage of mine residues

Despite the variability in chemical characteristics and mineralogical composition of tailing heaps, it was possible to classify tailings as oxidized and unoxidized (Figure 3). The oxidized tailing heaps (Tailings 3, 5 and the slope of Tailing 7) were characterized by acidic pH, high EC,

low contents of total carbonate and high concentrations of water and DTPA-extractable PTEs (except Pb and Mn). These residues were brown or yellowish, with relatively high occurrence of secondary minerals, high concentrations of Fe-ox and dissolved sulfate. The observations suggested that Tailings 3, 5, 8 and the slope of Tailing 7 are in active oxidation stages.

The unoxidized tailing heaps had neutral to alkaline pH, low redox potential and EC, low concentrations of water-soluble and DTPA-extractable PTEs, high contents of total carbonate, low concentrations of Fe-ox, and grey colour. Tailings that are considered unoxidized are 1, 2, 4, 6, 9 and the plateau of Tailing 7. It is interesting that in Tailing 7, the residues from the plateau were considered unoxidized, while in the slope of the same tailing the residues were oxidized. This situation could be explained by the addition of a fresh residue layer over the oxidized tailing heap.

It has to be mentioned that this classification, oxidized and unoxidized mine tailings, is temporary. Mine tailings systems are active and changing continuously.

Will PTEs release continue?

The characteristics of residues from the newest tailing heap (Tailing 9) reflect the initial condition of the mine residue: alkaline pH, high carbonate content (Table 2), the presence of non acid-producing sulfide minerals (galena, sphalerite) and neutralizing minerals, mainly calcite (Table 1). Under this situation it is reasonable to assume that acidic oxidation will not occur. However, our results suggested the opposite.

Normally, pyrite is not considered very reactive at pH higher than 7 (Dold, 2005). Nonetheless, sulfide minerals may be sensitive to environmental conditions (Frau, 2000; Sima et al. 2011). Precipitation and dissolution cycles of some minerals are strongly influenced by seasonal wetting and drying (Dold and Fontboté 2001), and oxidative dissolution take place when such minerals

are exposed to surface air and water (drought/flooding cycles). Moreover, the oxidation of sulfide minerals can also occur at circumneutral pH (Moses and Herman 1991; Emerson and Moyer 1997; Alakangas *et al.* 2012) and under the direct and indirect effect of microbial activity (Edwards et al, 2000; Southam and Sanders 2009; Templeton and Knowles 2009). These factors undoubtedly represent an interesting field for future research regarding oxidation, PTEs release and eventual remediation of PTEs-containing mine residues.

It was interesting that sulfide minerals were identified in tailing heaps with acidic pH (Table 1, 2). Usually at these pH sulfide minerals are dissolved (Akcil and Koldas 2006). The presence of such minerals suggests that even in highly oxidized mine tailings the persisting oxidation of sulfides will result in acidic pH and continuing release of PTEs. Additionally, at low pH values some secondary minerals may re-precipitate, and previously occluded PTEs will be liberated.

Factors affecting PTEs release from mine residues

Our results showed that differences in oxidation advance and PTEs release among tailing heaps did not correspond necessarily to the time of environmental exposition. Other factors intervene during the oxidation process to impact release of PTEs from tailing heaps. For instance, most mining companies, including this in study, manipulate pH and use surfactants, chelating agents or sequestering ligands to extract minerals from the ore. The specific flotation agents used during ore processing affect the activity of acidophilic bacteria present in mine residues and thereby oxidation rate and the type and amount of PTEs liberated from residues (Dong *et al*. 2011). However, in many cases information about used chemical reagents is not accessible.

The apparent inconsistency in finding calcite and sulfide minerals in acidic tailings, described in previous paragraphs, might have been partially impacted by residue handling. As mentioned in the Results section, sometimes a fresh residue layer was placed over the oxidized tailing heaps.

This action caused oxidized and unoxidized residues to mix, and then calcite and sulfide minerals occur simultaneously in acidic tailings with the mentioned consequences.

The tailing heaps were constructed as stair-step impoundments within a valley (Figure 1). This situation results in individual tailings deposits with different water flux, infiltration and drainage characteristics, leading to differences in wetting-drying cycles (EPA, 1994). Since the particles are separated by size during construction, even in the same tailing heap the oxygenation may be different inducing different oxidation rates.

Importance and implications of laboratory assessments to field observations

Characteristics determined in the laboratory are very important for assessing the current oxidation state of residue and the potential for PTEs release to the environment (Gleisner and Herbert 2002; Cappuyns and Swennen 2008). The relationship of these characteristics to residue handling and to the environmental and topographic conditions is especially relevant.

The processes occurring after residue deposition are diverse; they involve microbiological, hydrological, mineralogical and geochemical characteristics, and the climatic conditions, resulting in a complex system. Due to the different characteristics among tailing heaps in the same mine, each tailing heap may be considered as a specific system for remediation purposes. And in some cases a single tailing heap, the slope and the plateau should even be considered as different systems. Therefore, for future restoration/remedation plans it is necessary to emphasize the importance of the specific characteristics of tailing heaps. This specificity should be taken into account for the handling of residues, environmental risk assessment and technology transference.

Conclusions

This study compared the mineralogical compositions, chemical features and PTEs release characteristics of tailing heaps from the same ore mine but with environmental exposure times from <1 to 40 years. The chemical and mineralogical characteristics of the residues among the tailing heaps differed considerably. It was possible to identify the relative oxidation states of the individual tailing heaps. Due to their metal-sulfide rich mineralogical composition, the tailings now classified as unoxidized will likely proceed through an oxidation phase and eventually release PTEs into the environment.

The oxidation state, reflected as the extractable PTEs concentration, observed in mine tailings was not only in function of exposition time and mineralogical composition but also complex sediment deposition, management and hydrological factors. These findings further emphasize the importance of handling and environmental management of residues. In one tailing heap the plateau and the slope of the same pile had different characteristics. These differences could be explained by the construction method of the piles (separation by particle size), by the handling of residues (layers of fresh residues) and by the location of tailing heaps within the basin.

The specific characteristics of tailing heaps are relevant for environmental risk evaluation and remediation technology transference. For future remediation projects it is important to take into account these differences, because what may work for one tailing may not work for another one, even if they are originated from the same mine or in the same tailing heap.

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Supplemental material

Supporting information shows the total, water-, and DTPA-extractable concentrations of PTEs determined in mine residues.

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Table 1. Mineralogical composition of different tailing heaps from a mine in Zimapan, Mexico.

Minerals			Tailing heap										
			1	2	3	4	5	6	7p	7s	8	9p	9s
Silicates	Quartz	SiO_2	+	+	+	+	+	+	+	+	+	+	+
	Diopside	$CaMgSi_2O_6$	-	+	+	+	+	-	-	-	-	+	+
	Hedenbergite	$CaFeSi_2O_6$	+	-	-	+	-	+	-	-	-	-	+
Carbonates	Calcite	$CaCO_3$	+	+	+	+	+	+	+	+	+	+	+
	Dolomite	$CaMg(CO_3)_2$	+	+	-	+	-	-	+	-	-	+	-
	Ankerite	$Ca(Fe,Mg,Mn)(CO_3)_2$	-	+	+	-	+	+	+	-	-	-	-
Sulfide	Pyrite	FeS_2	+	+	+	+	+	+	+	+	+	+	+
	Arsenopyrite	FeAsS	-	-	-	+	-	+	-	-	-	+	+
	Pyrrhotite	$Fe_{(1-x)}S$	+	+	-	+	-	+	-	+	-	+	+
	Galena	PbS	-	+	-	+	+	+	+	+	+	+	+
	Sphalerite	(Zn,Fe)S	+	-	-	+	-	+	+	+	-	+	+
Oxides/ Oxyhydroxides	Goethite	FeO(OH)	+	+	+	+	-	+	+	+	+	+	-
	Hematite	Fe_2O_3	-	-	+	-	-	-	+	+	-	-	-
	Ferryhydrite	Fe_2O_3•$0.5(H_2O)$	+	+	+	-	+	-	-	+	-	-	-
Sulphate	Gypsum	$CaSO_4$	+	-	+	+	+	+	+	+	+	-	+
	Plumbojarosite	$PbFe_6(SO_4)_4(OH)_{12}$	+	-	+	-	+	-	-	+	+	-	-
	Jarosite	$KFe_3(SO4)_2(OH)_6$	-	-	+	-	+	-	+	+	+	-	-
	Anglesite	$PbSO_4$	-	-	-	-	+	-	-	-	+	-	-
Sulfosalts	Boulangerite	$Pb_5Sb_4S_{11}$	-	-	+	-	+	-	-	+	+	-	-
	Jamesonite	$Pb_4FeSb_6S_{14}$	-	-	+	-	+	-	-	-	+	-	-
	Freibergite	$Ag_6Cu_4Fe_2(Sb,As)_4S_{13}$	-	-	+	-	-	-	-	-	+	-	-
Others	Cerusite	$PbCO_3$	-	+	-	-	+	-	+	-	-	-	-
	Berlinite	$AlPO_4$	+	-	-	-	-	-	-	-	-	-	-
	Djerfisherite	$K_6Na(Fe,Cu,Ni)S_{26}Cl$	-	+	-	-	-	-	-	-	-	-	-

p: plateau of tailing heap; s: slope of tailing heap; minerals identified (+) or not identified (-) in different tailing heaps; *at least one of the sulfide minerals was identified in tailing heaps.

Table 2. Characteristics of different tailings heaps from a mine in Zimapan, Mexico.

Tailing Heap	Age (years)	pH	Redox Potential (mV)	EC ($\mu S\ m^{-1}$)	Total CO_3^- ($g\ kg^{-1}$)	Dissolved SO_4^- ($mg\ kg^{-1}$)	Iron oxides ($mg\ kg^{-1}$)	Color Dry	Color Wet
1	40	7.0±0.6	172.0±36	186700±1410	100±4	922±29	1214±91	7.5YR 4/4	7.5YR 3/2
2	30	6.9±0.5	191.4±22	173500±2830	107±19	910±23	755±99	7.5YR 4/2	7.5YR 3/1
3	30	3.6±0.3	410.7±54	225300±6310	45±9	1008±199	1041±92	7.5YR 5/6	7.5YR 4/4
4	3	6.6±0.3	295.7±41	114200±1810	140±51	421±98	265±41	7.5YR7/1	7.5YR5/1
5	18	2.4±0.1	521.8±87	188200±7970	51±17	40±16	1221±82	7.5YR6/8	7.5YR 6/8
6	14	7.1±0.4	215.6±66	185900±4860	202±78	882±57	631±105	7.5YR4/1	7.5YR 3/1
7p	8	7.2±0.9	238.4±52	169800±3850	220±60	816±44	799±56	7.5YR5/1	7.5YR 4/1
7s	8	1.7±0.7	557.2±87	477100±3650	50±14	2831±238	3032±87	7.5YR4/6	7.5YR 4/6
8	10	1.9±0.2	393.1±70	1627000±289200	50±10	1295±167	1100±43	10YR8/8	10YR 7/8
9p	2	7.4±1.1	143.9±42	135900±2410	268±30	171±54	449±31	7.5YR4/1	7.5YR 3/1
9s	2	8.4±1.0	137.8±38	51400±830	222±13	49±11	172±34	7.5YR5/1	10YR 4/1

Values are the average of three replicates ± standard deviation; p: plateau of tailing heap; s: slope of tailing heap; EC: electrical conductivity.

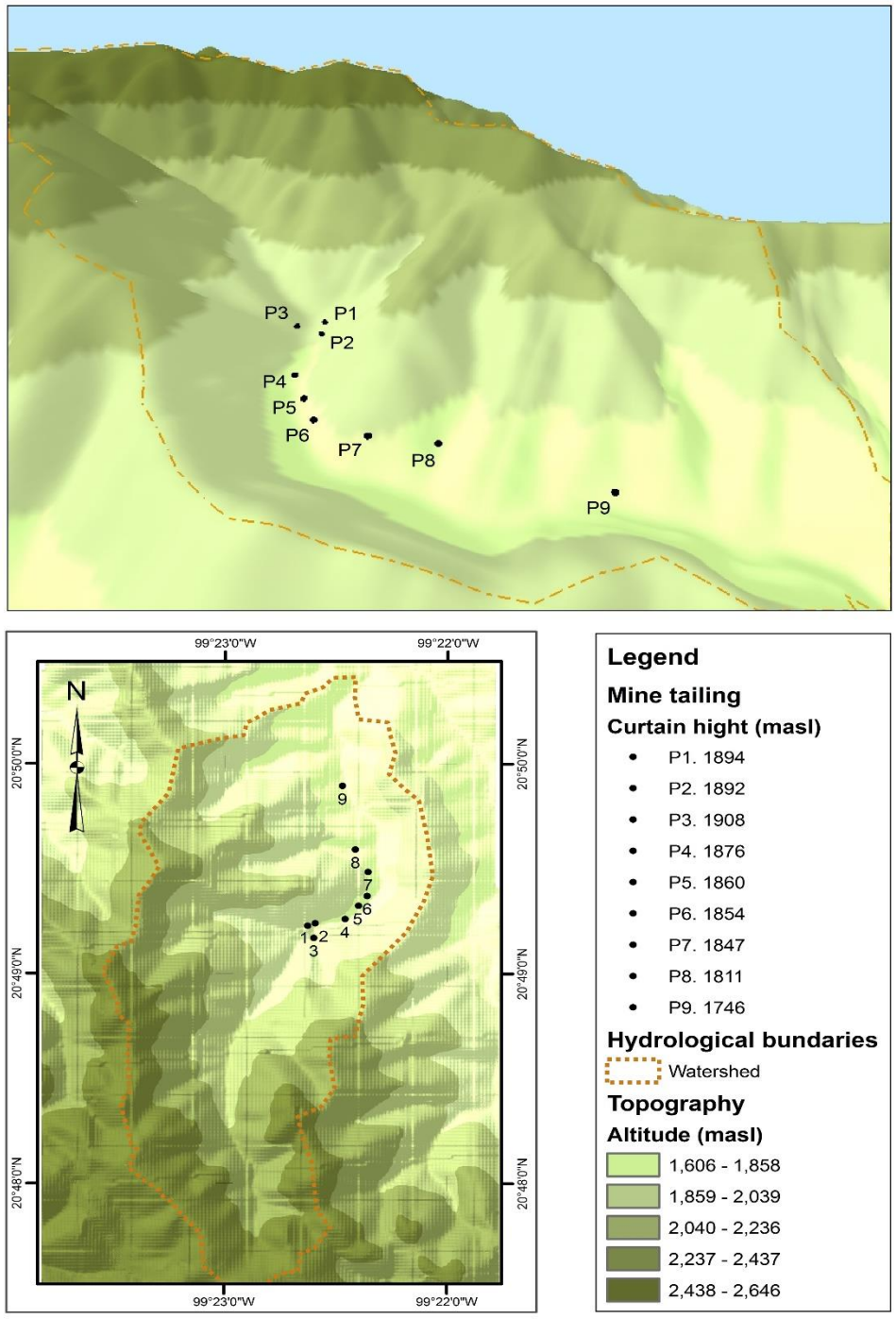

Figure 1. Distribution of tailing heaps along a hydrologic basin in Zimapan, Mexico.

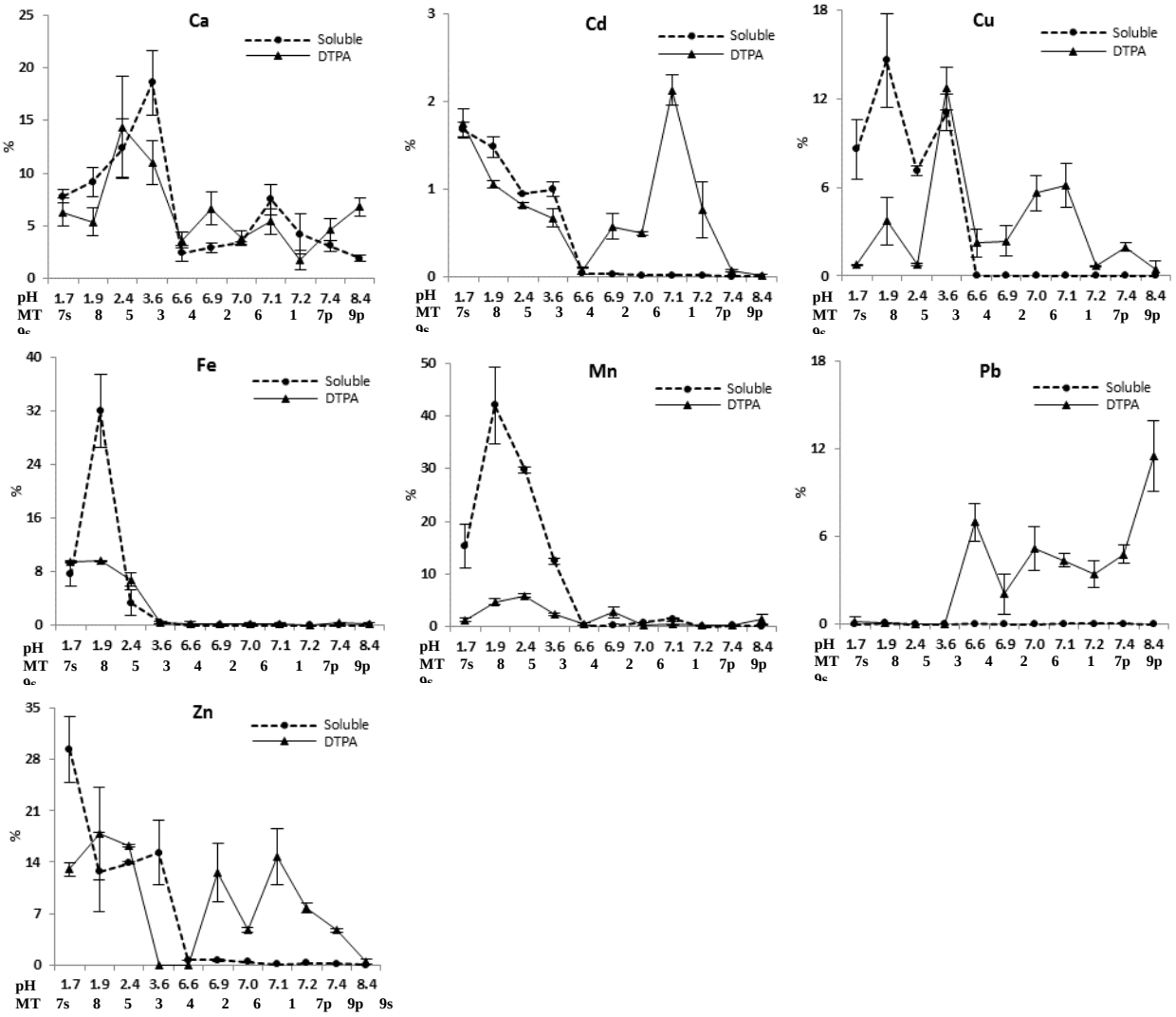

Figure 2. Soluble and DTPA-extractable percentage of PTEs in mine residues with different pH.

MT: number of mine tailing; p: plateau of tailing heap; s: slope of the tailing heap.

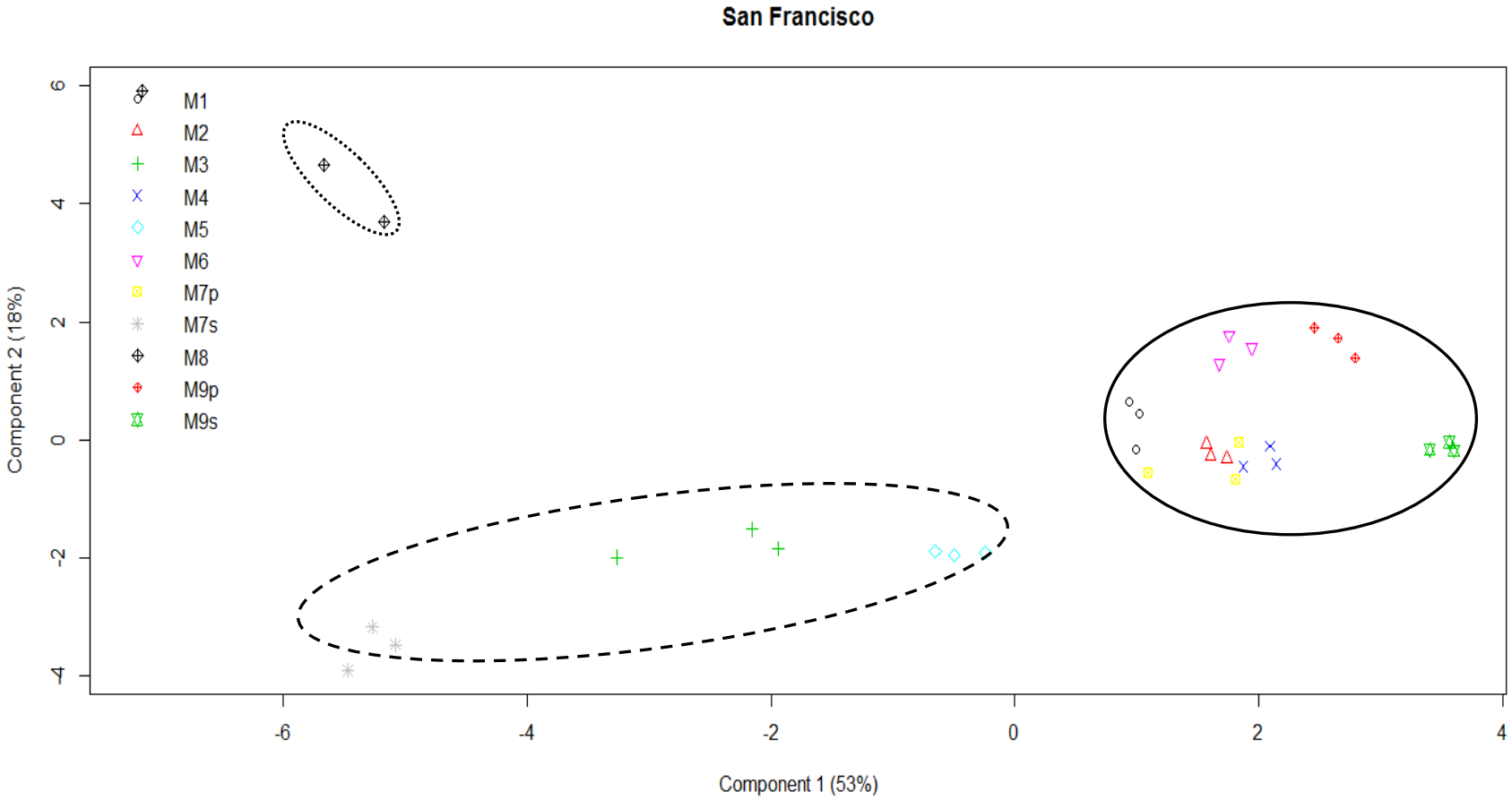

Figure 3. Plot of principal component analysis of chemical variables determined in different tailing heaps from a mine in Zimapan, Mexico (n=3). M: mine tailing; when posible both plateu (p) and slope (s) of mine tailing were sampled. Circles identified different grouping: non oxidated mine residues (continuos line); oxidized mine residues (non-continuos line).

Table S1. Total concentrations (mg kg^{-1}) of PTEs in tailing heaps from a mine in Zimapan, Mexico.

Tailing heap	Age (years)	Ca	Cd	Cu	Fe	Mn	Pb	Zn
1	40	78313±1935	533±21	850±27	26833±2371	307±47	2720±245	6757±208
2	30	60813±1603	777±92	1490±30	45500±621	457±95	4250±171	6623±210
3	30	33180±2121	757±90	1913±156	37600±211	217±21	10243±436	7263±249
4	3	122313±2496	776±23	1023±228	31767±231	883±94	2457±664	5723±812
5	18	32513±1794	887±51	320±44	22033±588	76±5	7283±509	6067±416
6	14	153513±2114	1013±21	740±154	45567±141	1143±123	4723±115	3673±527
7p	9	78980±3413	1123±6	653±21	48000±2426	1253±75	4193±530	4540±322
7s	9	44513±326	1190±82	847±29	116633±739	260±70	3777±660	4550±166
8	7	53446±836	1120±50	253±21	117367±3311	221±9	3630±496	2670±134
9p	In operation	108380±1521	977±45	433±51	45867±9250	573±57	3337±638	1503±42
9s	In operation	39680±210	1057±110	2967±125	145233±9314	1000±103	5117±907	6627±302
Range		32,513-153,513	533-1,190	320-2,967	22,033-145,233	76-1,253	2,457-10,243	1,503-7,263

Average of three replicates ± standard deviation; p: plateau of tailing heap; s: slope of tailing heap;

Table S2. Water-extractable concentrations (mg kg^{-1}) of PTEs in tailing heaps from a mine in Zimapan, Mexico.

Mine tailing	Age	Ca	Cd	Cu	Fe	Mn	Pb	Zn
1	40	5547±349	0.1±0.01	0.1±0.02	0.3±0.05	5.0±1.0	1.0±0.1	3±0.1
2	30	1815±222	0.2±0.03	0.1±0.02	0.3±0.03	1.0±0.2	1.0±0.2	39±1.0
3	30	4611±219	7.0±0.40	212±13	126±10.4	27.0±1.9	1.0±0.3	1096±49.1
4	3	2842±186	0.3±0.04	0.2±0.02	0.4±0.10	2.0±0.4	1.0±0.1	2.0±0.2
5	18	2976±172	1.0±0.10	23±2.12	678±29.6	7.0±0.7	1.0±0.2	236±5.1
6	14	5241±270	0.2±0.04	Nd	0.4±0.10	9.0±0.5	0.4±0.1	15±4.0
7p	9	2926±70	0.2±0.01	Nd	0.30±0.02	1.0±0.1	1.0±0.3	11±3.7
7s	9	3454±167	20.0±0.60	72	8833±152.7	38±1.5	1.0±0.2	2693±21.8
8	7	4819±95	17.0±1.27	102±10.43	57026±167.7	158±15.8	2.0±0.2	265±10.7
9p	In operation	3300±305	Nd	Nd	5.0±0.52	2.0±0.3	1.0±0.3	3±0.3
9s	In operation	758±86	Nd	Nd	0.5±0.02	1.0±0.1	0.4±0.1	1±0.1
Range		758-5547	Nd-20	Nd-212	0.3-57026	1-158	0.4-2.0	1-2693

Average of three replicates ± standard deviation; p: plateau of tailing heap; s: slope of tailing heap; Nd: no detectable

Table S3. DTPA-extractable concentrations (mg kg^{-1}) of PTEs in tailing heaps from a mine in Zimapan, Mexico.

Mine tailing	Age	Ca	Cd	Cu	Fe	Mn	Pb	Zn
1	40	4088±419	11.0±1.1	52±14	81±15	36.0+15.1	120±18	1006±46
2	30	3928±100	6.0±1.6	35±10	93±10	12.0+4.0	77±82	858±99
3	30	3008±144	5.0±1.5	242±10	117±5	5.0+0.1	0.4±0.1	Nd
4	3	4330±74	1.0±0.3	21±3	58±12	5.0+1.0	158±18	Nd
5	18	3618±474	1.0±0.2	3±0.1	1406±125	5.0+0.1	1±0.2	98±5
6	14	5885±607	2.0±0.3	41±5	121±6	4.0+0.7	233±26	175±26
7p	9	1773±290	9.0±0.1	4±0.3	47±7	3.0+0.1	143±37	352±13
7s	9	2797±251	21.0±1.5	6±0.7	1109±21	3.0+0.5	8±1	140±45
8	7	2797±221	12.0±03	8±0.7	45±8	10+1.3	3±1	428±30
9p	In operation	4973±346	1.0±0.2	8±0.2	148±16	1+0.4	157±12	71±2
9s	In operation	2696±330	Nd	15±2.1	103±22	8+0.1	565±63	22±4
Range		1773-5885	0.2-21	3-242	45-1406	1-36	1-565	Nd-1006

Average of three replicates + standard deviation; p: plateau of tailing heap; s: slope of tailing heap; Nd: no detectable.

CHAPTER 2. WILD FLORA OF MINE TAILINGS: PERSPERCTIVES FOR USE IN PHYTOREMEDIATION OF POTENTIALLY TOXIC ELEMENTS IN A SEMI-ARID REGION IN MEXICO

Abstract

The aim of this research was to identify wild plant species applicable for remediation of mine tailings in arid soils. Plants growing on two mine tailings were identified and evaluated for their potential use in phytoremediation based on the concentration of potentially toxic elements (PTEs) in roots and shoots, bioconcentration (BCF) and translocation factors (TF). Total, water-soluble and DTPA-extractable concentrations of Pb, Cd, Zn, Cu, Co and Ni in rhizospheric and bulk soil were determined. Twelve species can grow on mine tailings, accumulate PTEs concentrations above the commonly accepted phytotoxicity levels, and are suitable for establishing a vegetation cover on barren mine tailings in the Zimapan region. *Pteridium* sp. is suitable for Zn and Cd phytostabilization. *Aster gymnocephalus* is a potential phytoextractor for Zn, Cd, Pb and Cu; *Gnaphalium* sp. for Cu and *Crotalaria pumila* for Zn. The species play different roles according to the specific conditions where they are growing at one site behaving as a PTEs accumulator and at another as a stabilizer. For this reason and due to the lack of a unified approach for calculation and interpretation of bioaccumulation factors, only considering BCF and TF may be not practical in all cases.

Key words: phytoextraction, phytoaccumulation, phytostabilization

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Introduction

For hundreds of years mining has been an important economic activity in Mexico; during the last half century the production of mining wastes has strongly increased. This represents risks for the environment and the human population, mainly because of dispersion of potentially toxic elements (PTEs) (Armienta, Rodríguez and Cruz, 1997). One way to avoid or reduce PTEs dispersion and to remediate contaminated sites is by the establishment of a vegetation cover (Peuke and Rennenberg 2005). Establishment of well-developed vegetation decreases contaminants' leaching and prevents the dispersal of contaminants through wind and water erosion from formerly bare or sparsely vegetated sites (Vangronsveld *et al.* 1995, 1996, 2009). The development of a stable and self-perpetuating vegetation cover can progressively reduce the soil labile PTEs pool leading to an attenuation of the impacts of PTEs on the contaminated site and to adjacent ecosystems (Mendez and Maier 2008). The most important limitations for the establishment and development of a plant cover on mine tailings not only are the high concentration of PTEs, which are toxic for most plant species, but also the generally low contents of organic matter and nutrients as well as the low water retention capacity (Vangronsveld *et al.* 2009). Consequently, only a few species are adapted to these extreme edaphic conditions; such species, however, could be suitable for phytoremediation purposes (Barrutia *et al*. 2011; Mendez and Maier 2008). PTEs phytoremediation has two main approaches: to remove (phytoextraction) or reduce the bioavailability of PTEs (phytostabilization) (Arthur *et al*. 2005). For all phytoremediation approaches it is desirable to use native species, since these plants proved to be adapted to the specific soil and climatological conditions (Barrutia *et al.* 2011) and will not become invasive. Therefore, the objective of the present study was to identify wild plant species

suitable for phytoremediation and establishment of a plant cover on metalliferous mine tailings at Zimapan, Mexico.

Materials and methods

Sites of collection

The study area is located in the municipality of Zimapan, Hidalgo State, a semi-arid region in the central part of Mexico. The temperature ranges 12 - 20° C and the annual precipitation is approximately 700 mm (INEGI, 2009).

During the ore extraction procedure waste residues are discharged outside the mining site, and as a result, uncovered mine tailings are widely spread across the municipality. Due to their accessibility, two mine tailings sites, Santa Maria and San Francisco, were selected for the present study. Santa Maria tailing (SM) is located close to a populated area (20°44'8.89" N and 99°23'56.07"; approximately 11000 m^2). Here the residues are unoxidized and have a grey colour. The main minerals present are pyrite, galena, calcite and quartz (Moreno-Tovar *et al.* 2009). The San Francisco (SF) mine area (18000 m^2) is located between 20°49'32.5" N and 99°22'20.1"W. The residues are unoxidized (grey ones) and oxidized which have a red-orange colour. In the oxidized residues the most common minerals are ferric sulphates and iron oxides (Moreno-Tovar *et al.* 2009). Although only SM is close to habitation (<20 m), both mine tailings areas are uncovered, favourizing wind and water erosion; it further is common to find cattle searching for grazing or water. Therefore, these tailings are a significant environmental and public health concern (Moreno-Tovar et al, 2012; Armienta et al., 1997).

Plant and Tailings Sampling

At both sites 'plant covered islands' composed of several pioneer plant species were found, which were collected for identification. Bulk (non-rhizospheric) substrate samples from the tailings comprised five to eleven subsamples taken randomly from the heaps. According to Mexican regulation NOM-147-SEMARNAT/SSA1-2004, the rhizospheric substrate samples were taken from residues surrounding the roots of each plant species collected at a depth of 0-30 cm; three subsamples were combined to a composite sample for each plant species. All samples were air-dried and passed through a 2 mm sieve prior to PTEs extraction.

Plants analysis

Shoot samples were first rinsed with tap water, washed with P-free detergent, rinsed with distilled water, washed with diluted HCl 10% (15 min) and finally rinsed three times with deionized water (15 min). Root samples were treated according to the same protocol, but doubling the time for rinsing with tap water and diluted HCl. Root and shoot samples were dried at 65° C for 72 h, then ground in a stainless steel mill. Three replicates of dry ground shoot and root samples were acid-digested using 4 mL H_2SO_4:$HClO_4$ (4:1 v/v) after the prior addition of 1 mL H_2O_2 to 0.5 g of sample. All samples were allowed to pre-digest for 24 h and then heated (220° C) until the solution had a clear appearance. Each digest solution was filtered (Whatman 42) and diluted with deionized water. Concentrations of Zn, Cd, Pb, Ni, Cu and Co were determined by flame atomic absorption spectrometry (Perkin Elmer 3100). For control of the digestion procedure, blanks were included in triplicate. Internal reference standards were also incorporated. Certified standard stock solutions (1000 mg L^{-1}) were used for calibrating the instrument used for sample analyses. The quality of the analytical methods was controlled

considering detection limit and variation coefficient. The detection limit was calculated according to Wels and Sperling (2007).

Analysis of tailings substrates

For determination of total concentration of PTEs, substrate samples (both bulk and rhizospheric) were digested with HNO_3 using the USEPA 3050 method (EPA, 1992). Extractable PTEs were analyzed by diethylene triamine pentaacetic acid-triethanolamine-calcium chloride (DTPA-extractable) procedure in an extraction ratio 1:4 (w:v) substrate:solution (Lindsay and Norvell 1978). Water-soluble elements were analyzed after extraction for 16 h using 1:2.5 (w:v) substrate:deionized water (Shuman 1985). For quality control of the procedures, blanks for both extractions were included in triplicate. pH, electric conductivity, and available P were determined as mentioned by Ruiz-Olivares *et al.* (2013). Particle distribution was determined by Bouyoucos method. Organic matter was analyzed by Walkley and Black procedure (Nelson and Sommers, 1982).

The bioavalability index (BI) was calculated based on the quotient of DTPA-extractable and total concentrations of PTEs in the rhizospheric substrate (Chen, Shian, and Qian 1996). The quotients were multiplied by 100 to express the index in percentages. We use the quotient as an estimation of the plant-available fraction of PTEs in the rhizosphere. However, we prefer the term 'extractable' instead of 'available'.

$$BI(\%) = \frac{DTPA - extractable\ concentration\ of\ PTE\ in\ rizosphere}{Total\ concentration\ of\ PTE\ in\ rhizosphere} \text{x}100$$

Bioaccumulation factors

Two kinds of bioconcentration factors (BCF) were calculated in order to express the relationship between PTEs concentrations in shoot plants and in the substrate (Peuke and Rennenberg 2005), specifically in rhizosphere.

$$BCFT = \frac{PTE\ concentration\ in\ shoot}{Total\ concentration\ of\ PTE\ in\ rhizosphere}$$

$$BCFD = \frac{PTE\ concentration\ in\ shoot}{DTPA - extractable\ concentration\ of\ PTE\ in\ rhizosphere}$$

The translocation factor (TF), defined as the quotient of metal concentration in the shoots to the roots, is an estimation of the plant's capacity to translocate a PTEs from roots to the shoots (Yoon *et al.* 2006). A TF was calculated for each PTE.

Results

Identified plant species

On the mine tailing heaps in total 12 plant species were identified (Table 1). Four species occurred at both tailings: *Dalea bicolor* Humb. & Bonpl. Ex Willd., *Viguiera dentata* (Cav.) Spreng., *Brickellia veronicifolia* (Kunth) A. Gray and *Dichondra argentea* Willd. Species collected only from the SF tailings were *Cuphea lanceolata* Aiton, *Ruta graveolens* L., *Pteridium* sp. and *Juniperus* sp. Further, *Aster gymnocephalus* A. Gray, *Crotalaria pumila* Ortega, *Gnaphalium* sp. and *Flaveria trinervia* were only found on the SM.

Under normal conditions *B. veronicifolia*, *D. bicolor* and *R. graveolens* are shrubs with abundant foliage and commonly reach a size of 90 cm or higher (Rzedowsky and Rzedowsky 1985). At both sites, *B. veronicifolia* and *D. bicolor* were between 80 and 100 cm height, with abundant foliage. But, at the SF tailing, *R. graveolens* grew up to 20 cm. *Dichondra argentea* is a creeping species, which stems can grow up to 80 cm (Rzedowsky and Rzedowsky 1985), but the plants found were between 10 and 50 cm. It is commonly found in grasslands, xerophytic scrubs and dry sunny places (Sánchez-Sánchez, 1979). *Viguiera dentata* is a perennial herb that grows up to 2.5 m (Rzedowsky and Rzedowsky 1985), but on the mine tailings it reached an average of

1.3 m. At the SM, *V. dentata* was found together with *A. gymnocephalus* and *Gnaphalium* sp. *Juniperus* sp. and *Pteridium* sp. grew in clusters on SF (Figure S1).

At SM, *C. pumila* was the most abundant plant; 40 specimens were together forming a patch (Figure S1), they had an average height of 20 cm and produced abundant seeds. *Aster gymnocephalus* plants were about 20 cm high but under favourable conditions it can reach up to 50 cm (Rzedowsky and Rzedowsky 1985). This species is common on eroded soils.

Characterization of bulk and rhizospheric substrates of the tailings

The average pH of non-oxidized SF bulk substrate was 7.8; but, in oxidized conditions was very acidic (1.7). However, for all rhizospheric samples the pHs were close to 7 (Table 1). EC of non-oxidized tailing varied 1.6-4.1 dS m^{-1}, in oxidized increased up to 5 dS cm^{-1}. Organic matter content varied 8.2 to 80.5 g kg^{-1}, the predominant particles were sand (410–830 g kg^{-1}), followed by lime (140-340 g kg^{-1}) and clay (30-210 g kg^{-1}). P extracted was very low (15.6-23.7 mg kg^{-1}).

On SM, the pH of the bulk substrate was 7.6, which was higher than those in the rhizospheres (5.8 to 6.8). EC values of rhizospheric samples were below 4 dS cm^{-1}. Organic matter content ranged from 1.1 to 8.2 g kg^{-1}. Sand content varied from 390 to 570 g kg^{-1}; lime 240-340 g kg^{-1} and clay 90-270 g kg^{-1}. pH and EC in rhizosphere should not be limiting factors for plant development, but phosphorus do, with 1.8– 8.7 mg kg^{-1}.

At both sites, Cd and Pb total concentrations in the bulk substrates were higher than the Mexican reference limit for industrial soils (NOM-147-SEMARNAT/SSA1, 2004). While, only in oxidized bulk substrate from SF total Ni concentration exceed such regulation.

In general, total concentrations of PTEs in rhizospheric substrates were lower than those in non-rhizospheric bulk substrates (Table 1). Total Zn, Cd, Pb and Co concentrations in

rhizospheres were similar in the two tailings, but the range was greater in those from SF than in SM. The opposite was observed for Cu and Ni.

The difference between total concentrations in rhizospheric and bulk soil has different possible explanations. Waste has been mixed with other material during tailing manipulation and construction of the heap. Since chemical and mineralogical characteristics of the residues are variable, it is possible to observe pH changes in discrete points in mine tailings, because of the difference in solubility of the various minerals. Due to residue management, it is possible to find specific points where the chemical conditions are not so stressful for plants, allowing them set up and progressive colonization. Along time plants might remove PTEs from rhizospheric soil.

DTPA-extractable Cu concentrations were similar for the two tailings (Table 1). Zn and Pb DTPA-extractable concentrations in rhizospheric substrates from the SF site were higher than the concentrations in non-vegetated tailings. The rhizosphere of *Juniperus* sp. contained approximately 8x more Zn in comparison to the bulk substrate. At SM, the DTPA-extractable Zn concentrations in rhizospheric substrates were higher than those in bulk substrates, except for *C. pumila* and *D. argentea* rhizospheres. Concentrations of Co in rhizospheric substrates were 2-7 times higher than in the bulk substrate. The DTPA-extractable concentrations of Cd in the bulk substrates were higher than in rhizospheric samples.

Root exudates release by plants may modify the pH (Blossfeld et al., 2010; Bravin et al., 2009), increasing the minerals dissolution (Houben and Sonnet, 2012). The rhizospheric microorganisms (bacteria and mycorrhizal fungi) could contribute to this effect (Bravin et al., 2009); most of collected plants were associated with different arbuscular mycorrhizal fungal species (data not shown), which could have variation on the rhizosphere.

The highest concentrations of water soluble PTEs were observed for Zn in the rhizospheres of *Juniperus* sp. (3 mg kg^{-1}), *B. veronicifolia* (2 mg kg^{-1}), and *D. bicolor* (2 mg kg^{-1}). In general, the water soluble concentrations of PTEs were higher in bulk substrate than in rhizospheres (Table S1).

Zinc was the element with the highest BI; *F. trinervia* had 35%, followed by *D. argentea* (20%) from SF. Except for *C. pumila*, all BI-Cu were around 1 and 2%. The rhizosphere of *B. veronicifolia* from the SF site had the highest BI for Cd (9%), Ni (34%) and Co (3%). In the case of Pb, 5% was the highest BI observed in *Juniperus* sp., *A. gymnocephalus* and *Gnaphalium* sp. (Table S2).

PTEs concentrations in plant tissues

The shoot concentrations of Zn and Cu (Figures 1a and 1d) were variables among species. *Cuphea lanceolata,* from SF, and *Gnaphalium* sp. from SM had the highest Zn concentrations in shoots. This last species also had the highest Cu concentration. Excepting *R. graveolens, D. bicolor, V. dentata* and *D. argentea* from SM, the rest of plants exceeded phytotoxicity level (PL) for Zn. Only *D. bicolor* did not reach the Cu-PL. The PLs are those mentioned by Vamerali et al. (2010).

Cadmium concentrations among plants from SM were homogeneous. These values, were higher than those observed in SF. The same trend was identified for Pb. Plant species at both sites exceeded the PL for shoot Pb concentrations (Figure 1). All species collected from SM and *Juniperus* sp. from SF showed Cd shoot concentrations above the PL.

Shoot concentrations of Ni and Co were similar in all species at both sites. While all species investigated had Ni concentrations above PL; none species reached the PL for Co.

For all species, concentrations of Pb, Ni and Co were higher in roots than in shoots (Figure 1c, e, f), except for *A. gymnocephalus* and *Gnaphalium* sp. In species from the SF site Cd concentration were highest in roots, whereas for plants collected at SM, concentrations in roots and shoots were similar (Figure 1b). Concentrations of Zn and Cu in all species were higher in shoots than in roots (Figure 1a, d). Only *Pteridium* sp. roots contained about 5 times more Zn and Cu than the shoots of this species.

Bioaccumulation factors

BCFs were different when calculated from total and DTPA-extractable concentrations; the formers were obviously lower than the second ones. In many species and elements the BCFTs were zero, especially for Pb in SF and Ni in SM (Table 2). Total concentrations do not reflect bioavailable concentrations; therefore, the BCFDs are described here. In all elements and species from SM BCFDs were >1. Plants from SF had BCFD >1 in the case of Cu, Ni and Co; but the values were variable for Zn, Cd and Pb.

It is noteworthy that for individuals of the same species but originated from the two different sites under investigation, clearly distinct BCFDs were observed. *Dichondra argentea*, when growing at SM, had the highest BCFDs for Pb and Ni, being respectively 8 and 4 times higher than those from the SF tailing. In *B. veronicifolia*, *D. bicolor* and *V. dentata* from the SM, Zn-BCFDs were>1, in those from the SF tailings <1. Similar trends were observed for Pb in *B. veronicifolia* and Cd in *D. bicolor*.

In general, the order of TFs was Zn>Cu>Pb>Cd>Ni=Co (Table 2). The highest TFs for Zn and Cu were observed in *Gnaphalium* sp. (>20). *Aster gymnocephalus* demonstrated high TFs for Cd, Zn, Cu and Pb. In case of Cd, all plants originating from the SF tailing showed a TF <1, whereas the TFs for plants collected from SM were ≥1. For all species the Ni-TF was <1. Co-TF for all

species, except *Gnaphalium* sp., was <1. *Pteridium* sp. was the only species with a TF<1 for all elements studied.

Discussion

Some of the species identified in this study were already found on mine tailings in different Mexican semi-arid regions: *B. veronicifolia* (Flores-Tavizón *et al*., 2003), *D. bicolor* (González-Chávez *et al.* 2009; Ortega-Larrocea *et al.* 2010), *A. gymnocephalus*, *Juniperus* sp. (González and González-Chávez, 2006), *F. trinervia* and *V. dentata* (Franco-Hernández *et al.* 2010). The reported concentrations of PTEs in vegetal tissues and the BCFs are variable, but the presence of these plants in several mine tailings suggests that they possess a high PTEs tolerance and are adapted to the arid conditions prevalent at these sites. There were also identified other species that have not been reported previously as PTEs tolerants: *C. lanceolata, D. argentea, R. graveolens, and C. pumila.*

There were no hyperaccumulator species according to the criteria mentioned either by Brooks (2000) or more recently by van der Ent *et al.* (2012), but some species could be appropriate for use in phytoextraction or phytostabilization of PTEs contaminated substrates. Efficient phytoextraction depends basically on high PTEs concentrations in harvestable plant tissues (generally leaves and stems) (Vangronsveld *et al.* 2009; Barrutia *et al.* 2011), and high plant biomass production (Cortés-Jiménez *et al.* 2013). In this study there were species with relatively high PTEs concentrations (Figure 1) that might be suitable for phytoextraction purposes. However, Vangronsveld *et al.* (2009) mentioned important limitations of PTEs phytoextraction: (1) it can only be used for low to moderately contaminated soils, (2) its applicability is limited to soil surface (at rooting depth) which varies with the species used, but on average is not more than

50 cm. In case of some woody plants, the target zone can be in the range of one to several meters. Thus, phytoextraction does not sound a practical applicable technique for mine tailings. The application of fast growing high biomass species, also offers the possibility to combine PTEs extraction with the production of biomass for bioenergy production (Ruiz-Olivares *et al.* 2013; Schröder *et al*. 2008).

Aster gymnocephalus and *Gnaphalium* sp. showed high BCFDs and had concentrations of Zn, Cd, Pb, Ni and Cu higher than PL; thus they may be useful for phytoextraction purposes. As well as *F. trinervia* and *C. pumila* for Zn, Cd, Pb and Ni. Though, keeping in mind the limitations of phytoextraction mentioned before. It is also recommended that more studies on biomass production, extraction mechanisms, extraction efficiency and propagation techniques are performed in order to successfully apply this strategy in the field.

According to Arthur *et al.* (2005), phytostabilization establishes a plant cover in order to prevent PTEs dispersion by wind or water erosion, or by leaching. Phytostabilization does not necessarily depend on biomass production or PTEs accumulation in aerial plant tissues. The basis of phytostabilization is to decrease PTEs bioavailability by 'fixing' elements in the rhizosphere or in roots. PTEs immobilizing soil amendments can also contribute to this process (Vangronsveld *et al.* 2009). *Pteridium* sp. was the only plant where TFs for all PTEs were <1; by consequence it might be a useful species for phytostabilization of Zn, Cd, Pb, Ni, Cu and Co contaminated substrates.

It is remarkable that the same species growing on the two study sites has different BCFDs for certain elements (Table 2). For example, the BCFD for Zn of *D. bicolor* growing at SM was 2.7 whereas at SF it was 0.3. Similar observations were done in *V. dentata,* and in *D. bicolor* and *B. veronicifolia* for Cd. Like for the BCFDs also the TFs for the same species but collected at

different sites were distinct. *Dalea bicolor* and *V. dentata* growing at SM had a Cd-TF >1 but <1 when growing at SF. With this perspective, the same species can behave as an accumulator or as an excluder depending on the site characteristics where it is growing. The behavior of PTEs accumulation in plants seems to be affected by the environment (van der Ent *et al*., 2012). This is support by the results of correlation analysis. Shoot and roots PTEs concentration were significant correlated with different variables at each site, SF (Table S3) and SM (Table S4).

Taking into account the differences in total and extractable PTEs concentration in rhizosphere and bulk substrate, it is recognized the importance of rhizospheric processes in phytoremediation (Wenzel 2009); but at the same time it is accepted their complexity and heterogeneity. Through the release of different compounds it is possible that plants, and their associated microorganisms, modify their rhizospheres. Additionally, mine tailings have specific mineralogical and physicochemical characteristics leading to different PTEs exposure to plants. As a result, each plant species, its associated microorganisms, and the different PTEs formed a complex system with characteristics different from the bulk substrate.

Our results support the thesis that PTEs uptake and translocation from root to shoot not only depends on the plant species but that other factors. The differences in the plant strategy (accumulator or excluder) need further studies in order to determine which environmental, chemical, physical and biological factors are involved and determine which strategy is employed.

Comparing both BCF and TF is the main way to decide if a plant is a metal accumulator or excluder. The general consent is BCF and TF are higher than 1 is an accumulator plant, and with values lower than 1 is a metal excluder. However, it was observed that TF values (<1) indicate that a species does not translocate PTEs to the aboveground biomass; while the BCFD (>1) may indicate that the same species behaves as a PTEs accumulator. It is even more contrasting in the

case of BCFT. Additionally, it was observed that the highest BI did not correspond with the highest values of BCFD and PTEs concentrations in plant tissues.

It is therefore required to specify and validate which of both concentrations, total or extractable and by which methodology, is the best for BCF calculation. The different behavior of the same species under different conditions and the lack of an unambiguous approach for their quantification limit their usefulness as criteria for plant selection in phytoremediation work.

Conclusions

The species studied could grow on mixed/multiple PTEs contaminated sites. The plants able to grow on these sites involve annual species, shrubs, perennial herbs, and one creeping perennial species; all of them are adapted to semi-arid conditions. This allows the establishment of a plant cover that combats dispersion of PTEs from bare mine tailings throughout the year. *Dalea bicolor, A. gymnocephalus, V. dentata* and *Juniperus* sp. are all promising plants because they were also reported growing on mine tailings in other Mexican regions. *C. lanceolata*, *D. argentea, R. graveolens,* and *C. pumila* are species not reported previously.

Considering the BCFDs, TFs and PL, *Pteridium* sp. shows very suitable for phytostabilization of Zn and Cd. *Aster gymnocephalus* can be a candidate for Zn, Cd, Pb, and Cu phytoextraction in PTEs contaminated susbtrates, as well as *Gnaphalium* sp. for Cu, and *C. pumila* for Zn. Further work is needed regarding potential biomass production and propagation techniques of the aforementioned species.

For a better evaluation of plant potential for use in phytoremediation it is recommended that the methodology and approach for calculation and interpretation of BCFs and TFs is standardized. Further, more fundamental research still is needed to better exploit the metabolic

diversity of the plants themselves, but also to better understand the complex interactions between contaminants, soil, plant and micro-organisms (bacteria and mycorrhiza).

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Supplemental material

Supporting information shows water soluble concentrations of PTEs in bulk substrate, distribution of plants in vegetated islands, BI of PTEs determined in rhizosphere of all species, and significant correlations among variables.

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Table 1. Potentially toxic elements concentrations (mg kg^{-1}) in two mine tailings in Zimapan, Mexico.

Tailing/rhizospheric tailing	pH	EC[a]	Zn		Cd		Pb		Cu		Ni		Co	
		dS cm^{-1}	Total	DTPA	Total	DTPA	Total	DTPA	Total	DTPA	Total	DTPA	Total	DTPA
San Francisco non oxidized	7.8±1.3	3.4±1.5	4745±1613	283±357	157±310	5±3	1923±943	183±174	1045±490	69±57	60±22	1.3±1.1	52±17	1.5±1..3
San Francisco oxidized	1.7±0.3	5.0±0.5	5550±67	139±13	1190±81	21±2	5777±60	8±2	847±29	6±1	4550±66	17±1	1710±65	2±0.3
Rhizosphere of:														
Pteridium sp.	6.4±0.4	2.8±0.1	2631±59	316±19	60±7	2±0.4	4890±44	26±4	357±11	6±2	513±25	5±1	409±21	2±0.2
Juniperus sp.	5.8±0.2	1.8±0.1	4432±74	1168±23	127±6	7±0.3	3521±62	184±10	635±45	4±0.3	655±26	7±1	428±51	1±0.4
Cuphea lanceolata	6.5±0.2	1.8±0.1	2063±65	184±33	30±5	2±0.3	1598±63	33±5	188±36	3±0.4	795±12	19±3	231±48	2±0.1
Dichondra argentea	6.6±0.2	2.4±0.2	4703±40	948±19	80±6	4±0.1	2461±46	60±3	517±30	3±1	33±3	9±2	420±41	1±0.1
Brickellia veronicifolia	6.4±0.2	1.9±0.1	4642±64	754±20	72±13	6±1	2777±54	121±16	276±51	6±1	33±3	11±1	250±37	6±0.4
Ruta graveolens	6.8±0.1	1.8±0.1	4011±60	513±36	54±9	2±0.1	1889±56	40±2	315±39	3±1	57±6	9±0.4	374±45	1±0.2
Dalea bicolor	6.7±0.1	1.7±0.2	5368±71	754±20	105±14	8±2	1117±61	32±4	430±11	4±1	463±14	20±2	315±33	2±0.4
Viguiera dentata	6.8±0.1	1.6±0.2	2649±47	250±17	55±10	2±0.1	1959±78	24±7	178±37	4±0.4	495±19	14±1	182±27	2±0.4
Range	5.8-6.8	1.6-2.8	2063-5368	184-1168	30-127	2-8	4890-1117	24-184	178-635	3-6	33-795	5-20	182-428	1-6
Santa Maria	7.6±0.4	0.7±0.2.	4546±58	65±9	120±58	9±2	4183±67	188±21	1764±35	5±1	1112±60	6±0.4	972±58	0.4±0.1
Rhizosphere of:														
Aster gymnocephalus	6.4±0.2	0.5±0.1	4396±48	182±20	51±8	1±0.3	802±43	43±3	703±24	4±0.2	322±11	5±1	571±29	1±0.1
Gnaphalium sp.	5.8±0.3	0.5±0.1	4068±41	164±3	43±6	1±0.2	2080±62	104±10	1324±25	2±0.3	790±24	6±1	475±36	2±0.3
Viguiera dentata	6.5±0.1	0.4±0.1	4081±34	173±18	48±8	1±0.1	1493±38	26±3	527±13	4±0.2	687±28	3±1	444±44	1±0.2
Dalea bicolor	6.6±0.1	0.4±0.1	3799±41	77±14	40±4	1±0.3	2158±62	20±2	1154±17	3±1	230±13	3±1	381±27	3±0.1
Crotalaria pumila	6.4±0.3	0.4±0.1	326±36	8±1	24±4	1±0.2	1194±63	8±0.4	25±7	7±1	904±29	4±1	213±43	3±0.3
Brickellia veronicifolia	6.8±0.3	0.4±0.1	4381±32	174±17	60±7	1±0.3	1970±61	34±1	812±43	9±0.2	721±8	6±2	65±15	2±0.4
Flaveria trinervia	6.7±0.2	0.3±0.1	303±26	107±15	32±7	1±0.1	872±13	12±2	576±11	1±0.3	579±8	3±1	331±21	1±0.2
Dichondra argentea	6.8±0.2	0.3±0.1	275±17	4±1	33±4	1±0.1	932±15	8±0.3	618±22	2±0.2	443±9	2±1	60±12	1±0.1
Range	5.8-6.8	0.3-0.5	275-4396	4-182	24-60	1	802-2158	8-104	25-1154	1-9	230-904	2-6	60-571	1-3
TC-A/R/C					37		400				1600			
TC-I					450		800				20,000			

All the values are mean of three replicates ± standard deviation; n=3. [a] electrical conductivity: TC-A/R/C: Mexican reference for total concentrations in agricultural/residential/commercial soil (NOM-147-SEMARNAT/SSA1, 2004); TC-I: Mexican reference for total concentrations industrial soil (NOM-147-SEMARNAT/SSA1, 2004).

Table 2. Bioconcentration and translocation factors of wild plants growing on two mine tailings in Zimapan, Hgo.

Species	Zn			Cd			Pb			Cu			Ni			Co		
	BCFT[a]	BCFD[b]	TF[c]	BCFT	BCFD	TF	BCFT	BCFD	TF	BCFT	BCFD	TF	BCFT	BCFD	TF	BCFT	BCFD	TF
San Francisco																		
Pteridium sp.	0.1	0.7	0.2	0.0	1.1	0.1	0.0	2.6	0.2	0.2	10.2	0.4	0.1	7.7	0.4	0.1	14.0	0.4
Juniperus sp.	0.2	0.9	17.0	0.1	1.6	0.8	0.0	0.5	1.1	0.0	7.4	3.0	0.1	5.3	0.7	0.1	19.7	0.5
Cuphea lanceolata	0.8	7.7	6.7	0.3	4.6	0.6	0.1	2.4	0.6	0.4	29.6	3.9	0.0	1.6	0.5	0.1	169.5	0.6
Dichondra argentea	0.2	1.0	3.4	0.1	1.7	0.2	0.1	2.3	0.6	0.2	35.4	7.1	1.1	4.1	0.3	0.1	16.3	0.3
Brickelia veronicifolia	0.1	0.4	1.4	0.1	1.0	0.4	0.0	0.5	0.6	0.2	9.3	2.8	1.0	3.0	0.6	0.1	4.1	0.7
Ruta graveolens	0.0	0.4	2.5	0.1	2.4	0.3	0.1	2.2	1.0	0.1	10.6	2.9	0.8	4.8	0.8	0.1	19.5	0.7
Dalea bicolor	0.0	0.3	2.3	0.0	0.4	0.3	0.2	1.9	0.6	0.0	4.1	1.0	0.1	1.6	0.6	0.1	10.6	0.5
Viguiera dentata	0.1	0.6	0.9	0.1	2.0	0.2	0.0	2.6	0.5	0.2	10.2	1.2	0.1	2.6	0.6	0.1	10.1	0.5
Range	0.0-0.8	0.3-7.7	0.2-17	0.0-0.3	0.4-4.6	0.1-0.8	0.0-0.2	0.5-2.6	0.2-1.1	0.0-0.4	4.1-35.4	0.4-7.1	0.0-1.0	1.6-7.7	0.3-0.8	0.1-0.1	4.1-169.5	0.3-0.7
Santa Maria																		
Aster gymnocephalus	0.2	5.7	20.5	0.4	23	1.7	0.3	4.6	2.0	0.2	28.4	12.7	0.1	8.2	0.7	0.1	372.5	0.7
Gnaphalium sp.	0.4	8.6	20.8	0.4	27.2	1.2	0.1	1.7	1.6	0.2	125.0	27.7	0.1	7.3	0.8	0.1	30.5	1.2
Viguiera dentata	0.1	2.3	2.0	0.3	17.1	1.3	0.1	2.8	0.6	0.1	8.6	1.0	0.0	7.5	0.4	0.1	16.2	0.5
Dalea bicolor	0.1	2.7	3.3	0.4	32.4	1.3	0.0	4.6	0.9	0.0	8.0	1.6	0.1	8.6	0.5	0.1	7.8	0.5
Crotalaria pumila	0.9	26.9	11.6	0.6	19.7	1.4	0.1	12.3	1.1	2.0	6.6	9.2	0.0	7.4	0.6	0.1	9.8	0.6
Brickelia veronicifolia	0.1	2.2	4.2	0.3	16.4	1.4	0.1	4.2	1.3	0.0	3.8	1.4	0.0	4.7	0.5	0.4	12.4	0.5
Flaveria trinervia	1.7	4.8	10.9	0.5	74.6	1.1	0.1	8.6	1.0	0.1	55.0	5.6	0.0	10.7	0.5	0.1	44.7	0.6
Dichondra argentea	0.4	25.7	1.3	0.5	51.6	1.0	0.1	18.4	0.8	0.3	69.7	7.2	0.1	17.8	0.4	0.8	56.1	0.6
Range	0.1-1.7	2.2-26.9	1.3-20.8	0.3-0.6	17.1-74.6	1.0-1.7	0.0-0.3	1.7-18.4	0.6-1.6	0.0-2.0	3.8-125	1.0-27.7	0.0-0.1	4.7-17.8	0.4-0.8	0.1-0.8	7.8-372.5	0.5-1.2

BCF: bioconcentration factor calculated from [a]Total and [b]DTPA-extractable concentration of PTE in rhizosphere; [c]: translocation factor

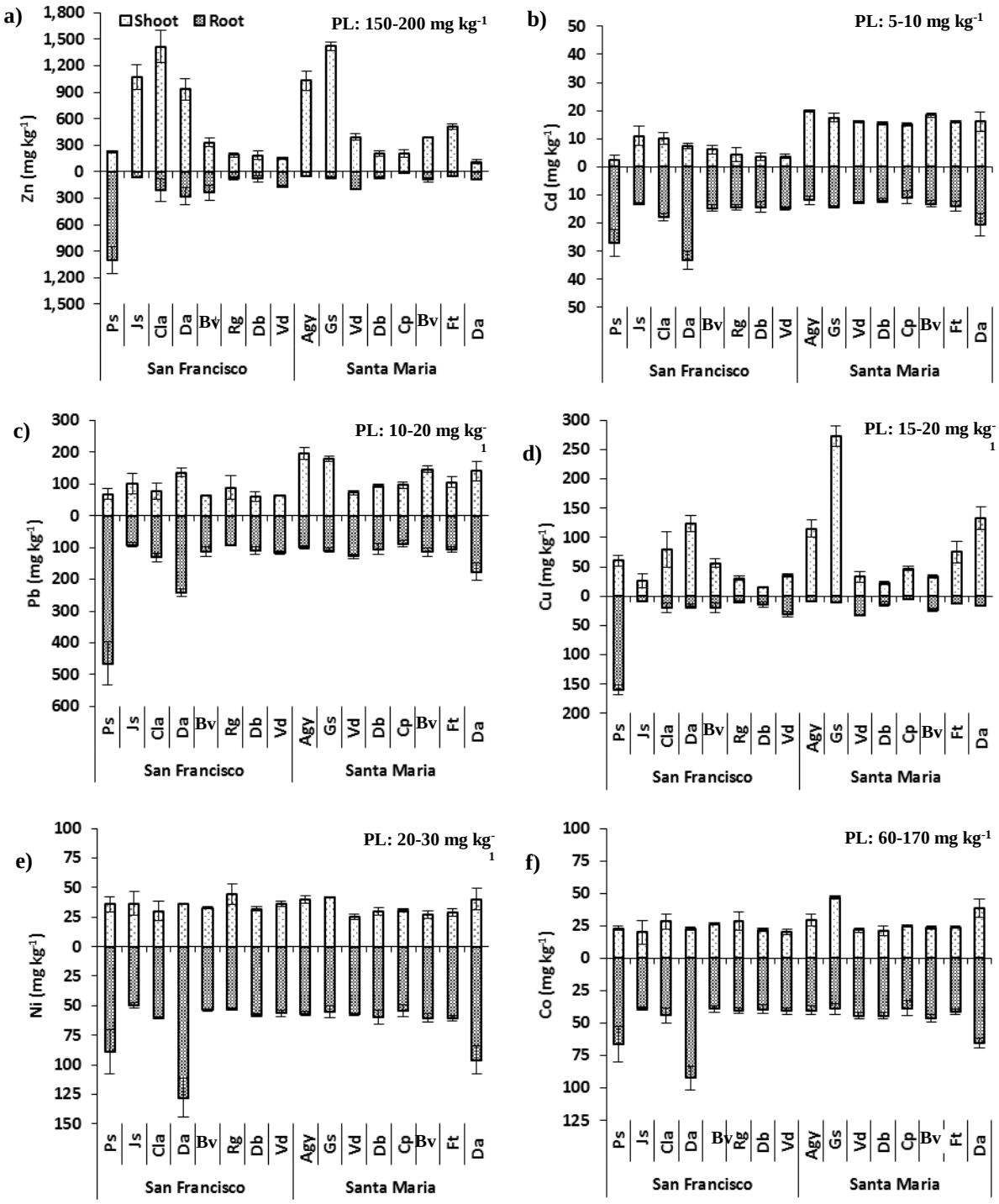

Figure 1. Concentrations of Zn (a), Cd (b), Pb (c), Cu (d), Ni (e), and Co (f) in shoots and roots of wild plants growing on two mine tailings at Zimapan, Mexico. Mean (n=3), error bars represent standard deviation. Identification species similar to those in Table 1. PL: phytotoxicity level (Vamerali *et al.* 2010).

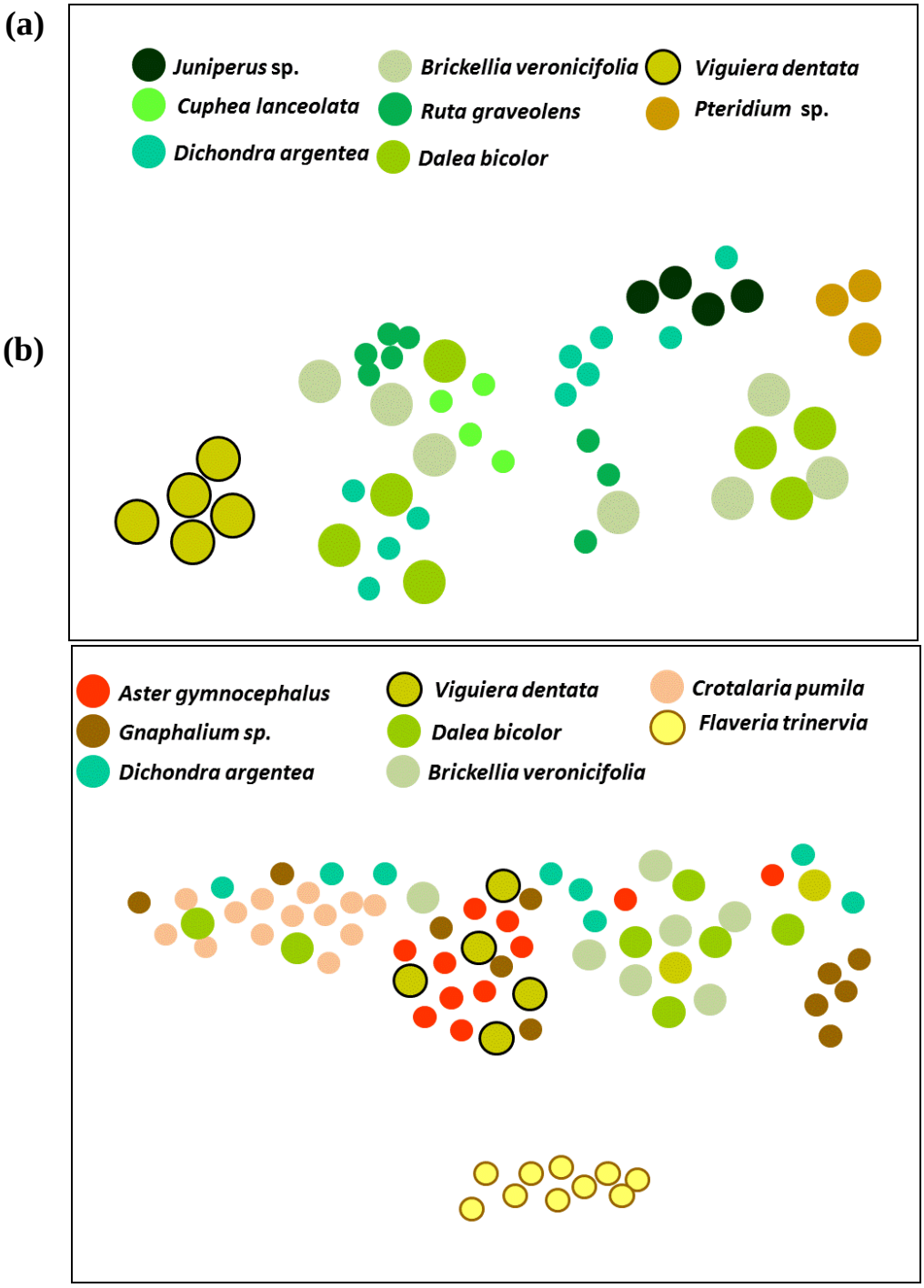

Figure S1. Distribution of vegetal species within the "plant covered islands" at San Francisco (a) and Santa Maria (b) sites. Small circles are herbaceous plants, big circles represent shrubs. Note: the figures only represent the distribution of plants, is not a scale figure.

Table S1. Water soluble concentrations (mg kg^{-1}) of PTEs in bulk substrate and rhizospheres of wild plants growing on mine tailings

	Zn	Cd	Pb	Cu	Ni	Co
San Francisco	2384±240	20±0.3	1.7±0.1	86±10	1.3±0.3	2.4±0.2
Pteridium sp.	0.2±0.02	0	1.0±0.1	0.02±0.02	0.3±0.2	0.1±0.03
Juniperus sp.	3.0±0.5	0.04±0.02	1.0±0.1	0.1±0.01	1.0±0.1	0.1±0.04
Cuphea lanceolata	0.3±0.03	0	0.3±0.1	0.1±0.02	0.4±0.1	0
Dichondra argéntea	0.6±0.1	0	0.4±0.1	0.1±0.003	1.0±0.1	0
Brickellia veronicifolia	2.0±0.2	0.1±0.03	1.0±0.2	0.1±0.004	0.5±0.03	0.1±0.1
Ruta graveolens	0.3±0.03	0.01±0.004	1.0±0.1	0.03±0.02	1.0±0.2	0.02±0.02
Dalea bicolor	2.0±0.1	0.1±0.01	1.0±0.2	0.1±0.01	1.0±0.1	0.1±0.01
Viguiera dentata	1.2±0.03	0.04±0.01	1.1±0.08	0.01±0.01	1.0±0.04	0.1±0.04
Range (in rhizosphere)	0.2-2.0	0-0.1	0.3-1.1	0.02-0.1	0.3-1.0	0-0.1
Santa Maria	0.04±0.01	0	0.5±0.02	1.0±0.1	1.1±0.1	1.7±0.1
Aster gymnocephalus	0.5±0.1	0.1±0.01	1.2±0.1	0.04±0.02	1.0±0.1	0.1±0.1
Gnaphalium sp.	2.5±0.8	0.1±0.02	1.2±0.1	0.1±0.05	1.0±0.1	0.1±0.1
Viguiera dentata	1.0±0.1	0.03±0.02	1.2±0.01	0.04±0.03	1.0±0.1	0.1±0.05
Dalea bicolor	0.3±0.1	0.03±0.02	1.2±0.01	0.03±0.03	1.0±0.1	0.2±0.1
Crotalaria pumila	0.1±0.1	0.01±0.01	1.1±0.1	0	1.0±0.1	0.2±0.04
Brickellia veronocifolia	1.1±0.2	0.07±0.02	1.2±0.01	0.1±0.07	1.0±0.01	0.2±0.1
Flaveria trinervia	1.0±0.1	0.01±0.01	0.7±0.01	0.02±0.01	0.3±0.02	0
Dichondra argéntea	1.0±0.1	0.02±0.003	0.1±0.1	0.1±0.01	0.4±0.1	0.1±0.04
Range (in rhizosphere)	0.1-2.6	0.01-0.1	0.1-1.2	0.02-0.1	0.3-1.0	0-0.2

Values are average of n=3 ± standard deviation.

Table S2. Percentage of DTPA-extractable concentration of PTEs in rhizosphere from wild plants growing on mine tailings

	Zn	Cd	Pb	Cu	Ni	Co
San Francisco						
Pteridium sp.	12±3	4±1	1±0.3	2±0.4	1±0.2	1±0.2
Juniperus sp.	4±1	5±1	5±1.0	1±0.3	1±0.3	1±0.4
Cuphea lanceolata	9±2	7±2	2±0.3	1±0.3	2±0.4	1±0.3
Dichondra argéntea	20±3	5±1	2±1.0	1±0.4	26±2.0	1±0.3
Brickellia veronicifolia	16±3	9±1	4±1.0	2±0.4	34±3.0	3±1.0
Ruta graveolens	13±2	3±1	2±0.4	1±0.2	16±3.0	1±0.2
Dalea bicolor	14±3	8±2	3±1.0	1±0.2	4±1.0	1±0.2
Viguiera dentata	9±3	4±1	1±0.3	2±0.4	3±0.4	1±0.4
Range	4-20	3-9	1-5	1-2	1-34	1-3
Santa Maria						
Aster gymnocephalus	4±1	2±1.0	5±1	1±0.3	2±0.3	1±0.4
Gnaphalium sp.	4±1	1±0.4	5±1	1±0.4	1±0.3	1±0.2
Viguiera dentata	4±1	2±0.4	2±1	1±0.3	1±0.3	1±0.2
Dalea bicolor	2±1	1±0.3	1±0.3	1±0.2	2±0.4	1±0.3
Crotalaria pumila	2±1	3±1.0	1±0.4	29±3	1±0.2	1±0.3
Brickellia veronocifolia	4±1	2±0.4	2±0.4	1±0.3	1±0.2	3±1.0
Flaveria trinervia	35±4	1±0.4	1±0.3	1±0.3	1±0.4	1±0.3
Dichondra argéntea	2±1	1±0.3	1±0.2	1±0.2	1±0.3	1±0.2
Range	2-35	1-2	1-5	1-29	1-2	1-3

Values are average of n=3 ± standard deviation.

Table S3. Significant correlations among PTEs concentration in plants collected from San Francisco site.

	Zn (shoot)	Pb (shoot)	Cu (shoot)	Cd (root)	Zn (root)	Pb (root)	Cu (root)	Co (root)
Cd (shoot)	0.834**	0.569 *						
Ni (shoot)		0.582*						
Cu (shoot)		0.567*						
Zn (root)				0.571*		0.959**	0.938**	
Cu (root)				0.512*				
Ni (root)		0.562*		0.974**		0.645*		0.814**
Co (root)		0.773**		0.800**				
Pb (root)				.735**			0.912**	
Cu (water soluble)				-0.559*				
Cd (water soluble)				-0-559*				
Ni (water soluble)					-0.639*	-0.539*		
Ni (DTPA-extractable)					-0.528*	-0.599*		
Pb (water soluble)			-0.596*					
Cu (water soluble)							-0.522*	

Table S4. Significant correlations among PTEs concentration in plants collected from Santa Maria tailings.

	Pb (shoot)	Cu (shoot)	Co (shoot)	Zn (root)	Ni (root)	Co (root)	Zn (water soluble)	Zn (DTPA-extractable)	Cd (water soluble)
Pb (shoot)									0.609*
Zn (shoot)	0.700*	0.801*	0.671*				0.731**	0.561*	0.646*
Cd (shoot)	0.723*								
Ni (shoot)	0.755**	0.756**	0.828**						
Cu (shoot)	0.657*		0.909**				0.704*		
Co (shoot)	0.651*						0.727**		
Cu (root)				0.917**					
Cd (root)					0.874**	0.760**			
Pb (root)					0.862**	0.845**			
Co (root)					0.916**				
Pb (water soluble)					-0.615*				
Pb (DTPA-extractable)		0.814**	0.713**						

CHAPTER 3. PHYTOBARRIERS: PLANTS REDUCE ATMOSPHERIC DISPERSION OF PARTICLES CONTAINING POTENTIALLY TOXIC ELEMENTS ORIGINATING FROM MINE TAILINGS IN SEMIARID REGIONS

Abstract

Retention of particles containing potentially toxic elements (PTEs) on aerial parts of plants spontaneously colonizing mine tailings was studied through analyses and comparison of washed and unwashed shoot samples. In both types of samples concentrations of Zn, Pb, Cd, Cu, Ni, Co and Mn were determined. Particles retained on leaves were examined by scanning electronic microscopy and energy dispersive X-Ray analysis. Particles containing PTEs were detected on both washed and unwashed leaves. This indicates that the thorough washing procedure did not eliminate all particles containing PTEs, leading to an overestimation of the concentrations of PTEs in plant tissues. Particularly trichomes and fungal mycelium were retaining particles. The quantity and composition of particles varied in function of plant species and place of collection. It is obvious that plants growing on toxic mine tailings form a physical barrier to particle dispersion and hence limit atmospheric PTEs spreading.

Keywords: particles deposition, mine tailings, air dispersion, phytoremediation

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Introduction

It is documented that mine tailings generally represent an environmental and public health issue mainly because of high concentrations of potentially toxic elements (PTEs) and their dispersion by wind and water erosion and percolation (Vangronsveld et al., 1995a, b; Jonathan et al., 2010). In arid and semi-arid regions the environmental conditions limit the development of a plant cover, promoting dispersion of particles containing PTEs (Mendez and Maier, 2008). This is the case in Zimapan, Mexico, where mine tailings are located within and around the populated zone. PTEs dispersion from mine tailings in the Zimapan area was observed up to 4 km from the tailings (Duarte-Zaragoza, 2013).

It is recognized that plant surfaces represent an important sink for pollutants in terrestrial environments, for instance of nitrogen and sulfur oxides (Bamniya et al., 2012; Nowak et al., 2006), polycyclic aromatic hydrocarbons (Terzaghi et al., 2013), particulate matter (Dzierżanowski et al., 2011; Popek et al., 2013) and toxic PTE associated to particulate matter (Fujiwara et al. 2011; Tomašević et al., 2005). Consequently, on mine tailings vegetation might contribute to reduce PTEs dispersion not only by accumulation in roots and shoots, but also by retention of particles on aerial plant parts. Investigations on vegetation as PTEs sink have been carried out mainly in big cities (Litschke and Kuttler, 2008; Tomašević et al., 2005) and industrial areas (Bamniya et al., 2012). Apparently, such studies were not performed in mining areas where dispersion of and also exposure to PTEs are conceivably high.

Results from works comparing particle retention of trees and shrubs in urban areas are variable among plant species (Dzierżanowski and Gawroński 2011; Dzierżanowski et al., 2011; Neinhuis and Barthlott, 1998; Popek et al., 2013; Sæbø et al., 2012; Tomašević and Aničić 2010; Wang et al., 2006) according to leaves characteristics. There is not a consensus which plant species or

which leaf structure is the most efficient for particle retention. Some authors mentioned that hairiness is the principal feature (Dzierżanowski and Gawroński 2011); others stated that is surface roughness (Freer-Smith et al., 2003; Sæbø et al., 2012; Wang et al., 2006); while others attribute retention capacity to cuticular waxes (Dzierżanowski et al., 2011; Popek et al., 2013).

Therefore, the objectives of this work were: (1) to document the retention of solid particles containing PTEs by different plant species spontaneously growing on mine tailings; (2) to investigate which leaf structure(s) of studied plants contribute(s) to retention of particles; and (3) to identify plant species with highest potential to retain particles containing PTEs arising from mine residues.

Materials and methods

Sites of collection

Plants were collected at the municipality of Zimapan in Hidalgo State, Mexico. Two mine tailings were selected, San Francisco (S1) and Santa Maria (S2) (Fig. 1). At both sites total concentration of Zn and Pb in mine resides are higher than 4000 mg kg^{-1} and above 800 mg kg^{-1} for Cu; while 157 and 120 mg Cd kg^{-1} were reported for S1 and S2, respectively (Sánchez-López et al., 2015). The mineralogy of the studied site is dominated by sulfide minerals such as pyrite (FeS_2), pyrrhotite ($Fe_{(1-x)}S$), galena (PbS), sphalerite ((Zn,Fe)S), arsenopyrite (FeAsS), chalcopyrite ($CuFeS_2$), bornite (Cu_5FeS_4), marcasite (FeS_2). Other common minerals are calcite ($CaCO_3$), dolomite ($CaMg(CO_3)_2$) and quartz (SiO_2). In oxidized mine residues, like S1, gypsum ($CaSO_4$), jarosite ($KFe_3(SO_4)_2(OH)_6$), goethite (FeO(OH)), hematite (Fe_2O_3), boulangerite ($Pb_5Sb_4S_{11}$) have been reported (Moreno-Tovar et al., 2009; 2012).

Site 1 is located between 20°49’32.5” N and 99°22’20.1”W. Heaps are constructed at the bottom of a basin; thus they are surrounded by hills. Despite of the fact that the site is away from the urban area, several houses are situated around the mine tailings and it is common to observe cattle grazing. In Site 2 (20°44’8.89” N and 99°23’56.07” W) the mine tailing is located in an open space close to the urban area. The houses are located along mine tailing, from only 20 m distance. Climate conditions at Zimapan are: average temperature is 18.9 ^{o}C; annual precipitation 445.9 mm with rainfall season from May to August, dry season from November to March. Predominant wind direction is from South, Southwest and Southeast. Wind speed is from 2 to 35 km h^{-1} (SMN, 2015).

Sampling and identification of plant material

Plants growing on mine tailings were collected during the dry season (November) of 2013. Plant species identification was done at the herbaria of Chapingo University and Mexico Autonomous National University. Samples for chemical analysis consisted of aerial part of plants. Leaf samples for particles counting were taken as follows: leaves from all sites of the canopy of different plants of each species were removed. Leaves with visible symptoms of disease or pest´ invasion were not taken; young or old leaves were neither collected. To avoid the possibility of contamination after sampling, the removed leaves were sealed and labeled separately in plastic containers until analysis.

Chemical analysis of plants

For chemical analysis shoot samples were divided into two: one part was kept as it was collected in the field (unwashed) and the other one was washed. The washing protocol consisted of 10 min tap water rinse, washing with P-free detergent Extran 2% for 5 min, 5 min rinse with distilled water, immersion in diluted HCl 10% during 10 min, and rinses with deionized water for 10 min.

Washed (W) and unwashed (UW) samples were dried until constant weight at 65°C. Before milling, 3 leaves were taken from W and UW samples for counting and analysis of particles.

From milled samples 0.5 g were digested in a mixture of 1 mL of H_2O_2 and 4 mL of H_2SO_4:$HClO_4$ (4:1) for 24 h. Subsequently, they were heated until the solutions got clear appearance. Then, the solution was replenished up to 25 mL and filtered (Whatman 42). Concentrations of Zn, Cd, Pb, Ni, Cu, Co and Mn in the digests were determined using flame atomic absorption spectrometry (FAAS-Perkin Elmer 3100). For control of the digestion procedure, quality assurance/quality control samples were included. Certified standard stock solutions (dilutions start from1000 mg L^{-1}) were used for calibrating the instrument. The detection limits were calculated according to Wels and Sperling (2007).

Counting of particles and trichomes on leaves surfaces

Washed and UW leaf segments of the collected plants were mounted each on a Scanning Electron Microscope (SEM) sample holder; subsequently, the samples were coated with a 15 nm gold and palladium film. Due to the diversity of collected plant species there was a high variation of leaf sizes and shapes. To standardize the analysis, segments from the leaves centers were cut out, avoiding main veins, to fit on the sample holder. The analysis was done on the JEOL JSM 6390 SEM facility in the Electron Microscopy Unit at Postgraduate College. An accelerating voltage of 15kV was applied during the analysis.

It was reported that the presence and abundance of trichomes is important in particles retention on leaves (Dzierżanowski and Gawroński 2011; Sæbø et al., 2012). Previous studies found that the major retention capacity, up to 6 times higher (Wang et al., 2006) is observed at the leaves upper side due to the surface roughness created by different structures (Ottelé et al., 2010; Tomašević et al., 2005). Therefore, in this work, we concentrated on the upper side of the leaves. Pictures

obtained in SEM (at 150x) were further analyzed by targeting each of the particles on the image (Image Pro-Plus Image Processing and Analysis System). The analysis area for 150x in SEM corresponded to 0.5 mm^2. Due to this magnification, only particles of at least 15 μm size were counted.

Two different procedures for particle counting were examined. The first one consisted of taking three leaves from the shoot of each plant species W and UW from both collection sites. Three images of each leaf were taken (total 9 images per sample). In the second procedure, only one image of each leaf was made. Once the images were obtained the particles were counted as explained above and results were analyzed. The analysis of variance applied showed no significant differences between the two methodologies. Therefore, the second one was chosen because of time-efficiency. This methodology was followed for counting trichomes too.

To determine the elemental composition of individual particles, they were analyzed with a SEM coupled with an energy-dispersive X-ray microanalyser (EDX-OXFORD Instruments INCA X-act). The counting time for the X-ray spectra was 60 s; detection limit was 0.1%. The elemental compositions of randomly selected particles into the 0.5 mm^2 area were analyzed from each sample.

Statistical analysis

Data were tested for normal distribution and for equal variance using Shapiro-Wilks and Bartlett test, respectively. To compare metal concentrations and for number of particles in W and UW samples in each plant species a paired *t*-test (p=0.05) was applied. Correlation analysis was performed by Pearson test. The experimental unit for counting particles consisted in a leaf section of each species in each site; each section was taken from different leaf. The number of replicates was three. The data were processed using Statistical Analysis System (SAS) version 9.0 software.

Results

PTEs concentration in plants

The highest Zn concentration was observed in UW *Viguiera dentata* collected on S1. However, W samples of the same plant contained the lowest shoot Zn concentration (Table 1). A similar tendency was observed for Zn in *Dichondra argentea* from S2. Unwashed samples of both species contained 14 times more Zn than W samples (Table 1). Regarding Pb, *Cuphea lanceolata* was the species that retained most Pb on its leaves, the difference between W and UW Pb concentrations being a factor of 5, followed by a 4 times difference observed in *Viguiera dentata*, *Brickellia veronicifolia, Ruta graveolens* and *Dalea bicolor.* Unwashed samples of *Viguiera dentata, Dichondra argentea, Flaveria trinervia* and *Crotalaria pumila* from S2 showed Pb concentrations 3 times higher than the W ones, respectively (Table 1). The observed differences in concentrations suggest that Pb is an element preferentially retained on leaf surfaces.

Significant differences in Cd concentrations were found between W and UW samples for all plant species from S1, except for *Juniperus* sp. Cadmium concentrations between W and UW samples were not significantly different on S2 (Table 1). This suggests that Cd is highly retained on leaves in S1. The species that retained Cd the most was *Viguiera dentata*, UW samples from S1 contained 5 times more Cd than W ones.

On S2 the differences of Cu concentrations between the two W and UW samples were significant for *Gnaphalium* sp., *Aster gymnocephalus* and *Dichondra argentea*. On S1 only *Viguiera dentata* exhibited significantly different Cu concentrations. Unwashed *Viguiera dentata* from S1 contained 6 times more Cu than the W ones (Table 1). For Ni in W and UW samples, the difference was only significant for *Brickellia veronicifolia* and *Viguiera dentata* from S1 and *Gnaphalium* sp. from S2 (Table 1).

On S1 most of the species showed significantly different Co concentrations between W and UW samples. On S2 only *Aster gymnocephalus, Gnaphalium* sp. and *Brickellia veronicifolia* showed significant differences (Table 1). Unwashed *Gnaphalium* sp. showed the highest Mn concentration followed by *Flaveria trinervia* and *Aster gymnocephalus*. However, the species with the greatest difference between W and UW samples was *Viguiera dentata* from S1; UW samples contained 24 times more Mn than W ones (Table 1).

Particles on surfaces of leaves

Particles were observed on both W and UW leaf samples. Considering both sites, the number of particles per 0.5 mm^2 was higher on the UW samples (22-111 particles) than on W samples (9-56 particles) (Fig. 2). All plant species, except *Dalea bicolor* from S2, showed significant differences in numbers of particles between W and UW samples (Fig. 2). *Brickellia veronicifolia* was the species with the highest number of particles on UW leaves at both sites. It contained 3 and 2 times more particles than the W samples from respectively S2 and S1. *Ruta graveolens*, *Juniperus* sp. and *Pteridium* sp. exhibited the lowest number of particles on both, W and UW leaves (Fig. 2).

According to EDX analysis the elemental composition of the particles varied (Fig. 3) between sites and species. In general, the most abundant elements in particles on both UW and W leaves at the two sites were Ca, Si, Al, K, and Fe. These elements were identified in more than 40% of the analyzed particles and were more commonly observed on W samples than UW ones. In UW samples Zn, Cu, Mn, S, As, Re and Po were more frequent than in W ones (Table S1). More particles containing Zn were observed on leaves of *Juniperus* sp. and *Gnaphalium* sp. Particles containing Cu were more abundant on leaves from S2 than those from S1. On S1 Cu was identified on UW *Pteridium* sp., *Juniperus* sp., *Cuphea lanceolata*, *Dalea bicolor* and *Viguiera*

dentata. On S2 Cu was detected in at least one particle on each analyzed leaf sample. Species with more particles containing Cu were *Brickellia veronicifolia* and *Dichondra argentea* (Table S2). Sulfur, As, Mn and Re were associated with more than half of the particles found on UW samples from S1. Unwashed *Brickellia veronicifolia* from S1was the species with most particles containing S, As and Mn. Particles containing Re were found mainly on UW leaves of *Cuphea lanceolata* (Table S2). Polonium was detected on UW *Cuphea lanceolata* from S1, and UW *Aster gymnocephalus* and W *Dalea bicolor* from S2. Bromine was identified on *Dalea bicolor* from S2 and *Viguiera dentata* at both sites. Scarce elements, detected in less than 1% of particles, were Mo and F. Both elements were found on UW leaf samples, Mo on *Brickellia veronicifolia* from S1, and F on *Viguiera dentata* from S2 (Table S2). EDX analysis did not detect Pb, Cd, and neither Co in any of the analyzed particles.

Leaves surfaces retaining particles and PTEs

The studied species differed in size and growth habit (Table S3). Leaves are diverse in morphology and size (Fig. S1); depending on the species, their surfaces are not smooth and they possess various structures that contribute to retain particles. In the case of *Brickellia veronicifolia*, the species with the highest number of particles on both, W and UW leaves, the veins look like gullies across the leaf where particles are retained (Fig. 4a). Particles were also observed 'sticking' on leaf glands of the aforementioned species (Fig. 3d). Important leaf structures regarding particle retention are trichomes. *Aster gymnocephalus* was another species with high number of particles; abundant trichomes were observed on the surfaces of the leaves (Fig. 4 b). *Dichondra argentea* trichomes were abundant and the longest observed, forming a kind of cover where particles of several sizes were retained (Fig. 4c). Other species in which trichomes obviously affected particle retention were *Gnaphalium* sp. (Fig 4d) and *Cuphea lanceolata*.

However, among the mentioned species only *Dichondra argentea* had abundant trichomes, 102 trichomes mm^{-2} (Table S3), and had significant correlation between amount of particles and trichomes (Table S4). For *Aster gymnocephalus and Gnaphalium* sp. the amount of trichomes was not significant for particles retention; but trichomes length and the roughness of both, trichomes surface and leaf itself, might affect retention too (Fig. 4d). Stomata retaining particles were observed on *Juniperus* sp. leaves (Fig. 4e).

Also other biological structures were observed to contribute to the retention of particles. *Flaveria trinervia* was retaining high quantities of particles too; this could be attributed to the presence of an abundant fungal mycelium forming a wide web that retained particles (Fig. 5a). Other species where fungal mycelium was observed to contribute to particle retention were *Dichondra argentea* (Fig. 5b), *Aster gymnocpehalus* (Fig. 5c), *Crotalaria pumila* (Fig 5d), *Viguiera dentata* (Fig 5e), *Gnaphalium sp.* (Fig 5 and *Cuphea lanceolata*. Fungal mycelium was observed at both W and UW samples, but more abundantly on the latter samples.

Discussion

It is recognized that plants play an important role in capturing particulate contaminants (Beckett et al., 2000; Nowak et al., 2006). At the level of the whole plant, particle deposition is affected by the shape and structure of the plant, while when considering individual leaves, surface structures and and structural features affect deposition and retention of particles (Litschke and Kuttler, 2008; Dzierżanowski et al., 2011; Dzierżanowski and Gawroński 2011). In this work differences in the numbers of particles retained by different plant species were observed (Fig. 2). Features like veins, protuberances, glands (Wang et al., 2006), wax cover (Dzierżanowski et al., 2011;

Popek et al., 2013), are affecting roughness, hairiness of leaves and thus the particle retention capacity (Sæbø et al., 2012; Wang et al., 2006).

Sæbø et al. (2012) mentioned that the presence of trichomes on leaf surfaces is positively correlated to the amount of retained particles. In our work, trichomes were found to be the main leaf features affecting retention of particles in several of the investigated species (Fig. 4b-d) but not only for the amount of such structures, also their length and their surface roughness participated. However, in *Brickellia veronicifolia* which was retaining the highest amount of particles, the gully formed by the veins seemed the main feature involved in particle retention (Fig. 4a). In species with few or no trichomes, such as *Juniperus* sp., other leaf structures, like stomata (Fig. 4f), retained particles. Besides the leaf characteristics themselves, it was remarkable that fungal mycelium also participated in retention of particles containing PTEs (Fig. 5). When sampling was done, healthy shoots and leaves were taken. It has been documented the participation of phyllosphere fungi in degradation of organic contaminants (Undugoda et al., 2013). The interaction of such microorganisms with PTEs is scarcely studied, there are some reports about how particulate matter (SantaRamJoshi and Rakshak, 2008) or PTEs in soil (Tóth et al., 2011) affect phyllospheric fungal community. But there is no information on possible participation of fungi in air PTEs remediation. The identification of fungi observed on leaves and retaining particles is being explored by our research group. This information will be useful for understanding the possible role of these organisms on the leaves particles retention capacity.

In some species like *Ruta graveolens* and *Juniperus* sp., the differences in numbers of particles between W and UW leaves were significant (Fig. 2), but the PTEs concentrations in the entire shoots were not (Table 1). It must be taken into account that counting and EDX analysis were performed at the upper side of the leaves, but also at the downside particles are retained (Ottelé et

al., 2010; Tomašević et al., 2005; Wang et al., 2006). Moreover, since they are considered as the main organs that retain particles (Freer-Smith et al., 2004) in our investigation the counting of particles was only done on leaves. It is however known that particles can also be retained by other structures like stem, bark (Fujiwara et al., 2011; Suzuki, 2006), flowers, fruits (Petroff et al., 2008) or seeds. All these particles also contribute the total PTE burden of the aerial parts. Future work will explore particle accumulation on these plant structures.

The species that retained the highest amount of particles at both locations was *Brickellia veronicifolia* (Fig. 2). Though, only at S1 this species also showed the highest variety of PTEs retained on its leaves (Table S2). The species studied in this work have not been analyzed before for particles and pollutants collection. However, the PTEs retention capacities found here are higher than those reported previously for other species in urban areas. Nicola et al. (2003) compared PTEs concentration in W and UW samples of *Quercus ilex*. They found that differences between W and UW samples did not reach twice for Zn, Cu and Pb. In this work, UW samples contained until 14 times more Zn, 6 times more Cu and 5 time more Pb than W ones. Concentrations of PTEs in plant shoot reported in this work are higher as well. For instance Cu, the mentioned authors reported 13.8 and 9.05 mg kg^{-1}in UW and W samples, respectively. While, in the present work concentrations were between 15 -274 mg Cu kg^{-1} in W samples, and between 16 -544 mg Cu kg^{-1} in UW samples (Table 1). In the case of Zn, the lowest concentration in UW samples was 184 mg kg^{-1} (Table 1); whilst, Nicola et al. (2003) reported 32.13 mg Zn kg^{-1} in UW samples. Since methodologies for PTEs retention on leaves are variable is not possible to make a wider comparison.

Regarding particles composition, as Re is not a common element its presence was outstanding (Table S1 and S2). The geology reported in the studied area, organic-rich shales (Carrillo and

Suter, 1982) and the presence of molybdenite (Simons and Mapes, 1987), is a natural source of Re during weathering (Prouty 2011; Golden et al., 2013).

We also observed that at one location PTE concentrations in shoots were different between W and UW samples while for the same species on the other site this was not the case. This was observed for Zn, Cd, Cu, Ni, Co and Mn concentrations in *Viguiera dentata*, for Zn, Cd, Ni and Cu in *Brickellia veronicifolia*, for Cd, Co and Cu in *Dichondra argentea*, and for Cd, Co and Mn in *Dalea bicolor* (Table 1). The differences between the 2 locations may be explained by the specific mineralogy and chemical characteristics of the mine residues on which the plants were growing. Moreover, S1 is not close to inhabited area (Fig. 1) and mine tailings are surrounded by hills. This limits the possibility of entrance of particles from other origin than the mine tailings. Site 2 is located in an open zone near to an urban area (Fig. 1) and close to unpaved roads where traffic is relatively busy. Due to the semi-arid weather nearly the whole year around, dust and particle dispersion by wind is occurring almost continuously. It is likely that on S2 not all particles containing PTEs originate from mine residues. Particles containing PTEs are emitted from different sources, such as vehicles along road traffic (Fernández-Espinosa and Oliva, 2006). The negative correlation observed between PTEs concentrations in UW samples from S2 (Table S4) may result from this particular situation. Furthermore, environmental factors, such as rainfall, wind speed, humidity, *etc*. (Beckett et al., 2000; Litschke and Kutller 2008) lead to seasonal and spatial variations in particle deposition (Keane et al. 2001; Oliva and Rautio, 2005; Protonotarios et al., 2002; Tomašević and Aničić 2010), as well as variations in particle composition (Mingorance and Oliva, 2006; Petaloti et al. 2006). Therefore, it is important to take into account environmental factors, climate and season of sampling for future studies.

Lead and Cd concentrations were different between W and UW plant samples (Table 1); however, these elements could not be detected by EDX examination (Table S1). It is possible that these elements were present but at concentrations lower than detection limit. Additionally, only particles from at least 15 µm diameter were counted and analyzed; although present, smaller particles were not considered. It has been reported that, in urban and industrial areas, Pb and Cd are mainly associated with particles smaller than 15 µm (Fernández-Espinosa and Oliva, 2006; Petroff et al., 2008; Suzuki, 2006; Tomašević et al., 2010) or even smaller than 1 µm diameter (Niu et al., 2010). Undoubtedly, these small particles may affect concentrations of PTEs in plant tissue. Other authors have counted smaller particles (< 10 µm) on leaf surfaces using magnifications such as 250x, 500x (Ottelé et al., 2010), 1000x, 5000x (Lorenzini et al., 2006). During the standardization of the methodology of the present research we tried to count particles at different magnifications. At magnifications higher than 150x some particles appeared so big that they filled the entire observation field. Therefore, a magnification of 150x was chosen. The next step of this research will take into account higher magnifications to explore smaller particles retained on the plant leaves.

The presence of particles containing PTEs on leaf surfaces certainly influenced the concentrations that were determined for these elements in aerial tissue. A striking example is UW *Gnaphalium* sp., which exceeded the Cu hyperaccumulation level of 300 mg kg^{-1} (van der Ent et al., 2013), whilst in the W sample of the same species the Cu concentration was below this level (Table 1). The results illustrate that the washing protocol does not necessarily eliminate all leaf surface retained PTEs. However, it was described that rinsing with distilled (Ataabadi et al., 2012) or deionized water should be sufficiently adequate washing protocols to remove dust or PTEs retained on leaves. Oliva and Valdés (2004) reported that after washing there was a reduction in

PTEs concentrations of plant shoots; the results varied according to the species and the element. They mentioned that washing is necessary only for Al, Cr, Fe, Pb, and V when analyzing metal accumulation in leaves of plants. Peryea (2005) reported that detergent washing is critical for eliminating contamination introduced by deposition on leaves surfaces. Our results indicate that even if the samples are washed following a thorough protocol (including, water, detergent, and diluted acid) there possibility of overestimating PTE concentrations taken up through the roots still exists (Table 1) due to the presence of PTEs deposited and retained on the surface of aerial parts (Fig. 3). This situation is more likely to occur when plants are collected from the field or in root samples (van der Ent et al., 2012).

The application of an adequate washing protocol is critical in studies searching for plants suitable for phytoextraction since they use concentrations of PTEs in shoots to decide about eventual applicability of a certain species to extract PTEs from contaminated soils. In several of these studies the plant samples were washed with distilled or deionized water after tap water rinses (Barrutia et al., 2011; Lorestani et al., 2011; Moreno-Jimenez et al., 2009; Wang et al., 2008) or only rinses with distilled or deonized water (Conesa et al., 2006; Gupta and Sinha, 2008; Xue et al., 2014; Yoon et al., 2006). Our results demonstrate that this most probably is not sufficient. Inadequate washing leads to an overestimation of PTEs concentrations in plant tissues (Table 1) and thus to an incorrect estimation of the metal accumulating or hyperaccumulating capacities of a certain species. Therefore it is suggested that, in addition to water, detergent, and diluted acid rinses, also sonication and/or shaking should be included in washing protocols.

Our results presented above indicate two main points. Firstly, the importance of an adequate washing protocol in studies regarding PTEs concentrations in plant tissues. Secondly, plants represent a physical barrier that reduces dispersion of PTEs from mine tailings as possible main

source. Vangronsveld et al. (1995a, b, 1996, 2009) mentioned that phytostabilization includes the use of plants to stabilize PTEs in soils and prevent erosion and airborne transport. The term phytostabilization is more related and understood as the stabilization of contaminants in roots and rhizosphere (Arthur et al., 2005; Mendez and Maier 2008). Therefore, it is proposed to term the function of plants as a physical obstacle for spreading of particles as phytobarriers. This represents a way to diminish dispersion of PTEs mainly from uncovered mine tailings in arid and semiarid regions. This is the first attempt in which the role of leaves of plants growing spontaneously on mine tailings acting as a barrier for the dispersion of PTEs is documented.

We found a variety of herbaceous plants, shrubs (*Brickellia veronicifolia* and *Dalea bicolor*) and even a tree (*Juniperus* sp.) that were all participating in retention of particulate matter (Fig. 2). All these plant species are able to develop and contribute to a vegetation cover on the bare mine tailings, thus reducing aerial dispersion of PTEs.

Conclusions

The aim of this work was to document the retention of particles containing PTEs by leaves of plant species spontaneously establishing on mine tailings. For this purpose, we compared PTEs contents in W and UW plant samples and performed a SEM study of the leaf surfaces. Particles containing PTEs were observed at both, W and UW plant leaves; thus, all the investigated species were retaining PTEs on their leaf surfaces. The species with the highest particle retention capacity was *Brickellia veronicifolia*. The leaf structures that contributed most to particle retention in the majority of the species were the trichomes, although other structures such as veins, epidermal glands, stomata, and fungal mycelium also contributed to retention of PTEs containing particles. This confirms that besides PTEs accumulation inside plant organs, leaves of plants growing on

mine tailings represent a significant physical barrier that diminishes dispersion of PTEs containing particles from mine tailings. Among the studied PTEs, Cu and Zn were the most occurring elements associated to particles retained on leaf surfaces. Despite of the adoption of a thorough washing protocol, many particles containing PTEs were still present on leaf surfaces. This leads to an overestimation of concentrations of the mentioned elements in plant tissues.

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Table 1. Potentially toxic elements concentrations (mg kg^{-1}) in washed (W) and unwashed (UW) shoot samples of plants growing on two mine tailings.

Species	Zn		Pb		Cd		Cu		Ni		Co		Mn	
	W	UW	W	UW	W	UW	W	UW	W	UW	W	UW	W	UW
S1 (San Francisco)														
Pteridium sp.	225±14	613±11^{s}	68±16	222+11^{s}	3±1	14±3^{s}	61±10	78±10^{ns}	36±7	40±4^{ns}	23±2	30±3^{s}	22±2	77±14^{s}
Juniperus sp.	1067±141	1665±11^{s}	100±53	207+11^{ns}	11±4	12±1^{ns}	26±7	28±2^{ns}	37±14	38±3^{ns}	21±9	26±3^{ns}	36±11	38±2^{ns}
Cuphea lanceolata	1415±181	1406±25^{ns}	77±25	352+25^{s}	10±2	15±1^{s}	79±29	94±28^{ns}	30±8	35±2^{ns}	28±6	30±1^{ns}	54±9	56±14^{ns}
Dichondra argentea	935±120	1436±9^{s}	135±14	372+9^{s}	7±1	19±1^{s}	124±13	130±7^{ns}	36±1	37±1^{ns}	23±1	28±1^{s}	43±1	82±2^{s}
Brickellia veronicifolia	333±48	585±14^{s}	62±1	245+14^{s}	6±2	18±2^{s}	56±7	67±13^{ns}	33±1	38±1^{s}	26±1	30±2^{s}	46±5	64±5^{s}
Ruta graveolens	190±23	323±75^{ns}	88±36	341+75^{s}	4±2	13±2^{ns}	31±4	36±9^{ns}	44±9	47±12^{ns}	29±7	38±10^{ns}	21±1	33±5^{ns}
Dalea bicolor	185±52	184±31^{ns}	60±16	230+31^{s}	3±1	10±1^{s}	15±1	16±2^{ns}	32±2	34±4^{ns}	22±1	28±4^{s}	21±2	21±3^{ns}
Viguiera dentata	156±12	2231±29^{s}	62±1	264+29^{s}	4±1	21±3^{s}	36±2	215±42^{s}	36±2	43±1^{s}	20±2	31±4^{s}	8±1	189±57^{s}
S2 (Santa Maria)														
Aster gymnocephalus	1030±106	1302±30^{s}	195±19	366+30^{s}	20±1	21±1^{ns}	115±14	289±33^{s}	40±3	44±3^{ns}	30±5	50±4^{s}	92±12	228±21^{s}
Gnaphalium sp.	1419±46	2012±45^{s}	178±9	411+45^{s}	17±2	18±1^{ns}	274±27	544±79^{s}	42±1	52±4^{s}	47±1	60±4^{s}	206±22	338±36^{s}
Viguiera dentata	393±34	394±15^{ns}	73±7	265+15^{s}	16±1	16±1^{ns}	33±10	35±5^{ns}	25±2	29±1^{ns}	22±2	24±5^{ns}	38±4	41±5^{ns}
Dalea bicolor	207±27	211±22^{ns}	93±6	223+22^{s}	16±1	16±1^{ns}	22±3	26±1^{ns}	30±3	34±4^{ns}	21±4	28±3^{ns}	19±3	133±24^{s}
Crotalaria pumila	207±46	256±38^{ns}	96±9	258+38^{s}	15±1	16±1^{ns}	47±4	54±5^{ns}	31±2	35±4^{ns}	25±1	27±1^{ns}	37±3	48±10^{ns}
Brickellia veronicifolia	389±10	391±5^{ns}	145±10	340+5^{s}	18±1	20±2^{ns}	33±2	47±3^{s}	27±3	34±5^{ns}	24±1	30±1^{s}	45±1	65±5^{s}
Flaveria trinervia	514±30	538±24^{ns}	104±17	285+24^{s}	16±1	16±1^{ns}	75±18	102±12^{ns}	29±3	33±3^{ns}	24±1	26±3^{ns}	98±35	209±24^{s}
Dichondra argentea	113±25	1617±25^{s}	140±50	419+25^{s}	16±4	21±2^{ns}	132±19	319±75^{s}	40±9	51±2^{ns}	39±7	42±8^{ns}	50±8	288±3^{s}

Mean (N=3) ± standard deviation; according to *t* test ($p<0.05$): significant (^{s}) or non-significant (^{ns}) difference between element concentrations in washed (W) and unwashed (UW) samples of each species.

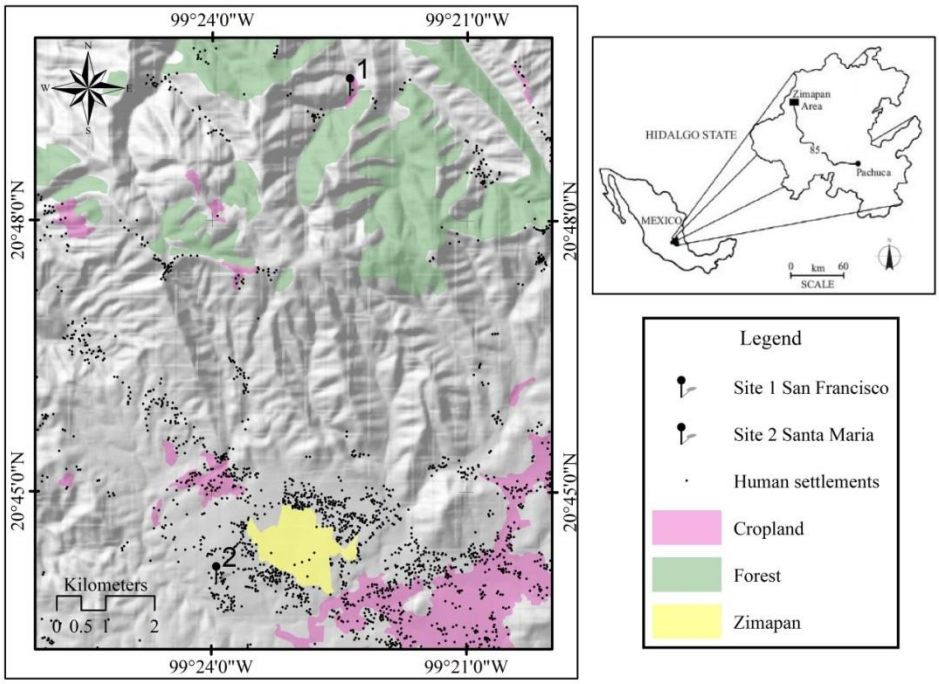

Fig. 1. Location of mine tailing sites where plants were collected.

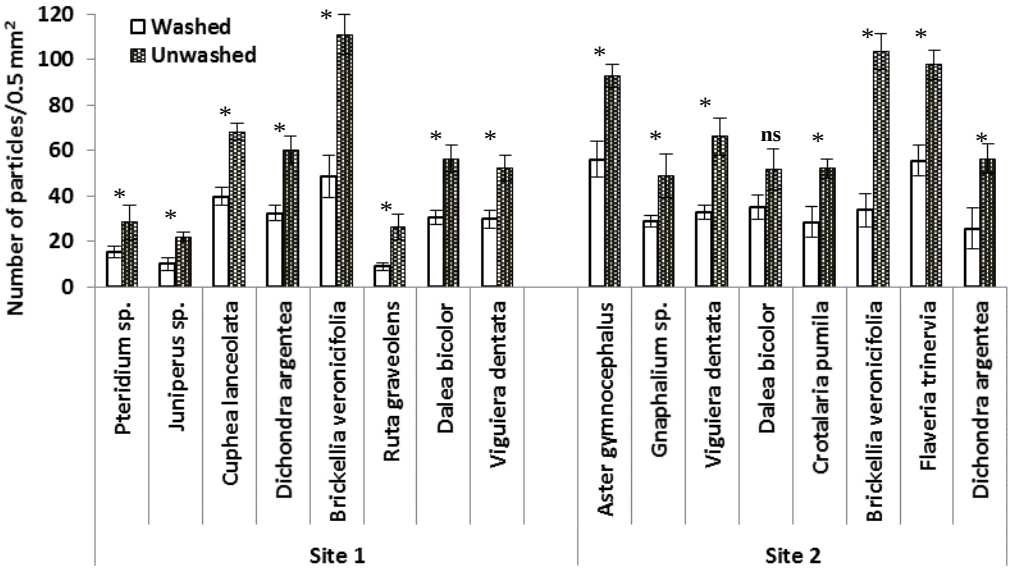

Fig. 2. Solid particles on washed (W) and unwashed (UW) leaves of plants growing on mine tailings. Mean and standard deviation (N=3). According to *t* test ($p<0.05$), significant (*) or non-significant (ns) difference between numbers of particles on W and UW samples of each species.

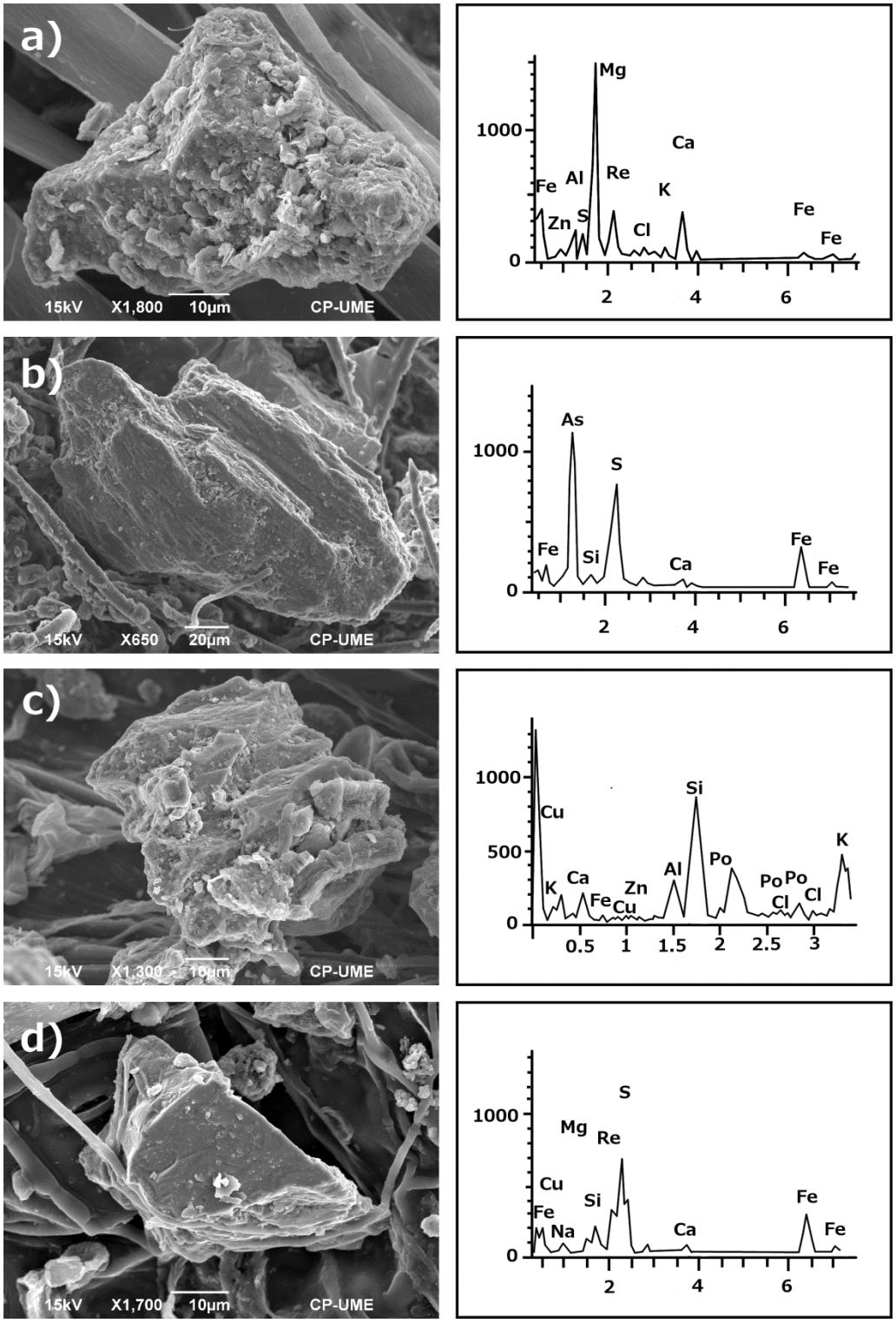

Fig. 3. EDX-spectrum of particles retained on W *Dichondra argentea* from S1 (a), W *Dalea bicolor* from S1 (b) and S2 (c), and UW *Brickellia veronicifolia* growing on S1.

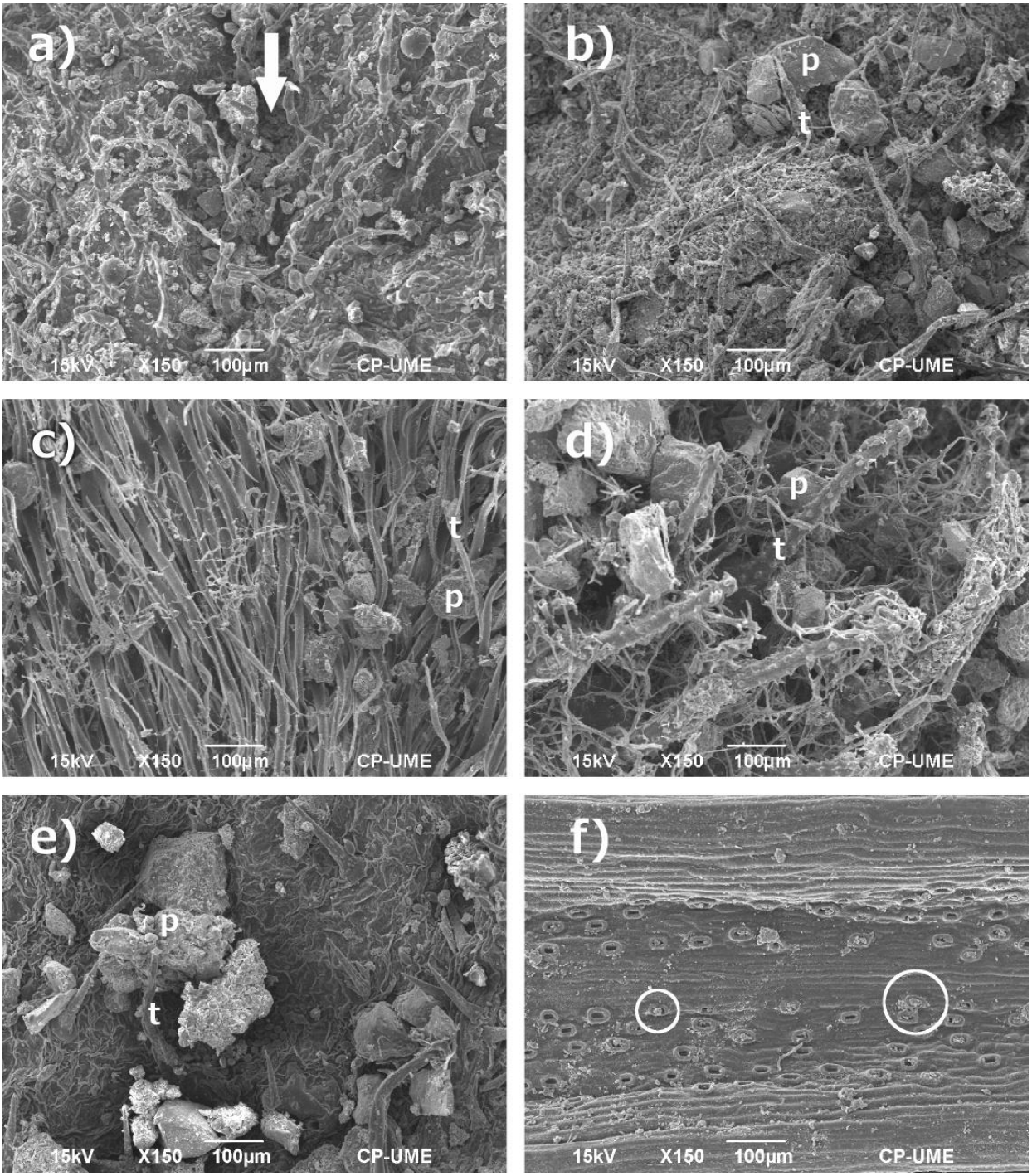

Fig. 4. SEM images of leaf structures retaining particles. a) W *Brickellia veronicifolia,* the arrow indicates the vein bottom where the particles are retained; particles (p) retained by trichomes (t) on b) W *Aster gymnocephalus*, c) UW *Dichondra argentea*, d) UW *Gnaphalium* sp. and e) UW *Cuphea lanceolata*, f) particles retained by stomata on UW *Juniperus* sp.

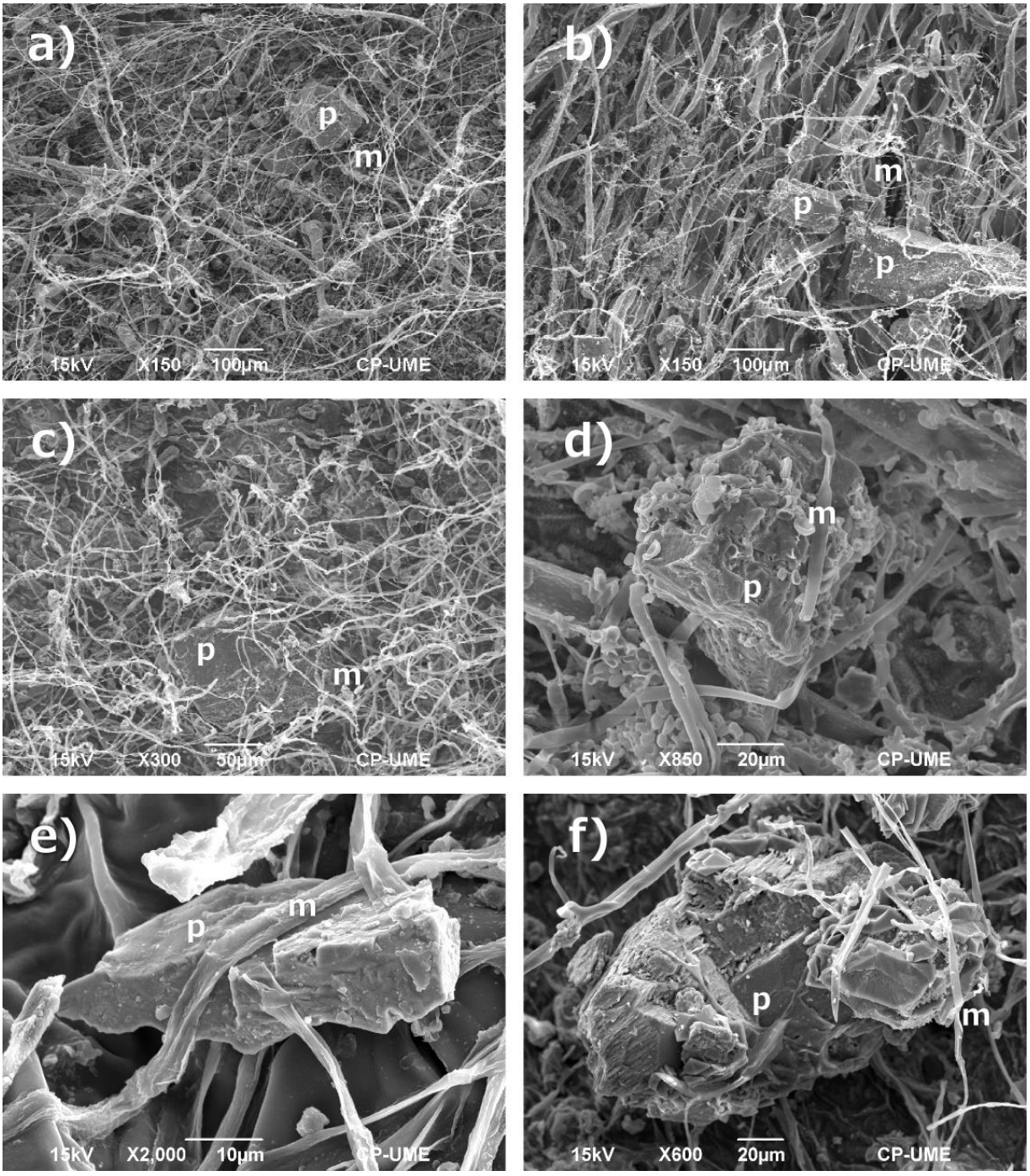

Fig. 5. Fungal mycelium (m) trapping particles (p) on leaves of a) UW *Flaveria trinervia*, b) UW *Dichondra argentea*, c) W *Aster gymnocephalus*, d) UW *Crotalaria pumila*, e) W *Viguiera dentata* and f) W *Gnaphalium* sp.

Table S1. Potentially toxic elements occurrence (%) in particles retained by washed and unwashed vegetal samples.

Sample	Elements detected																	
	Ca	K	Si	Fe	Al	Cl	Zn	Cu	Mg	S	As	Re	Mn	Na	Po	Br	F	Mo
Particles on washed shoot samples (%)	52	49	51	51	53	83	35	41	54	33	25	33	29	50	33	67	0	0
Particles on unwashed shoot samples (%)	48	72	49	49	47	17	65	59	46	67	75	67	71	50	67	33	100	100

%: percentage of analyzed particles containing certain element. For instance: 52% of particles present on washed samples contained Ca; while in unwashed samples 48% of analyzed particles contained such element.

Table S2. Frequency (%) of different elements detected by EDX on particles deposited on leaves surface of plants growing on mine tailings.

Species		Elements detected																	
Site 1		Ca	Si	K	Fe	Al	Cl	Zn	Cu	Mg	S	As	Re	Mn	Na	Po	Br	F	Mo
Pteridium sp.	W	67	67	33	56	11	22	0	0	11	0	0	0	0	0	0	0	0	0
	UW	56	56	44	44	33	0	11	11	0	0	0	0	0	11	0	0	0	0
Juniperus sp.	W	56	44	11	67	33	22	22	11	0	0	0	0	11	0	0	0	0	0
	UW	44	56	44	56	44	0	33	22	0	11	0	0	0	0	0	0	0	0
C. lanceolata	W	44	33	33	44	56	11	22	11	22	0	0	11	11	0	0	0	0	0
	UW	67	33	56	56	44	0	22	22	11	11	11	22	0	0	11	0	0	0
D. argéntea	W	56	67	56	44	44	22	11	0	22	0	0	11	0	0	0	0	0	0
	UW	56	67	44	67	44	0	22	0	33	33	22	11	0	0	0	0	0	0
B. veronicifolia	W	56	78	67	44	11	0	11	0	22	11	11	11	0	0	0	0	0	0
	UW	78	44	67	56	11	0	22	0	11	44	33	11	22	11	0	0	0	0
R. graveolens	W	78	89	44	33	22	11	0	0	11	22	0	0	0	0	0	0	0	0
	UW	67	44	22	44	44	0	0	0	11	0	11	0	0	11	0	0	0	0
D. bicolor	W	44	44	44	33	33	22	11	0	0	11	11	0	0	11	0	0	0	0
	UW	56	56	67	33	56	0	22	22	11	0	22	0	11	0	0	0	0	0
V. dentata	W	44	56	33	56	11	11	0	11	0	0	11	0	0	0	0	11	0	0
	UW	89	56	44	78	11	0	0	22	11	22	22	11	0	0	0	0	22	0
Site 2																			
A. gymnocephalus	W	56	67	56	44	56	56	11	11	22	0	0	0	0	0	0	0	0	0
	UW	56	44	67	33	67	0	22	22	11	11	0	0	11	0	11	0	0	0
Gnaphallium sp.	W	67	56	44	67	33	22	0	11	11	11	0	0	0	0	0	0	0	0
	UW	78	33	22	22	11	0	33	22	0	11	11	0	0	0	0	0	0	0
V. dentata	W	67	56	56	56	67	56	22	11	33	33	11	0	0	0	0	0	0	0
	UW	56	44	67	33	11	11	22	11	22	0	0	11	0	0	0	11	0	0
D. bicolor	W	56	67	56	33	67	67	22	11	11	0	0	0	11	0	11	11	0	0
	UW	78	44	67	44	33	11	0	22	22	22	0	0	11	11	0	0	0	0
C. pumila	W	56	56	22	44	22	22	11	11	22	0	11	0	11	0	0	0	0	0
	UW	89	67	44	22	44	0	22	11	0	11	0	0	0	0	0	0	0	0
B. veronicifolia	W	67	78	67	44	78	22	0	33	11	11	0	0	0	11	0	0	0	0
	UW	44	56	44	22	11	0	11	11	0	11	0	0	0	0	0	0	0	11
F. trinervia	W	67	78	78	56	67	11	0	22	11	0	0	0	0	0	0	0	0	0
	UW	89	78	67	67	78	0	11	33	22	11	11	0	11	0	0	0	0	0
D. argentea	W	89	56	67	33	0	0	0	22	11	0	0	0	0	11	0	0	0	0
	UW	44	22	33	56	11	56	11	11	22	0	0	0	4	0	0	0	0	0
Average		63.0	56.0	48.9	46.5	36.4	14.2	12.7	12.7	12.7	9.3	5.8	3.1	3.6	2.4	1.0	1.0	0.7	0.3

W: washed leaves; UW: unwashed leaves; percentage of analyzed particles containing certain element. For instance: 67% of particles present on washed samples of *Pteridium* sp. contained Ca; while in unwashed samples of the same species 456% of analyzed particles contained such element.

Table S3. Size and characteristics of studied plant species.

Species	Height (at sampling)	Canopy diameter (at sampling)	Growth habit	Trichomes / 0.5 mm^2 *	Other characteristics
Pteridium sp.	27-42 cm	36-47 cm	Erect buds	5	Large (30 cm), highly divided leaves
Juniperus sp.	40-53 cm	11-28 cm	Tree	0	Evergreen tree with needle-like leaves.
Cuphea lanceolata	18-33 cm	8- 14 cm	Annual herbaceous	24	Sticky leaves
Dichondra argentea	3-6 cm	10-50 cm	Perennial creeping, climbing plant	102	Hairy leaves; long trichomes. Branched stems
Brickelia veronicifolia	78-95 cm	33-68 cm	Evergreen shrub	27	Very branched shrub; slightly pubescent leaves.
Ruta graveolens	14-21cm	4-11 cm	Evegreen shrub	2	Branched shrub, divided leaves.
Dalea bicolor	71-103 cm	45-63 cm	Evegreen shrub	115	Compound, hairy leaves.
Viguiera dentata	57-130 cm	40-60 cm	Perennial herbaceous plant	21	Sparse foliage, slight hairy leaves
Aster gymnocephalus	17-33 cm	11-20 cm	Biannual herbaceous plant	14	Leaves are narrow, sparse foliage
Gnaphalium sp.	18-27 cm	25-46 cm	Herbaceous plant	10	Vey hairy (wolly) leaves and stems
Crotalaria pumila	12-32 cm	16-30 cm	Annual herbaceous plant	2	Leaves and stems are glabrous
Flaveria trinervia	23-35 cm	14-42 cm	Annual herbaceous plant	22	Sparse foliage; generally glabrous leaves.

*: trichomes counting following methodology described for particles counting in Material and Methods section. Average N=6.

Table S4. Pearson correlation between number of particles and PTEs concentrations in unwashed samples from Site 2.

	Number of trichomes	Mn UW	Cd UW	Pb UW	Ni UW
Number of Particles UW	0.96** *Dichondra argentea* (Site 1) 0.91** *Dichondra argentea* (Site 2)	-0.99** *A. gymnocephalus*	-0.99** *Dalea bicolor*	-0.97* *Gnaphalium* sp.	
Co UW		*-0.94** *Dalea bicolor*	*-0.99*** *Gnaphalium sp.*		*-0.99*** *Gnaphalium sp.*
Ni UW		*-0.99*** *Crotalaria pumila*			

UW: unwashed shoot samples; number in each cell is *r* value (Pearson coefficient); *: *p* value $\leq$ 0.05; ** *p* value $\leq$ 0.001; N=3.

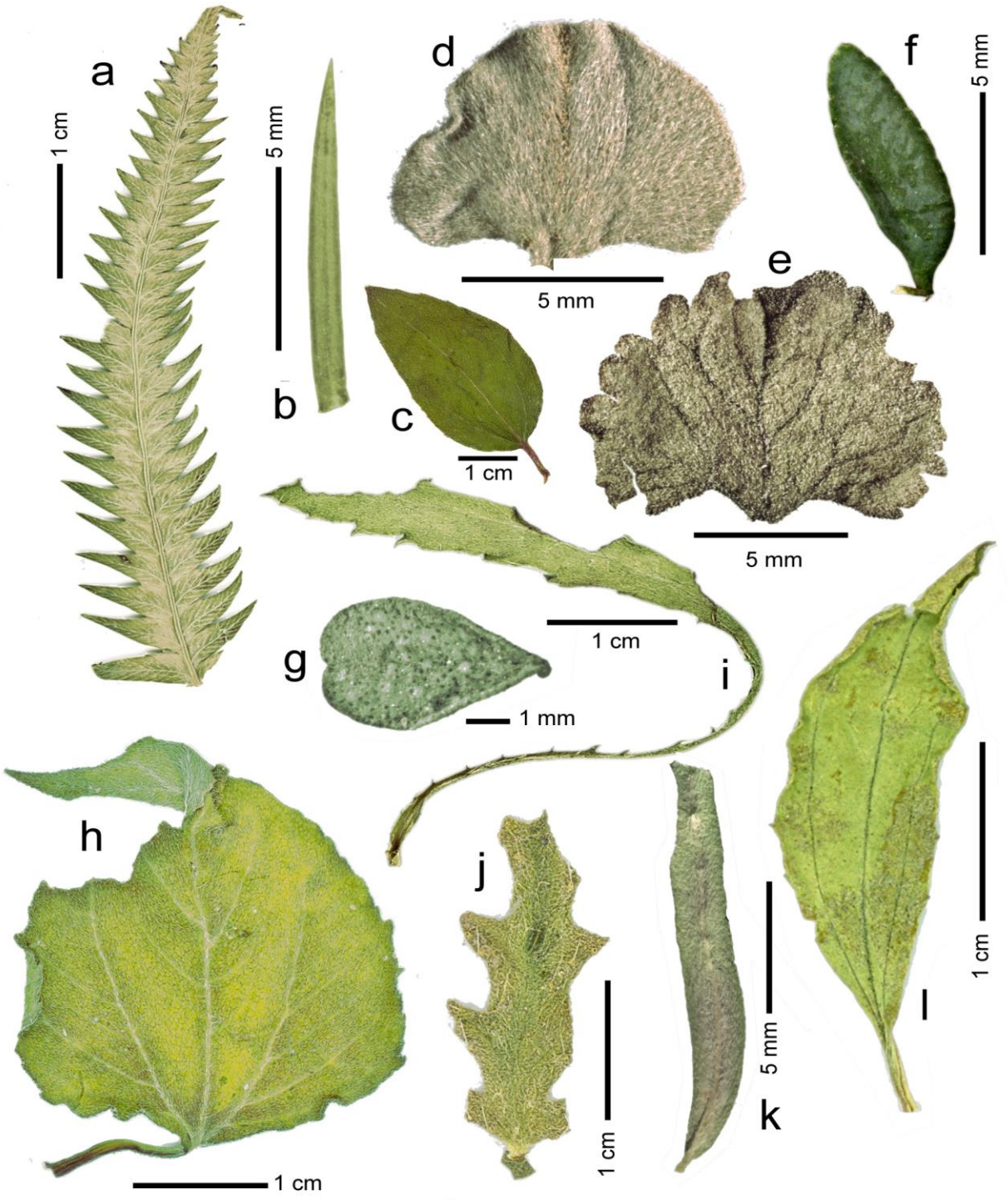

Figure S1. Leaves of the studied plants. A: *Pteridium* sp.; b: *Juniperus* sp.; c: *C. lanceolata*.; d: *D. argentea*; e: *B. veronicifolia*; f: *R: graveolens*; g: *D. bicolor*; h: *V. dentata*; i: *A. gymnocephalus*.; j: *Gnaphalium* sp.; k: *C. pumila*; l: *F. trinervia.*

CHAPTER 4. SEED ENDOPHYTIC BACTERIA OF A PIONEER PLANT SPECIES COLONIZING MINE RESIDUES IN A SEMI-ARID REGION: TRANS-GENERATIONAL CHARACTERIZATION

Abstract

Composition and functional characteristics of cultivable seed endophytic bacteria of *Crotalaria pumila* colonizing potentially toxic elemensts (PTEs)-containing mine residues were compared among three consecutive generations. These communities were compared to cultivable seed endophytes of the same species collected on a non-contaminated reference site. Differences in composition, functional traits of cultivable endophytes and metabolic fingerprints of the total community were observed between seeds from the non-contaminated site and the mine residues. Thirty-three strains belonging to 13 bacterial genera were isolated from mine residue seeds; *Methylobacterium* sp. Cp2, *Methylobacterium* sp. Cp3, and *Sphingomonas* sp. Cp1 were present in the three generations of cultivable endophytic communities. From control seeds 11 isolates classified in 7 genera were identified. Phytate mineralization, 1-aminocyclopropane-1-carboxylate deaminase activity, phosphate solubilization and PTEs tolerance were common functional traits of mine residue seed isolates. Phosphate solubilization, organic acids and Indol Acetic Acid production were common traits in cultivable endophytes from seeds originating from the non-contaminated site. Data suggest that cultivable endophytic bacteria that are transmitted via seed possess beneficial functional traits for germination, stress alleviation, subsequent growth and proliferation of *C. pumila* on PTEs containing-mine residues. These results are promising for field application of endophyte-stimulated phytoremediation of PTEs-contaminated soils.

Key words: metals, plant growth promotion, *Crotalaria pumila*

Introduction

Mine tailings originating from extraction and processing of mineral ores may affect the environment and public health due to the risk of potentially toxic elements (PTEs) dispersion. Phytoremediation is an alternative to avoid or reduce wind and water erosion and also PTEs percolation and to remediate PTEs contaminated sites (Vangronsveld, Colpaert, and van Tichelen 1996; Vangronsveld *et al.* 2009). Despite there exist a number of reports describing native plants that can accumulate or immobilize PTEs, the benefits that associated microorganisms generate to their host plants should be taken into account to better understand how these plants overcome the adverse environmental factors commonly found on polluted sites. *Crotalaria pumila* (Fabaceae) is a species found in the semi-arid mining region of Zimapan (central Mexico). It can accumulate PTEs to concentrations higher than phytotoxicity levels, complete its full life cycle, produce seeds within pods and thus increase the number of plants colonizing bare mine tailings year after year (Sánchez-López *et al*. 2015). The persistence of *C. pumila* in such adverse environments can be explained by the transmission (Johnston-Monje and Raizada 2011; Hardoim *et al*. 2012) and presence of seed endophytic bacteria during germination and early seedling development (van Oevelen *et al*. 2003; Truyens *et al*. 2013, 2014a). These microorganisms produce to their host several benefits similar to those described for plant growth-promoting bacteria (Hardoim, van Overbeek, and van Elsas 2008); moreover, they might lower PTEs phytotoxicity and increase tolerance to PTEs (Weyens *et al*. 2009a). The interaction between endophytes and plants suitable for phytoremediation is an important study area, *C. pumila* growing on mine tailings in Zimapan provides a very interesting case for the study of seed endophytic bacteria thriving in a contaminated environment as a mechanism for increased tolerance of their host to PTEs and semi-arid conditions. Thus, the objectives of this work were: to compare the metabolic

fingerprints of the total endophytic communities, as well as the composition and functions of the cultivable endophytic communities of three consecutive generations of *C. pumila* seeds from plants growing on multi-PTE contaminated mine residues. These aspects were compared to endophytes of the same plant species growing on a non-contaminated reference site.

Material and methods

Seed material

Seeds of *C. pumila* were collected from the Santa Maria mine residues, Zimapan, Mexico (20°44'8.89"N, 99°23'56.07"W) in 2011, 2012, and 2013. Mine residues pH was 7.6, total concentrations of Zn, Cd, Pb are 4546 mg kg^{-1}, 120 mg kg^{-1}, 4183 mg kg^{-1}, respectively (Sánchez-López *et al*. 2015). Reference seeds (from a non-PTEs contaminated site), samples were collected from an abandoned cropland in San Joaquin, Mexico (20°55'18.52"N, 99°34'03.21"W) in 2013. This soil has a pH 6.3 and total PTEs concentrations are 143 mg kg^{-1}, 1 mg kg^{-1}, and 4 mg kg^{-1} of Zn, Cd and Pb, respectively. The seeds from both sites were collected within pods in the field, and then the pods were opened under laboratory conditions.

Optimization of the surface sterilization protocol

To obtain as many different cultivable seed endophytes as possible, several surface sterilization protocols were tested (Table S1). First seeds were washed and rinsed with a P-free detergent and tap water. Subsequently, seeds were either immersed in ethanol (70%) or not immersed, several immersion times were tested. Later, seeds were submerged in NaClO solution supplemented with 0.1% Tween 80. Two concentrations of NaClO were tested, 0.1% and 1%. Afterwards, seeds were thoroughly rinsed in sterile deionized water (8 x 100 mL).

The effectiveness of surface sterilization was checked by incubating 100 µL of the last rinsing water and ten "surface sterilized" seeds on 869 solid medium (Mergeay *et al*. 1985). The remaining seeds were crushed in a sterile mortar after adding 500 µL of sterile 10 mM $MgSO_4$ solution. Further, 2.5 mL of the mentioned solution were added and a total volume of 3 mL was obtained. Dilutions 1/10 and 1/100 were done for each sample. A 100 µL aliquot of the undiluted sample and from the both dilutions was plated on 869 1/10 diluted medium. Five replicates were used. The plates were incubated one week at 30° C in darkness.

Isolation of endophytic bacteria

100 mg of seeds were surface sterilized with the optimal protocol (not including ethanol and immersing the seeds during 10 seconds in NaClO 0.1%). Seed endophytes were isolated as described above. Isolation of seed endophytes in liquid culture was also tested; 100 µL of surface sterilized seeds slurry were added to 100 mL of liquid medium (869 diluted 1/10) and incubated at 28°C and 120 rpm in darkness. After 5 days incubation, 100 µL of the liquid culture were plated on the solid medium. Subsequently, colony forming units (cfu) were counted and calculated per gram of seed. Each morphologically different colony type was chosen for further analyses.

Functional characterization of seed endophytic bacteria

Before tests performance, strains were grown in 869 liquid medium and then washed twice and resuspended (10^7 cfu mL^{-1}) with sterile 10 mM $MgSO_4$. All tests were done in triplicate. All purified bacterial isolates were screened for phosphate solubilization in National Botanical Research Institute's phosphate growth solid medium (Nautiyal, 1999) and for phytate mineralization (Jorquera *et al*. 2008). Nitrogen-fixing capacity was tested in a semi-solid malate-sucrose medium with bromothymol blue as a pH indicator (Xie *et al*. 2006). The same medium

supplemented with 0.12 g L^{-1} NH_4Cl was used as a positive control. After one week a colour change from blue to yellow indicated nitrogenase activity. Bacterial siderophore production was evaluated after 5 days incubation in 284 medium with C mix (Weyens *et al.* 2009b) by adding chrome azurol reagent (Schwyn and Neilands 1987); orange colour was considered positive for siderophore production. For organic acid production, sucrose tryptone medium was used, 5 days later 0.1% alizarine red was added; yellow colour indicated organic acids production (Cunningham and Kuiack 1992). Bacterial Indol-Acetic Acid (IAA) production capacity was verified in 1/10 869 medium with 0.5 g L^{-1} tryptophan. After 5 days incubation, cultures were centrifuged (3220 g, 15 min), and the supernatant was mixed with Salkowski's reagent (Gordon and Weber 1951); a pink colour was considered positive for IAA production. To detect strains that produce acetoin the protocol proposed by Romick and Fleming (1998) was used. The 1-aminocyclopropane-1-carboxylate (ACC)-deaminase activity was evaluated inoculating bacteria in salts minimal medium with 0.5 M ACC as only nitrogen source according to Belimov *et al.* (2005).

PTEs tolerance

The isolates were tested for their PTEs tolerance using 284 liquid selective medium pH 7 (Weyens *et al.* 2009b) with the addition of 0.6, 1.0, 2.5 and 5 mM of Zn ($ZnSO_4$), 0.4, 0.8 and 1.6 mM of Cd ($CdSO_4$), 0.4, 0.8, 1.6 mM of Cu ($CuSO_4$), 1, 3, and 5 mM of Ni ($NiCl_2$), or 0.5 mM of Pb ($Pb(NO_3)_2$). The metal salts were added to the medium after heat sterilization.

Genotypic characterization of seed endophytes

The DNA of all isolated purified bacteria was extracted using the DNeasy 96 Blood and Tissue Kit (Qiagen, Venlo, The Netherlands). Polymerase chain reaction (PCR) amplification of the 16S rRNA genes was performed on aliquots of the extracted DNA using the universal primers, 16S-

prokaryotic-R (5′-ACGGGCGGTGTGTRC-3′) and 16S-prokaryotic-F (5′-AGAGTTTGATCCTGGCTCAG-3′). Cycling conditions were: 5 min at 95 °C, 35 cycles of 1 min at 94 °C, 30 s at 52 °C and 3 min at 72 °C, and a final incubation step of 10 min at 72 °C. For the amplified 16S rDNA restriction analysis (ARDRA), 20 µL of the PCR products were digested with the HpyCH4IV enzyme, the resulting fragments were separated and visualized on a 1.5% agarose gel during 2 h at 90 V. Bacteria with the same ARDRA pattern were grouped and one representative isolate of each group was selected for sequencing. Purified PCR products of 16S rDNA were sequenced by Macrogen (Seoul, Korea). Consensus sequences were determined using the Staden package (Staden, Beal, and Bonfield 1999) and identification was carried out by comparing the obtained sequences with those of reference strains in the Ribosomal Database Project (RDPII). The diversity index (*H*) was calculated according to the Shannon index (Spellerberg and Fedor 2003)

Metabolic fingerprint of seed endophytic community

Seeds were weighted (500 mg) and surface sterilized, then crushed in a sterile mortar with 1 mL of phosphate buffer (per liter: 8 g NaCl, 1.805 g $Na_2HPO_4 \cdot H_2O$, 0.24 g KH_2PO_4, final pH 7.2); 14 mL of the same buffer were added to the mixture in order to obtain a final volume of 15 mL. The solution was filtered (GF/C Whatmann filters), and 140 µL were transferred to each well in the Biolog GN2 Micro Plates™. The plates were incubated at 30°C, 120 rpm in darkness. Optical density at 590 nm was read on a plate reader (Molecular Devices, Inc., Sunnyvale, CA, USA) at 0, 24, 48, 72, 120, 144, and 168 h. To analyze results from metabolic fingerprint, C sources were classified as proposed by Zak *et al*. (1994); metabolic indexes (average well colour development -AWCD-, diversity index, substrate richness, and substrate evenness) were calculated as mentioned by fabricant instructions and by Insam and Goberna (2004).

Statistical analysis

Biolog metabolic index values (twelve independent replicates for each kind of seed) were treated by analysis of variance, means were separated according to Tukey´s comparison test ($p<0.01$). Principal component analysis was applied on Biolog™ AWCD. Free software R 3.1.0 was used for statistical analysis.

Results

Isolation of seed endophytic bacteria

The highest diversity of cultivable seed endophytes was obtained after culture in solid. However, some endophytes could only be isolated through culture in liquid media (Table 1). Bacterial growth was observed in plates to which the seed extract was added directly, without any dilutions. The average amount of cfu per gram of seeds in each generation and in control seeds is presented in Table 1, values do not include strains isolated from liquid culture. Seeds collected in 2011 showed the lowest number of isolates; while 2012 seeds had the highest amount of isolates. The quantity of cfu was similar between control seeds and mine residue seeds collected in 2013. Despite of the high amount of cfu observed in 2012, the diversity of isolates was the lowest (*H* 1.65), whilst, the *H* for seeds harvested in 2013 indicated a high diversity (Table 1).

Genotypic characterization of cultivable endophytic bacteria

Identification of bacteria until genus level was performed using ARDRA. The relative abundance of each isolate was expressed as a percentage of the total number of cultivable isolates. In seeds from plants growing on mine residues 13 bacterial genera were identified (Figure S1). The most abundant was *Sphingomonas*, followed by *Staphylococcus*, *Brachybacterium*, *Variovorax* and *Methylobacterium*, while *Streptomyces*, *Caulobacter*,

Burkholderia, *Curtobacterium*, *Mycobacterium*, *Arthrobacter*, *Kocuria* and *Bacillus* each represented 3% of the cultivable community. The cultivable endophytic community from seeds of the non-contaminated reference site consisted of 7 cultivable genera (Figure S1). 32% of them belonged to the genus *Curtobacterium*, followed by *Bacillus* and *Sphingomonas*. *Microbacterium, Saccatothrix, Staphylococcus* and *Brachybacterium* represented each 9% of the cultivable endophytes of seeds from the non-contaminated substrate (Figure S1).

Composition of cultivable endophytic community in consecutive seed generations

Figure 1 presents the endophytic community composition of the three consecutive generations of seeds collected from mine residues and the seed community from the non-contaminated reference site. *Curtobacterium* sp., followed by *Bacillus* sp., composed the major part of the cultivable endophytic community of reference seeds. *Sphingomonas* sp. isolates were the most abundant in seeds from mine residues; at least 4 different strains were identified in each generation, except in 2011. In 2012, the cultivable seed endophytic community was the less diverse (Table 1, Figure 1), but there were bacterial genera that were not identified in other generations such: *Streptomyces*, *Arthrobacter* and *Mycobacterium*. In the seeds from 2013 *Brachybacterium* and *Sphingomonas* were the most abundant; *Caulobacter* and *Burkholderia* were isolated only from these seeds.

Some isolates were common among the three seed generations from mine residues (Figure 1): *Methylobacterium* sp. Cp2, *Methylobacterium* sp. Cp3 and *Sphingomonas* sp. Cp1. *Variovorax* sp. Cp5 and *Sphingomonas* sp. Cp4 were both found in 2011 and 2012. *Staphylococcus* sp. Cp6 was common in mine residue seeds from 2011 and 2013, while *Sphingomonas* sp. Cp7 was observed in 2012 and 2013. One *Sphingomonas* sp. Cp33 was identified in both, seeds from mine

residues collected in 2013 and seeds from plants growing on the non-contaminated soil (Figure 1).

Functional characterization of cultivable endophytic bacteria

A summary of the functional characteristics of cultivable seed endophytic bacteria of *C. pumila* is presented in Table 1. The percentage of isolates that produced IAA, organic acids and solubilize phosphate was higher in reference seeds than in those collected from plants growing on mine residues. Isolates able to fix N were observed only in seeds from the reference site. The opposite was observed for phytate mineralization: there were no positive results for isolates from reference seeds, while 30% of strains from mine residues seeds showed positive for this trait. The amount of strains producing ACC-deaminase and siderophores was higher in mine residue seed endophytes than in those from reference seeds (Table 1). The percentages of isolates producing acetoin were similar for both conditions.

Comparing only endophytes of the three consecutive generations of seeds collected from mine residue plants, the percentages of acetoin producing isolates were similar in 2011 and 2012, but in 2013 the amount was higher. Similar observations were done for ACC-deaminase activity. The highest fractions of isolates able to mineralize phytate and to produce siderophores were detected in 2011; in the next two generations these percentages were lower.

The majority of reference seeds isolates were able to solubilize phosphate, produce IAA and organic acids. In the case of endophytes from mine residues seeds the most general functional characteristic was ACC-deaminase activity, followed by phosphate solubilization. The same characteristics were observed for seed endophytes among the different seed generations from mine residues (Table 1).

PTEs tolerance of cultivable endophytic bacteria

In general bacteria from mine residues seeds were more tolerant to Zn, Cd, Cu, Ni and Pb than those from reference seeds (Table 2). Ten isolates from mine residues seeds were tolerant to 5 mM Zn, while only one strain from reference seeds showed tolerant to this concentration. For Cd and Cu, isolates from both conditions, mine residue and reference seeds, grew at the lowest concentration (0.4 mM). However, at higher concentrations strains from reference seeds did not grow. Some isolates from mine residue seeds were tolerant to 2 mM Cd or Cu. *Variovorax* sp. Cp13, *Variovorax* sp. Cp5 and *Methylobacterium* sp. Cp3, the first two were isolated from 2013 seeds and were tolerant to all tested PTEs concentrations. *Methylobacterium* sp. Cp3 was tolerant to 5 mM Zn and 1.6 mM Cd, and it was observed in the three seed generations collected from mine residues (Table 2). Considering the three consecutive seed generations collected from mine residues the results were variable, but endophytes from the youngest seeds (2013) were the most tolerant to Cu and Ni (Table 2). We observed that some genera like *Staphylococcus*¸ *Mycobacterium and Microbacterium* did not show any PTE tolerance.

Metabolic fingerprint of total endophytic community

According to the ANOVA and Tukey tests, there was no significant difference in any metabolic fingerprint index (H, substrate richness and evenness, and AWCD) between both mine residues and non-contaminated site (Table S2). However, principal component analysis indicated some trends. The plot in Figure 3 shows that the first two components accounted for most of the variance (75%), seed endophytic communities from the three generations collected from mine residues were separated from control seeds. Thus, C sources were determinant to differentiate endophytic communities at both conditions. Seed endophytes from 2011 and 2012 showed a similar behavior; however, components of 2013 endophytes were more variable and could form a

different group. Statistical analysis indicated that for the seed endophytes of 2013 substrate richness was the highest of the three generations investigated, but no significant differences were detected for H or substrate evenness (Table S2).

Discussion

Cultivable seed endophytic community: mine residues vs non-contaminated site

Some earlier studies mentioned that under natural conditions most of flowers and fruits did not harbor any cultivable endophytic bacteria or only very low amounts (Hallmann, 2001; Lopez, Bashan, and Bacilio 2011; Puente, Li and Bashan 2009a). However, there exist several reports concerning isolation of seed endophytic bacteria from different plant species, reviewed in Truyens *et al*. (2014b). Studies about seed endophytes and PTEs exposure are scarce, but Truyens *et al*. (2013) reported $37x10^6$ cfu g^{-1} of *Arabidopsis thaliana* seeds exposed to Cd during 8 generations. In the present work the quantity of cfu was lower, seed endophytes were found from 8.7 to $22.5x10^3$ cfu g^{-1} in seeds collected from plants growing on mine residues, and $18.6x10^3$ cfu g^{-1} in reference seeds from a non-contaminated area (Table 1).

It should be pointed out that, although no formal identification of bacteria based on 16S rRNA gene sequence is accepted, these data provide good indications concerning the phylogeny (Moore *et al*. 2006). According to ARDRA results there were differences between cultivable endophytes of seeds from mine residues and the non-contaminated site. The diversity of endophytes was higher in the seeds from the mine residues, than in reference seeds (Figure 1). However, it should be mentioned that from mine residues three consecutive generations of seeds were studied and only seeds of one year from the non-contaminated site. Truyens *et al*. (2014b) mentioned that among the most common genera reported as seed endophytes of different plant species are

Bacillus and *Staphylococcus*; both genera were also isolated from *C. pumila* seeds at both sampling sites, PTEs contaminated and non-contaminated (Figure 1). More different strains of *Bacillus* were observed in seeds from the non-contaminated site, while *Staphylococcus* strains were more numerous in mine residue seeds (Figure 1). However, other genera were dominant in *C. pumila* seeds: *Sphingomonas* and *Brachybacterium* predominated in the seed endophytic community from mine residues, whereas *Curtobacterium* were the most abundant isolates from reference seeds. *Sphingomonas* has been reported as a seed endophyte of *Eucalyptus* sp. (Ferreira *et al.* 2008); *Zea* spp. (Johnston-Monje and Raizada, 2011), *Oryza sativa* (Mano *et al*. 2006; Kaga *et al*. 2009), *Phaseolus vulgaris* (López-López *et al*. 2010), *Arabidopsis thaliana* exposed to Cd (Truyens *et al*. 2013) and in the Cd-hyperaccumulator *Solanum nigrum* (Chen *et al.* 2014). *Brachybacterium* and *Curtobacterium* have been isolated from bean (López-López *et al*. 2010) and rice seeds (Mano *et al.* 2006; Hardoim *et al*. 2012), respectively.

When considering only endophytes from plants growing under PTEs exposure the most common genera reported in earlier studies are *Bacillus*, *Pseudomonas* and *Microbacterium* (Barzanti *et al*. 2007; Sheng *et al*. 2008; Sun *et al*. 2010; Chen *et al*. 2014; Truyens *et al*. 2013, 2014a). These bacteria were mainly isolated from roots and stems. It is remarkable that these three bacterial genera were also isolated from Ni- (Barzanti *et al*. 2007) and Cd-hyperaccumulating plant species (Chen *et al.* 2014). However, in the present work, most of the *Bacillus* strains and the only *Microbacterium* in *C. pumila* were isolated from reference seeds.

The composition of the seed endophytic community of both seed types, from mine residues and a non-contaminated site, is different. Nevertheless, there was one *Sphingomonas* strain present in both seeds types (Figure 1). Croes *et al*. (2013) concluded that plants of the same

species but growing on different sites contained similar obligate endophytes possibly derived from a common seed endophytic community.

The presence of contaminants should affect the composition, as well as functions and PTEs tolerance of bacterial endophytes (Siciliano, Fortin, and Mihoc 2001; Truyens *et al*. 2014a). In the present work, except for acetoin and IAA production, it was possible to observe some trends that may be contaminant-dependent effects on the functional characteristics of seed endophytes. Additionally, metabolic fingerprinting of the total endophytic community (Figure 2) suggested differences in metabolic activity differences as a consequence of PTEs exposure.

In agreement with the results obtained by Truyens *et al*. (2013), production of organic acids and N fixation seem to be more important for endophytes of seeds that were not exposed to PTEs (Table 1). Phosphate solubilization appears a common trait for most cultivable seed endophytes from both mine residues and non-contaminated soils (Table 1). Phosphorus indeed often is a limiting growth factor for terrestrial plants and by consequence phosphate solubilizing bacteria are common in rhizosphere environments (Rajkumar, Ae, and Freitas 2009). However, it has been reported that endophytic isolates also solubilize phosphates, suggesting that endophytic bacteria can enhance phosphate availability to the host plant (Kuklinsky-Sobral *et al*. 2004; Puente, Li and Bashan 2009b; Johnston-Monje and Raizada 2011). Additionally, since phytate is the most abundant stock of phosphorus in seeds (López-López *et al*. 2010) phytate mineralizing endophytic bacteria found in mine residue seeds (Table 1) might be important for mineral nutrition during germination and early stages of seedling development.

PTEs tolerant endophytes can be equipped with diverse mechanisms that can decrease PTEs availability inside plants and thus lower phytotoxicity (Lodewyckx *et al*. 2002; Weyens *et al*. 2009a). We observed that in comparison to those from the non-contaminated site higher

percentages of endophytes from mine residue seeds were able to produce siderophores (Table 1). Endophytic bacteria equipped with this trait can assist their host to improve nutrient acquisition and reduce PTEs toxicity (Rajkumar *et al*. 2009). Another functional trait connected with alleviation of PTEs toxicity is ACC-deaminase activity. This characteristic was the most predominantly observed in seed endophytes of *C. pumila* growing on mine residues (Table 1). Increased ethylene production in response to PTEs stress was reported for several plant species (Mertens *et al*. 1999; Schellingen *et al*. 2014). It is known that an increasing ethylene production can inhibit plant development and accelerate senescence and abscission processes (Vandenbussche *et al*. 2012). Bacterial ACC deaminase can decrease ethylene levels in plants by metabolizing its precursor ACC into ketobutyric acid and ammonia (Glick, 2005). Consequently, plants associated with bacteria that are equipped with ACC deaminase activity can regulate their ethylene levels and thus show improved growth and development under stress conditions (Arshad, Saleem, and Hussain 2007; Hardoim *et al*. 2008) such as exposure to toxic concentrations of PTEs. ACC-deaminase activity and high PTEs tolerance are reported as characteristics of endophytes of plants in contact with PTEs (Arshad *et al*. 2007; Mastretta *et al*. 2009; Ma *et al*. 2011; Xianxian *et al*. 2011; Truyens *et al*. 2013; Croes *et al*. 2013; Janssen *et al*. 2015). In the present research endophytic bacteria tolerant to PTEs were isolated from seeds of *C. pumila* growing on mine residue; *Variovorax* sp. Cp29 and *Variovorax* sp. Cp30 were tolerant to the highest concentrations of Zn (5 mM), Cd (1.6 mM), Cu (1.6 mM) and Ni (5 mM); while *Methylobacterium* sp. Cp3 and *Sphingomonas* sp. Cp9 tolerated the highest Zn and Cd concentration (Table 2). All these isolates, except *Sphingomonas* sp. Cp9, presented ACC-deaminase activity too (Table 1). The mentioned isolates tolerated Zn, Cd and Cu concentrations higher than previously reported for endophytes of seeds from PTEs exposed tobacco and

Arabidopsis, 0.6 mM Zn, 1.5 mM Cd, and 0.4 mM Cu (Mastretta *et al*. 2009; Truyens *et al*. 2013, 2014a). They tolerated concentrations of Zn and Cd similar to those reported for shoot and root endophytes of willow growing on a PTEs-contaminated field (Weyens *et al.* 2013).

Endophytic communities in consecutive generations of seeds

Despite the fact that the composition of endophytic bacterial communities seems to be unpredictable (Hardoim *et al*. 2012), there exists evidence for the vertical transmission of bacterial endophytes in different plant species (van Oevelen *et al*. 2003; Johnston-Monje and Raizada 2011; van Overbeek *et al*. 2011; Hardoim *et al*. 2012; Croes *et al*. 2013). Our results revealed some variations in the cultivable endophytic community of three consecutive generations of seeds of *C. pumila* growing on mine residues (Figure 1).

Variations in density and composition of endophytic communities can be attributed to plant genotype, plant age, tissue type, capacity of colonization, season of sampling, and soil type (Zinniel *et al*. 2002; Conn and Franco 2004; Kuklinsky-Sobral *et al*. 2004; Hardoim *et al*. 2012; Rosenblueth *et al.* 2012). Despite of the aforementioned variations two strains of *Methylobacterium* and one of *Sphingomonas* that were common in the three consecutive generations of *C. pumila* seeds and moreover, they showed the same functional characteristics (Figure 1, Table 1).

Strains that are transmitted from one generation to the next might contribute to the establishment of the new generations of plants under limiting or stressful conditions (Truyens *et al*. 2013, 2014a), and they continue providing benefits to their host during plant development (Liu *et al*. 2012). Green (2006) mentioned that the genus *Methylobacterium* exhibits resistance to several stressful conditions. It also already has been reported as endophyte in plants exposed to PTEs (Truyens *et al*. 2013; Chen *et al*. 2014). *Methylobacterium* spp. were found to be dominant

as endophytic colonizers of the metallophyte *Thlaspi goesingense* (Idris *et al*. 2004), and have been reported to be PTEs tolerant (Dourado *et al*. 2012). In our study on seed endophytes of *C. pumila*, *Methylobacterium* sp. Cp3 showed tolerant to Zn and Cd (Table 2), it produced IAA and organic acids, could solubilize phosphate and possessed ACC-deaminase activity (Table 1). However, *Methylobacterium* sp. Cp2 was not PTEs tolerant and had no any positive result for the other functional traits tested. *Sphingomonas* sp. Cp1 was found in the three seed generations of *C. pumila* growing on mine residues (Figure 1). *Sphingomonas* has been reported as stem and root endophyte of other plant species exposed to PTEs (Sun *et al*. 2010; Truyens *et al*. 2013; Chen *et al*. 2014). In the present study, *Sphingomonas* sp. Cp1 could produce IAA, solubilized phosphate and had ACC-deaminase activity (Table 1). However, it did not show PTE tolerance.

The similarity of the seed endophytic communities among consecutive seed generations collected on the mine residues was not only regarding functional traits. Metabolic fingerprinting of the total endophytic communities suggested that the communities among the consecutive seeds generations are also physiologically similar (Figure 2). However, seeds collected in 2013 could be grouped separately. This might be due to the fact that these seeds were the youngest at them moment of the study. Cankar *et al*. (2005) and Mastretta (2007) also found a decreasing diversity with increasing storage time in seeds of *Picea abies* and *Nicotiana tabacum*, respectively. Further, as mentioned previously, in general the endophytic community changes in function of the age of tissues from which endophytes are isolated.

Cultivable seed endophytes of *C. pumila* were found at low densities, but it seems that these low numbers suffice for further vertical transmission from one generation to the next. Most seeds spend part of their life in soil, thus seed endophytes may play a role in diminishing decay of seed and preparing the surrounding soil environment for germination. The conserved endophyte

functional traits that we observed may reflect needs of plant seeds during germination and establishment (Johnston-Monje and Raizada 2011; Truyens *et al.* 2014). In this work functional traits common among endophytes of consecutive seed generations (ACC-deaminase activity and phosphate solubilization) could be an indicator of selection of endophytic bacteria by plants growing on PTEs contaminated substrates. Undoubtedly, improving plant nutrition by solubilization of phosphate and reducing levels of the stress hormone ethylene are, if not essential, very important factors for plant establishment in limiting or stressful conditions. These traits together with PTEs tolerance and the functional characteristics of other seed endophyte isolates may explain the success of *C. pumila* to germinate and to complete its life cycle on a multi-PTE contaminated substrate, tolerating high PTEs concentrations and the inherent environmental restrictions of a semi-arid climate.

We must mention that here we only investigated the cultivable seed endophytic bacteria. Undoubtedly also non-cultivable bacteria and other endophytes, *e.g*. fungi, contribute to plant survival in adverse conditions. For a better understanding of endophytes and their relation with plants in PTEs contaminated environments, the use of cultivation independent techniques is currently being performed. This knowledge will allow a better selection and application of bacteria that can be used to improve or optimize phytoremediation of PTEs contaminated soils, not only by phytoextraction, also promoting a plant cover establishment that diminish dispersion of PTEs. Future work should also explore interactions between endophytes.

Conclusions

In this study the composition and functions of the cultivable seed endophytic community of *C. pumila* were characterized in three consecutive generations of seeds collected from PTEs-

containing mine residues and they were compared to those isolated from reference seeds harvested on a non-contaminated site. The community composition, functional traits and metabolic fingerprint of mine residue seed endophytes were different from reference seed endophytes. *Sphingomonas* was the dominant genus in mine residues seeds, while *Curtobacterium* predominated in control seeds. Phytate mineralization, ACC-deaminase activity and PTEs tolerance were the main functional traits of mine residues seed isolates, suggesting that such endophytic community is adapted to the restrictive environmental conditions observed in mine residues. When comparing cultivable endophytes among consecutive generations of seeds collected in mine residues there were three strains common in through generations: *Methylobacterium* sp. Cp2, *Methylobacterium* sp. Cp3 and *Sphingomonas* sp. Cp1. Except the first one, the other two isolates were able to solubilize phosphate, they possessed ACC-deaminase activity and were PTEs tolerant, and all of them have functional traits that might be useful for their host plant in adverse environments. Similar metabolic fingerprints among the total seed endophytic community in three consecutive generations was observed. These results suggest that vertical transmission of seed endophytes is not a random process but that the selected seed endophytes possess functional traits that are beneficial during germination and further growth of *C. pumila* in the adverse growth environment of mine residues.

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Supplemental material

Supporting information shows the outline of surface sterilization protocols tested for *C. pumila* seeds, relative abundance of genera of bacterial isolates and metabolic index of seed endophytes.

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Table 1. Colonies forming units (cfu) and functional characteristics of *Crotalaria pumila* seed endophyte isolates.

Seed	Isolate	cfu g^{-1} seed	*H*		IAA	Sid	N_2	PO_4	Act	Pht	OA	ACC
MR:11/12/13	*Sphingomonas* sp. Cp1	$1.5x10^3/3x10^2/4x10^2$			+	-	-	+	-	-	-	+
	Methylobacterium sp. Cp2	$8x10^1/2x10^2/2x10^3$			-	-	-	-	-	-	-	-
	Methylobacterium sp. Cp3	$2x10^2/1x10^3/3x10^3$			+	-	-	+	-	-	+	+
MR:11/12	*Sphingomonas* sp. Cp4	$1.3x10^2/1.7x10^4$			-	+	-	+	+	-	-	+
	Variovorax sp. Cp5	$1x10^2/2.2x10^2$			-	+	-	+	-	-	-	+
MR:11/13	*Staphylococcus* sp. Cp6	$5.3x10^2/9.3x10^2$			-	-	-	+	+	-	+	+
MR:12/13	*Sphingomonas* sp. Cp7	$3.1x10^2/2.2x10^2$			+	-	-	+	-	-	-	+
MR:11	*Sphingomonas* sp. Cp8	$3.3x10^3$			+	-	-	-	+	+	-	+
	Sphingomonas sp. Cp9	$3.1x10^2$			-	+	-	-	-	+	-	-
	Staphylococcus sp. Cp10	$1.5x10^3$			+	-	-	+	+	-	-	+
	Staphylococcus sp. Cp11*				-	-	-	+	-	+	+	+
	Staphylococcus sp. Cp12*				+	+	-	-	-	-	+	-
	Variovorax sp. Cp13	$0.4x10^2$			-	-	-	-	-	+	+	+
	Curtobacterium sp. Cp14	$1x10^3$			-	-	-	-	-	-	+	+
	Kocuria sp. Cp15*				+	+	-	+	-	+	-	+
		Total $8.7x10^3$	1.96	%	43	36	0	57	29	36	43	79
MR:12	*Sphingomonas* sp. Cp16	$1.5x10^3$			-	-	-	-	-	-	-	-
	Mycobacterium sp. Cp17	$5x10^2$			-	+	-	+	-	+	-	+
	Streptomyces sp. Cp18	$5x10^2$			-	-	-	+	+	+	-	+
	Bacillus sp. 1 Cp19*				-	-	-	-	-	-	+	+
	Arthrobacter sp. Cp20*				+	+	-	+	+	-	+	+
		Total $22.5x10^3$	1.65	%	36	36	0	73	27	18	27	82
MR:13	*Sphingomonas* sp. Cp21	$4x10^2$			-	-	-	+	+	-	-	+
	Sphingomonas sp. Cp22	$2.2x10^2$			+	-	-	-	+	-	-	+
	Sphingomonas sp. Cp23	$6.4x10^3$			+	+	-	-	-	-	+	+
	Staphylococcus sp. Cp24	$4x10^2$			-	+	-	+	+	-	-	-
	Brachybacterium sp. Cp25	$7.1x10^2$			-	-	-	+	-	-	-	+
	Brachybacterium sp. Cp26	$1x10^3$			-	-	-	-	+	-	+	+
	Brachybacterium sp. Cp27	$2.2x10^2$			-	-	-	+	+	+	+	+
	Brachybacterium sp. Cp28	$2.2x10^2$			-	-	-	+	+	-	-	+
	Variovorax sp. Cp29	$1.2x10^3$			-	+	-	+	+	-	-	+
	Variovorax sp. Cp30	$4x10^2$			-	+	-	+	+	+	+	+
	Burkholderia sp. Cp31	$0.2x10^2$			+	+	-	+	-	+	-	+
	Caulobacter sp. Cp32	$0.2x10^2$			+	-	-	-	+	-	-	+
		Total $19x10^3$	2.25	%	44	28	0	67	61	17	33	89
MR:13/R	*Sphingomonas* sp. Cp33	$7.1x10^2/2.5x10^3$			+	-	-	+	+	-	-	+
R	*Sphingomonas* sp. Cp34	$4.9x10^3$			-	-	-	+	-	-	-	+
	Staphylococcus sp. Cp35	$1x10^3$			-	-	-	+	-	-	+	+
	Brachybacterium sp. Cp36	$1.8x10^3$			-	-	-	-	-	-	+	+
	Curtobacterium sp. Cp37	$2.2x10^2$			+	-	+	-	-	-	+	+
	Curtobacterium sp. Cp38	$2.9X10^3$			-	-	-	+	+	-	+	+
	Curtobacterium sp. Cp39	$1.3x10^3$			+	+	-	+	-	-	+	+
	Bacillus sp. Cp40	$5.3x10^3$			+	-	-	+	+	-	+	+
	Bacillus sp. Cp41	$4x10^2$			+	-	-	+	-	-	+	+
	Microbacterium sp. Cp42	$6.2x10^2$			-	-	+	+	+	-	+	+
	Sacchatothrix sp. Cp43	$0.12x10^2$			-	-	-	-	+	-	-	-
		Total $18.6x10^3$	1.93	%	45	9	18	73	45	0	73	55

Mine residue seeds (MR) collected in 2011(11), 2012(12) and 2013(13); Reference seeds (R); *bacteria isolated from liquid culture; H: Shannon index; %: percentage of isolates in each generation that have certain functional trait (taking into account the total amount of isolates in each generation); production of Indol Acetic Acid (IAA), siderophores (Sid); nitrogen fixation (N_2); phosphate solubilization (PO_4); acetoin production (Act); phytate mineralisation (Pht); organic acids production (OA); 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity.

Table 2. PTEs tolerance in *Crotalaria pumila* seed endophyte isolates.

Seed	Isolate	Zn (mM)				Cd (mM)			Cu (mM)			Ni (mM)			Pb (mM)
		0.6	1.0	2.5	5.0	0.4	0.8	2.0	0.4	0.8	1.6	1.0	3.0	5.0	0.5
MR:11/12/13	*Sphingomonas* sp. Cp1	−	−	−	−	−	−	−	+	−	−	+	−	−	+
	Methylobacterium sp. Cp2	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Methylobacterium sp. Cp3	+	+	+	+	+	+	+	+	−	−	+	+	−	+
MR:11/12	*Sphingomonas* sp. Cp4	+	+	+	+	+	+	−	+	+	−	+	+	+	+
	Variovorax sp. Cp5	+	+	+	+	+	+	−	+	+	−	+	+	−	+
MR:11/13	*Staphylococcus* sp. Cp6	+	−	−	−	−	−	−	+	−	−	+	−	−	−
MR:12/13	*Sphingomonas* sp. Cp7	+	+	+	−	+	+	−	+	−	−	−	−	−	+
MR:11	*Sphingomonas* sp. Cp8	+	−	−	−	−	−	−	−	−	−	−	−	−	−
	Sphingomonas sp. Cp9	+	+	+	+	+	+	+	−	−	−	+	+	−	+
	Staphylococcus sp. Cp10	+	−	−	−	−	−	−	−	−	−	−	−	−	−
	Staphylococcus sp. Cp11	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Staphylococcus sp. Cp12	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Variovorax sp. Cp13	−	−	−	−	−	−	−	−	−	−	−	−	−	+
	Curtobacterium sp. Cp14	+	+	−	−	−	−	−	+	−	−	+	−	−	−
	Kocuria sp. Cp15	+	−	−	−	+	−	−	+	−	−	+	−	−	+
	%	64	36	29	29	36	29	14	50	14	0	57	29	7	50
MR:12	*Sphingomonas* sp. Cp16	+	−	−	−	+	−	−	+	+	−	+	−	−	+
	Mycobacterium sp. Cp17	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Streptomyces sp. Cp18	−	−	−	−	−	−	−	−	−	−	+	−	−	−
	Bacillus sp. Cp19	+	+	+	−	+	−	−	+	−	−	+	−	−	−
	Arthrobacter sp. Cp20	+	+	−	−	+	+	−	+	+	−	−	−	−	+
	%	67	56	44	33	67	56	11	78	44	0	67	33	11	78
MR:13	*Sphingomonas* sp. Cp21	+	+	+	+	−	−	−	+	−	−	+	+	+	+
	Sphingomonas sp. Cp22	+	+	+	+	−	−	−	−	−	−	+	−	−	+
	Sphingomonas sp. Cp23	+	+	+	−	+	+	−	−	−	−	+	−	−	−
	Staphylococcus sp. Cp24	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Brachyobacterium sp. Cp25	+	−	−	−	+	−	−	−	−	−	−	−	−	−
	Brachyobacterium sp. Cp26	+	−	−	−	−	−	−	−	−	−	−	−	−	+
	Brachyobacterium sp. Cp27	+	+	+	+	+	−	−	+	−	−	+	−	−	+
	Brachyobacterium sp. Cp28	+	+	+	+	+	−	−	+	−	−	+	+	+	+
	Variovorax sp. Cp29	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Variovorax sp. Cp30	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Burkholderia sp. Cp31	−	−	−	−	−	−	−	+	−	−	+	+	+	+
	Caulobacter sp. Cp32	+	+	+	−	+	−	−	+	+	−	+	−	−	+
	%	75	55	55	35	55	25	15	60	15	10	65	30	25	60
MR:13/R	*Sphingomonas* sp. Cp33	+	−	−	−	+	−	−	−	−	−	−	−	−	−
R	*Sphingomonas* sp. Cp34	+	+	+	+	+	−	−	+	−	−	+	+	+	+
	Staphylococcus sp. Cp35	−	−	−	−	−	−	−	−	−	−	+	−	−	−
	Brachyobacterium sp. Cp36	+	−	−	−	−	−	−	−	−	−	+	−	−	−
	Curtobacterium sp. Cp37	+	−	−	−	+	−	−	+	−	−	+	−	−	+
	Curtobacterium sp. Cp38	+	+	−	−	+	−	−	+	−	−	+	−	−	+
	Curtobacterium sp. Cp39	+	−	−	−	+	−	−	−	−	−	−	−	−	−
	Bacillus sp. Cp40	+	+	−	−	−	−	−	+	−	−	+	−	−	−
	Bacillus sp. Cp41	+	+	−	−	−	−	−	−	−	−	−	−	−	−
	Microbacterium sp. Cp42	−	−	−	−	−	−	−	−	−	−	+	−	−	−
	Sacchatothrix sp. Cp43	+	−	−	−	−	−	−	−	−	−	+	−	−	−
	%	82	36	9	9	45	0	0	36	0	0	73	9	9	9

Mine residue seeds (MR) collected in 2011(11), 2012(12) and 2013(13); Reference seeds (R); +: growth compared to non-PTEs supplemented control cultures; -: no growth; %: percentage of isolates in each generation that are tolerant to certain PTE concentration (taking into account the total amount of isolates in each generation).

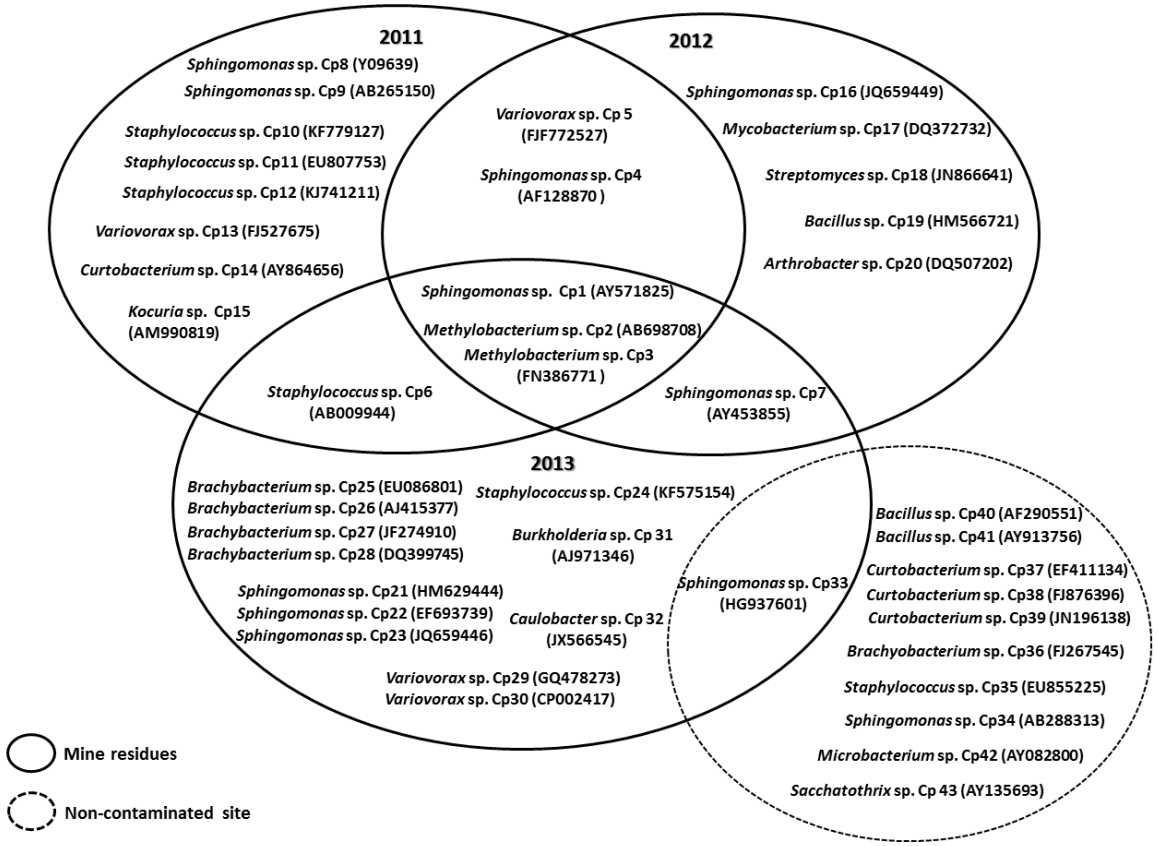

Figure 1. Composition of endophytic community in consecutive generations of *Crotalaria pumila* seeds collected in mine residues and non-contaminated site. Accession number in parenthesis.

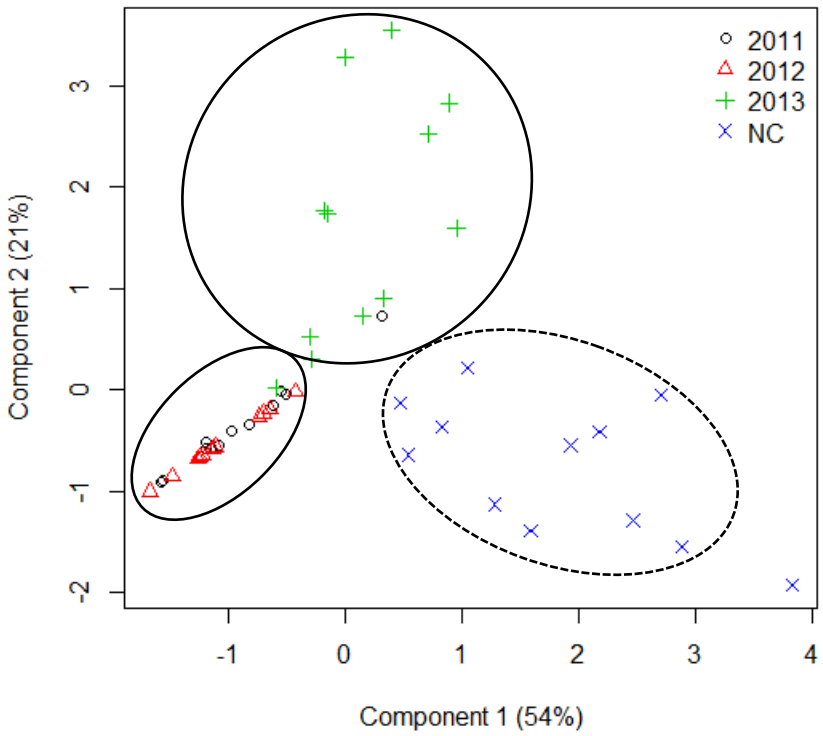

Figure 2. Plot of principal component analysis of Biolog® NG2 Microplates in total seed endophytic communities of three consecutive generations of *Crotalaria pumila* seeds (2011, 2012, 2013) and in non-contaminated site (NC) (*n*=12). Continuous line is grouping seed endophytes from mine residues and dotted line those from non-contaminated site.

Table S1. Outline of surface sterilization protocols tested for *Crotalaria pumila* seeds.

	NaClO		Morphologically distinct bacteria	cfu g^{-1} seed
70% ethanol	%	time		
3 min	1	1 min	0	-
3 min	1	30 s	0	-
3 min	0.1	1 min	1	29
3 min	0.1	1 min	1	58
1 min	0.1	1 min	2	67
1 min	0.1	30 s	2	98
30 s	0.1	1 min	3	199
30 s	0.1	30 s	4	284
No	1	30 s	2	430
No	0.1	3 min	3	710
No	0.1	1 min	2	540
No	0.1	30 s	6	8470
No	0.1	10 s	42	17223

Table S2. Metabolic index of *Crotalaria pumila* seed endophytes.

Seed	**Shannon's diversity Index**	**Substrate richness**	**Substrate evenness**	**AWCD**
Control seeds	*2.44*	*5.42*	*3.42*	*0.75*
Mine residues (3 generations)	*2.42*^{ns}	*3.67*^{ns}	*5.12*^{ns}	*1.19*^{ns}
2011	2.54 a	2.25 b	5.86 a	1.24 ab
2012	2.33 a	3.33 ab	5.39 a	1.54 a
2013	2.38 a	5.42 a	4.10 a	0.80 b

AWCD: average well colour development; ^{ns}: no significant differences between mine residues and control seeds (italic). Means with the same letter in each indicates not significant statistical difference among three generations (2011, 2012, 2013) of seeds collected in mine residues according to Tukey test (p<0.01).

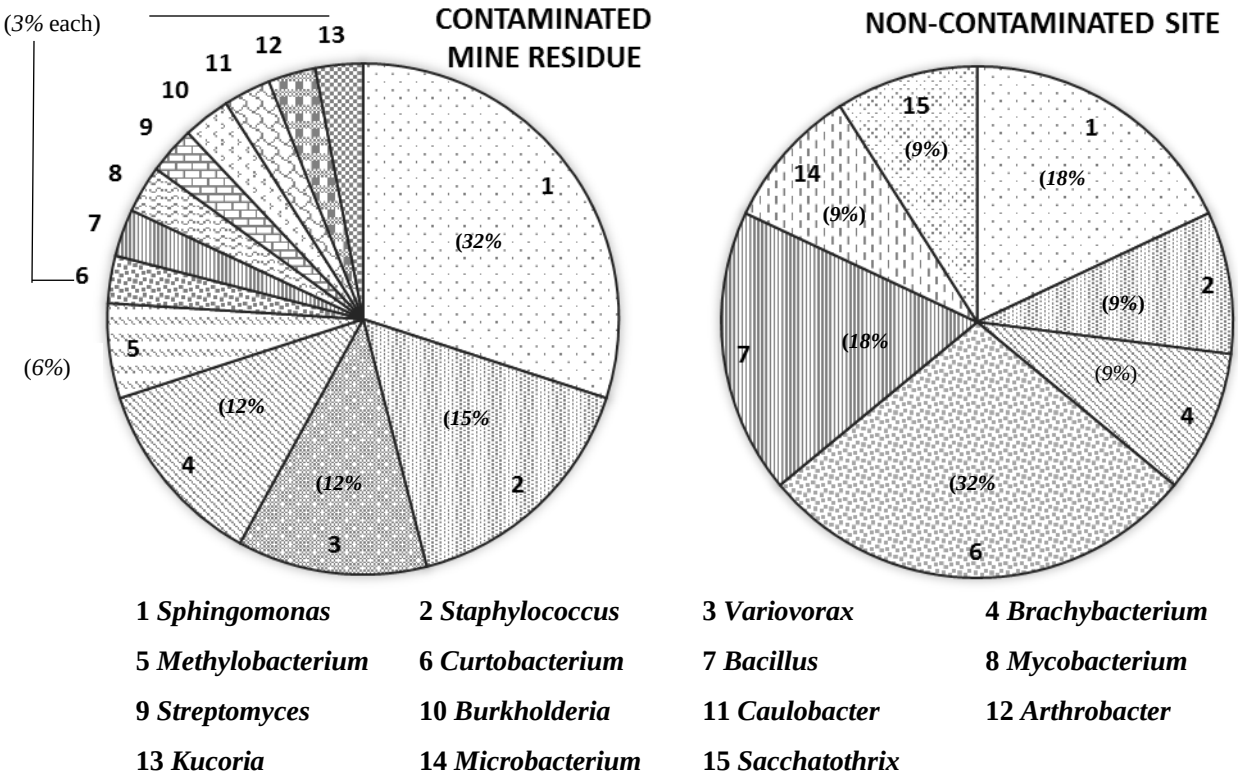

Figure S1. Genera of endophytic bacteria isolated from *Crotalaria pumila* seeds collected from mine residues and non-contaminated site.

CHAPTER 5. SEED ENDOPHYTIC BACTERIA FROM *Crotalaria pumila* AND THEIR POTENTIAL TO PROMOTE REMEDIATION OF SOILS CONTAMINATED BY POTENTIALLY TOXIC ELEMENTS

Abstract

Among a collection of seed endophytic bacteria isolated from *Crotalaria pumila* Ort. growing on potentially toxic elements (PTEs) containing mine residues, ten strains were selected based on their *in vitro* plant growth promoting characteristics and PTEs tolerance. Their capability to promote plant establishment and growth on PTEs-containing soil was tested on two batches of *C. pumila* seeds, one batch collected from plants growing on mine residues and the other one from a non-contaminated site. *Crotalaria pumila* plants from the different seed batches responded differently to seed endophyte inoculation. Plants from mine residue seeds showed a higher survival rate and PTEs accumulation than reference seed plants. *Methylobacterium* sp. triggered the highest survival rate and *Variovorax* consortium the highest biomass production. Inoculation with *Sphingomonas* sp. had negative effects on plant biomass production. All inocula except *Bacillus* sp. diminished PTEs translocation compared to non-inoculated plants. Results suggested differential responses to endophyte inoculation in the same plant host species originating from different environmental conditions. *Crotalaria pumila* and its seed endophytes can improve revegetation of PTEs-contaminated sites through phytostabilization.

Key words: phytoremediation, *Methylobacterium*, *Variovorax*, plant growth promoting bacteria

Introduction

The extensive and continuous industrialization has led to environmental problems; due to the hazard of dispersion affecting public health and risk of contaminants' introduction to food chains, potentially toxic elements (PTEs) contamination of soils is one of the major concerns (Rajaganapathy *et al*. 2011). Therefore, the development and application of sustainable remediation strategies, like phytoremediation is imperative. However, effective *in situ* phytoremediation comprises a number of difficulties, such as toxic levels of contaminants and other adverse environmental conditions for plants leading to slow growth and low biomass production (Vangronsveld *et al*. 2009; Mench *et al*. 2010). Synergistic use of plants and their associated microbes, especially bacteria, has been investigated to overcome these problems (Rajkumar, Ae, and Freitas 2009; Weyens *et al*. 2009). Plant associated bacteria can improve plant establishment and further growth (Rajkumar *et al*. 2009). Bacteria may also enhance PTEs phytoextraction (Sheng *et al.* 2008) or promote phytostabilization by reducing PTEs availability in rhizosphere (Ma *et al*. 2011). Endophytic bacteria possess plant growth promoting mechanisms: mobilization of unavailable nutrients, such as phosphorus, through production of organic acids or synthesis of phytohormones (Hardoim, van Overbeek, and van Elsas 2008) and other plant-growth promoting substances, for instance acetoin (Bailly and Weisskopf 2012). Additional traits present in endophytic bacteria that are helpful when plants are exposed to PTEs are 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity and PTEs tolerance. ACC deaminase plays an important role in alleviation of stress induced by PTEs (Arshad, Saleem, and Hussain 2007). There are different mechanisms involved in PTEs tolerance of endophytes, which can affect the solubility and availability of PTEs to plants, and certainly modify PTEs uptake by plants and by consequence possible toxic effects (Rajkumar *et al*. 2009). But, scarce information

about the difference between endophytic bacteria isolated from plants exposed to multi PTE polluted environment and those from non-polluted soils has been reported. Therefore a simple question remains until the present study: have endophytic bacteria isolated from anywhere the same effect on plants?

Crotalaria pumila has been observed growing on mine residues in a semi-arid region in Mexico. This plant species, which is able to complete its life cycle on multi-PTE highly contaminated mine residues, is a good candidate for phytostabilization of Cd, Pb and Zn contaminated substrates (Sánchez-López *et al.* 2015). Previously, seed endophytic bacteria were isolated from *C. pumila* and characterized according to their plant growth promoting traits and PTEs tolerance. Among these bacteria, seven strains were selected to be tested in an experiment using a Zn and Cd contaminated substrate. The objectives of this work were: i) to investigate the possible beneficial effects that endophytic bacteria isolated from *C. pumila* seeds growing on mine residues bring to their host in the presence of PTEs; ii) to test whether seed endophytic bacteria keep their plant growth promotion and PTEs tolerance traits in a seed batch of *C. pumila* seeds originating from a non-contaminated soil; iii) to select strain(s) which might be useful to improve efficiency of phytostabilization of PTEs contaminated soils.

Materials and Methods

Plant material

In 2013 seeds were collected within pods from *Crotalaria pumila* plants colonizing two different locations: a non-contaminated reference site and a mine residue containing high PTEs concentrations (Sánchez-López *et al.* 2015). To increase and homogenize germination rate, seeds were soaked in H_2SO_4 during 30 minutes as mentioned by Linding-Cisneros and Lara-Cabrera

(2004) and rinsed 4x with distilled water. Afterwards, the following surface sterilization protocol was applied: 1 min in ethanol 70%, 1 min in NaClO 0.1%, 8x rinses with sterile distilled water. To verify the surface sterilization protocol effectiveness 100 µL of the last rinse water were plated in 869 1/10 diluted medium (Mergeay *et al.* 1985) and incubated one week at 30° C in darkness.

Surface sterile seeds were put in sterile Petri dishes at 27° C in the darkness. An aliquot of a bacterial inoculum (5 mL) was added to the seeds in the Petri dishes. Seeds were kept in such conditions during 72 h, until the radicle had completely developed; then they were transplanted to 1 L pots containing Zn-Cd-contaminated soil.

Bacterial inoculum

Ten bacterial strains were selected from a collection of *C. pumila* seed endophytes previously identified and characterized (data not published yet). Five strains were inoculated individually and 2 bacterial consortia were prepared. Strain selection was done based on their plant growth promotion characteristics and PTEs tolerance formerly tested (Table 1). The strains were grown at 30 °C in 869 liquid medium on a rotary shaker (Mergeay *et al.* 1985). Cells were harvested after approximately 12 h, subsequently washed by centrifugation with sterile distilled water and resuspended at a density of 10^8 colony forming units (cfu) mL^{-1}. Bacterial consortia were prepared by mixing individual cultures in equal parts and adjusted to the aforementioned density. Prior to preparing the bacterial consortia, the compatibility of the strains was tested by plating them in a Petri dish containing 869 1/10 diluted medium. Individual strains or combinations were used to inoculate the surface sterilized *C. pumila* seeds. Subsequent inoculations were done at 10 and 25 days after transplantation.

Plant growth conditions

The soil was collected from a contaminated field in Lommel, Belgium. Soil pH was 6.6, electrical conductivity 247 µS cm^{-1}. Total PTEs concentrations were 6.9 mg Cd kg^{-1}, 217 mg Pb kg^{-1} and 429 mg Zn kg^{-1}. $Ca(NO_3)_2$ extractable PTEs concentrations were 0.43 mg Cd kg^{-1}, 0.3 mg Pb kg^{-1} and 21.3 mg Zn kg^{-1}. Prior to use, soil was sieved (2 mm). Pots were kept in a greenhouse for 2 months at 25 °C, 8:16 day-night period. Pots were irrigated twice per week with distilled water.

Variables

Plant survival percentage. Six inoculated seedlings were transplanted into pots filled with 750 g of PTEs contaminated soil. After 10 days the number of alive plants was counted and the percentage of survival was calculated. Then, if necessary, plants were removed to obtain a final amount of two plants per pot.

Biomass production. After 2 months all plants were harvested and shoot and root fresh weight was determined. Samples were first washed with a P-free detergent, rinsed with distilled water, washed with diluted HCl 10% (15 min) and finally rinsed 3x with deionized water (15 min). Root samples were treated according to the same protocol, but doubling the time for rinsing with tap water and diluted HCl. Dry weight was obtained by oven-drying at 60 °C for 3 d.

PTEs concentration in plants. The dried samples were ground to a fine powder using mortar and pestle. The samples were digested in a heat block with in HNO_3 (70%) and HCl (37%) (Weyens *et al.* 2010). The concentrations of Zn, Cd and Pb were determined by inductively coupled plasma – atomic emission spectrometry (ICP-OES Agilent Technologies 700 Series). Blanks and certified reference material (spinach, Standard Reference Material® 1570a, National Institute of Standards and Technology, USA Department of Commerce) were included for quality

control. From these data translocation factors (TF) were calculated as the quotient of metal concentration in the shoots to the roots.

Antioxidative enzymes activity. For enzymes analyses fresh clean leaves and roots were excised from plants, immediately snap-frozen in liquid nitrogen and stored at -80 °C until analysis. Frozen plant tissue was crushed and homogenized with cold ice 0.1 M Tris-HCl buffer at pH 7.8, containing 1mM EDTA and 1mM dithiotreitol and 4% insoluble polyvinylpyrrolidone (1 mL and 2 mL of buffer per gram of root and shoot fresh weight, respectively). The homogenate was centrifuged for 10 min at 20,000 rpm and 4 ˚C. The enzyme activity was determined in the supernatant. Activities of guaiacol peroxidase (GPOD) and super oxide dismutase (SPOD) were determined according to Bergmeyer, Gawehn, and Grassl (1974) and Imberty, Goldberg, and Catesson (1984), correspondingly. Superoxide dismutase (SOD) determination was done according to McCord and Fridovich (1969). Analysis of glutathione reductase (GR), glutamate dehydrogenase (GDH) and catalase (CAT) activities were performed as described by Bergmeyer *et al*. (1974).

Statistical analysis

Seed origin (mine residues and non-contaminated site) and bacterial inoculum (8 bacterial inocula) were the two studied factors, in total there were 16 combinations or treatments. Analysis of variance (ANOVA), and in case of any difference, Tukey test was applied ($p<0.05$). Six replicates were used in each treatment; one replicate was a pot with two plants. Percentage values of survival rate were arcsin transformed before statistical analysis. For analysis of data SAS program V 9.1 was used.

Results

Seedling survival

The germination rates of both seed batches, the reference one (98%) and the one collected from mine residues (96%), was not affected by surface seed sterilization. Ten days after transplanting survival rate was checked; results varied as a function of the seed origin and bacterial inoculum (Figure S1). Survival rate varied from 64% to 100%; the lowest rates were observed for plants from control seeds inoculated with *Brachybacterium* sp. or the *Sphingomonas* consortium. In contrast, the same bacterial consortium inoculated to plants from mine residues showed the highest survival rate. Based on factorial analysis, mine residues seedlings demonstrated higher percentages of survival (82%) compared to control seedlings (62%). Plants inoculated with *Methylobacterium* sp. exhibited higher survival rate in comparison to the other bacterial inocula. The lowest survival rates were observed for control plants without bacterial inoculation (Table 2).

Biomass production

When comparing all bacterial inocula and seed origin combinations significant differences on fresh shoot and root weights were observed (Figure 1). Plants inoculated with the *Variovorax* consortium showed root and shoot biomasses that were three times higher than plants inoculated with *Sphingomonas* sp., which had the lowest values (Table 2). The origin of the seed did not have significant effects on dry and fresh weights of shoot; for root dry weight, however, a significant difference was observed. The biomass of non-inoculated plants was similar to biomasses of plants inoculated with *Arthrobacter* sp., *Bacillus* sp., *Brachybacterium* sp. and *Methylobacterium* sp. (Table 2).

PTEs concentration in plants

Concentrations of Cd, Pb and Zn determined in plant tissues are presented in Figure 2. For the three PTEs, significantly different concentrations were observed in shoots. Cadmium shoot concentrations varied from 2.3 to 4.8 mg kg^{-1}; Pb varied between 3.2 and 9.3 mg kg^{-1}. Plants originating from mine residue seeds accumulated the highest concentrations of Cd and Pb in shoots when they were inoculated with *Methylobacterium* sp. and *Sphingomonas* sp., respectively. Inoculation with *Methylobacterium* sp. on mine residue plants lead to Cd shoot concentrations up to 2 times higher than the other treatments. However, in comparison to other treatments, control seed plants inoculated with *Sphingomonas* sp. contained up to 3 times higher Pb concentrations in their shoots (Figure 2). Accumulation of Zn was enhanced by *Sphingomonas* sp. inoculation to control seed plants (Figure 2).

Cadmium and Pb concentrations in shoots depended on the origin of seeds; plants from mine residue seeds accumulated higher concentrations than those grown from control seeds. Concentrations of Cd and Zn in roots were up to 50% and 20% higher in control seed plants, respectively (Table 2).

Plants tended to accumulate most Zn in shoots when no inoculum was added (340.6 mg kg^{-1}), followed by plants from control seeds inoculated with *Sphingomonas* sp. and the *Variovorax* consortium. Inoculation of mine residue seeds with *Arthrobacter* sp. led to the lowest Zn shoot concentration (161.8 mg kg^{-1}). Cadmium uptake was increased when plants were inoculated with *Methylobacterium* sp. and *Bacillus* sp.; the opposite was observed after inoculation with *Sphingomonas* sp. However, *Sphingomonas* sp. induced accumulation of Pb and Zn in shoots.

For roots, differences were detected only in Cd and Zn. Plants originating from mine residue seeds and inoculated with *Sphingomonas* consortium contained the highest Cd (19.2 mg kg^{-1}) and

Zn concentrations (179.8 mg kg^{-1}). Taking into account the effect of the bacterial inoculum, inoculation of the *Sphingomonas* consortium and *Arthrobacter* sp. led to up to 2 times higher Cd concentration; the *Sphingomonas* consortium also induced the highest values of Zn in roots. The *Variovorax* consortium diminished Pb accumulation in root (Table 2).

Regarding Pb, all TF values were lower than 1. In case of Cd the highest TF was 1.05 observed in plants from mine residue seeds without bacterial inoculation (Table S1). Translocation factors higher than 1 were observed only for Zn; plants from mine residue seeds without inoculation presented a TF of 2.64, followed by plants from reference seeds inoculated with *Bacillus* sp. (1.84). Seed origin did not have an effect on TF of any of the studied PTE; however, the bacterial inoculum had obvious effects on TF. Non-inoculated plants tended to translocate higher amounts of Zn and Cd to the aboveground plant part, followed by those inoculated with *Bacillus* sp. (Table 2).

Antioxidative enzymes activity

The activities of antioxidative enzymes varied among treatments (Figure 3). With the exception of GPOD in some treatments, enzymes had higher activities in shoots than in roots with all bacterial inocula tested.

No significant differences were observed for the activities of SOD and GR in shoots (Figure 3). CAT and GDH activities were up to 3 and 8 times higher, respectively, when mine residues plants were inoculated with *Sphingomonas* sp.

In roots, no significant differences were observed for activities of GPOD and CAT. Activities of GPOD and SOD increased twice in control seed plants when *Arthrobacter* sp. was inoculated (Figure 3). When *Brachybacterium* sp. was inoculated to plants grown from reference seeds

SPOD activity in roots was observed to increase 4 times. Plants from mine residue seeds showed the highest values for GDH and GR when *Sphingomonas* sp. was added (Figure 3).

Activities of SPOD and GPOD in roots of plants from reference seeds were higher than the activities determined in roots of plants from mine residue seeds. The opposite trend was observed for GDH (Figure 3). Regarding the effects of bacterial inocula, *Sphingomonas* sp. increased the activities of CAT, GDH, SPOD, and GPOD in shoots and GR in roots (Table 2). The activity of SPOD was enhanced by the *Variovorax* consortium, *Arthrobacter* sp. and *Bacillus* sp. In roots SPOD activity was enhanced by *Brachybacterium* sp. The lowest activities of GPOD and SPOD in shoots were found in non-inoculated plants. However, inoculation with *Methylobacterium* sp. diminished CAT activity in shoots, while *Bacillus* sp. led to low activities of antioxidative enzymes (CAT, GR and SPOD) in roots (Table 2).

Discussion

The survival rates of plants were verified after 10 days of growth on a PTEs containing soil. The origin of the seed clearly was responsible for differential effects: plants grown from reference seeds had a lower survival rate (Table 2). This might be due to the fact that seeds collected from mine residues underwent a selection process that made plants more tolerant to PTEs (Truyens *et al.* 2013). Inoculation with any of the tested endophytes was beneficial for the survival rate; the best result was observed after inoculation with *Methylobacterium* sp. (Figure S1; Table 2). The ACC-deaminase activity that was present in all bacterial inocula (Table 1) might explain these positive effects. Increased ethylene production in response to PTEs induced stress is considered as a major limitation in improving phytoremediation efficiency (Rajkumar *et al.* 2009). Bacterial ACC deaminase regulates ethylene levels in plants by metabolizing its immediate precursor ACC

into ketobutyric acid and ammonia; thus, plants inoculated with ACC deaminase equipped bacteria will have lower ethylene levels and consequently better withstand stress conditions (Arshad *et al.* 2007; Cheng, Park, and Glick 2007; Dell'Amico, Cavalca, and Andreoni 2008; Hardoim *et al.* 2008).

The origin of the seeds was a determining factor for root dry weight, but not for shoot biomass either fresh or dry. A lower root dry biomass was observed in plants grown from reference seeds (Table 2); this most likely is due to the fact that the root was in continuous contact with the PTEs and, as mentioned in the above paragraph, plants from reference seeds were less PTEs tolerant. Inoculation also affected biomass production. Most often, increases of root and shoot growth were reported after inoculation of plants growing in PTEs contaminated soil (Madhaiyan, Poonguzhali, and Sa 2007; Sheng *et al*. 2008; Sun *et al*. 2010; Truyens *et al.* 2014; Babu *et al*. 2015). In some cases, biomass of inoculated plants was similar to that of non-inoculated plants (Nejad and Johnson 2000; Mastretta *et al.* 2009). Higher fresh and dry weights of shoots and roots of *C. pumila* were observed when plants were inoculated with the *Variovorax* consortium (Table 2; Figure 1), possibly due to a synergistic effect of the strains. The two strains composing this inoculum possessed all plant growth promotion features tested except IAA production (Table 1). Synergistic effect of seed endophyte consortia has been observed in tobacco plants exposed to Cd (Mastretta *et al*. 2009). Nonetheless, the *Sphingomonas* consortium tested in this work did not have a big effect on biomass production (Table 2; Figure 1). Indeed, we observed about 80% reductions of shoot and root biomass in plants from mine residue inoculated with *Sphingomonas* sp. Their biomass was two times lower than for non-inoculated plants (Table 2). *Sphingomonas* sp. was not PTEs tolerant (Table 1); though. *Arthrobacter* sp. and *Bacillus* sp. were not PTEs

tolerant either (Table 1) after inoculation they did not cause reductions in biomass production (Table 2).

An important factor for effects of endophytic bacteria on plants may be the initial concentration of bacteria in the inoculum (Nejad and Johnson 2000). Most investigations concerning effects of root or shoot endophytic bacteria on plant growth, generally use concentrations of 10^6 (Wang et al., 2013), 10^8 (Sheng *et al.* 2008; Mastretta *et al*. 2009; He *et al.* 2013; Babu *et al*. 2015), or 10^9 (Lopez, Bashan, and Bacilio 2011; Lopez *et al*. 2012) cfu mL^{-1}. Truyens *et al*. (2014) applied a lower concentration, 10^5 cfu mL^{-1}, when using seed endophytic bacteria. It indeed has to be taken into account that endophytic bacteria are found at lower concentrations than rhizospheric bacteria (Compant, Clément, and Sessitsch 2010), especially in case endophytes from seed or reproductive plant organs are considered (Hallman, 2001; Rosenblueth *et al.* 2010). It is possible that for some bacterial inocula, like *Sphingomonas* sp., a concentration of 10^6 cfu mL^{-1} was too high at early stages of plant development (germination) and disturbed the balance of the endophytic community, causing non beneficial effects to inoculated plants (Nejad and Johnson 2000; Paszkowski, 2006). Some endophytes indeed are more aggressive colonizers and can displace others (Rosenblueth and Martínez-Romero 2004; Verma *et al.* 2004). Colonization of endophytes may result in different kind of interactions, ranging from mutualism to parasitism (Kogel, Franken, and Hückelhoven 2006; Partida-Martínez and Heil 2011; Zamioudis and Pieterse 2012). There are reports indicating endophytes can become parasitic under certain conditions (Schulz and Boyle, 2005; Paszkowski, 2006). The basis for the conversion from one kind of interaction to another one still remains little understood (Long, Schmidt, and Baldwin 2008).

The highest Cd (5 mg kg^{-1} of dry weight) and Pb (9 mg kg^{-1} of dry weight) shoot concentrations found in this work were lower than those reported previously for *C. pumila* growing on mine residues, 15 and 12 mg kg^{-1}, respectively (Sánchez-López *et al*. 2015). However, Zn concentrations in shoots of all treatments, except plants from mine residue seed inoculated with *Arthrobacter* sp. (Figure 2), were higher than concentrations observed in field grown plants (206.5 mg kg^{-1}) and higher than the threshold value for Zn phytotoxicity (200 mg kg^{-1}) as reported by Vamerali, Bandiera, and Mosca (2010).

In the case of roots, except non-inoculated plants and those inoculated with the *Variovorax* consortium, inoculation with seed endophytes lead to Pb root concentrations higher than 88 mg kg^{-1}, the value reported for *C. pumila* growing *in situ* on mine residues (Sánchez-López *et al.* 2015). Plants from mine residue seeds inoculated with the *Sphingomonas* consortium, *Sphingomonas* sp., *Arthrobacter* sp., *Methylobacterium* sp. and the *Variovorax* consortium had higher Cd concentrations in their roots (Figure 2) than concentrations reported for the same species growing on mine residues (10.8 mg kg^{-1}) (Sánchez-López *et al.* 2015). All treatments, except plants from control seeds inoculated with *Bacillus* sp. and non-inoculated plants from mine residue seeds, accumulated Zn in roots at concentrations higher than previously found (63.2 mg kg^{-1}) for *C. pumila* on PTEs containing substrate (Figure 2). It should be mentioned that these variations could be attributed to differences in the physico-chemical characteristics of the substrates and also due to the fact that the previously reported concentrations were obtained from adult plants growing *in situ* on mine residues.

Regarding TF, values calculated in this work (Table S1) were till 30, 9 and 7 times lower than earlier mentioned values for Pb, Zn and Cd, respectively (Sánchez-López *et al*. 2015). The same authors found that *C. pumila* may be considered as an accumulator of Zn and excluder of Pb and

Cd. Obtained results showed that inoculation of seed endophytes, either from control seeds or mine residue seeds, on *C. pumila* induced this species to act as a Cd and Pb excluder (Figure 2; Table 2). Only non-inoculated plants or those inoculated with *Bacillus* sp. behaved as Zn accumulator (Table 2). Interestingly, *Bacillus* sp. was isolated from seeds collected on a non-contaminated site (Table 1). It means that inoculation with seed endophytic bacteria affected PTEs translocation to above-ground plant part (Sheng *et al.* 2008; Sun *et al.* 2010; Babu *et al.* 2015), and that they had different effects in function of the origin of the plant they were isolated from. Mostly, inoculation with endophytic bacteria isolated from plants growing on PTEs contaminated soils resulted in increases of PTEs uptake by plants (Sheng *et al.* 2008; Mastretta *et al.* 2009; Sun *et al.* 2010; He *et al.* 2013; Wang *et al.* 2013; Babu *et al.* 2015). However, Janssen *et al*. (2015) mentioned that enhancements in PTEs extraction potential of willow after inoculation with plant associated bacteria were variable and not always occurred. In the present work, *C. pumila* plants inoculated with seed endophytes isolated from the same plant species growing on mine residues, diminished PTEs translocation to aboveground plant (Figure 2; Table 2). Similar results were observed by Madhaiyan *et al*. (2007) for tomato plants. The decreased accumulation of PTE might be due to bacterial immobilization of PTE in rhizosphere (Sheng *et al.* 2008).

Despite variations in PTEs concentrations, plants from mine residue seeds generally accumulated higher concentrations of Cd and Pb in shoots than plants from reference seeds (Table 2). The effect of bacterial inoculation varied depending on the element analyzed and plant tissue, but in general inoculation with seed endophytes diminished PTEs translocation (Table 2). This makes bacterial inoculation advantageous for phytostabilization purposes. Future work should include evaluations along the complete life cycle of plants under field conditions.

Besides effects on plant growth, endophytic bacteria can change plant metabolism in such a way that after exposure to PTEs, the plants are able to tolerate the contaminants and thus can better withstand stress conditions (Weyens *et al.* 2009). The antioxidative capability plays an important role in the tolerance and accumulation of PTEs in plants. Among the enzymes involved in antioxidative defense mechanisms, SOD, GPOD and SPOD showed the highest activities in shoots (Figure 3). Plants protect themselves from superoxide radical stress by elevated SOD activity; these enzymes convert the O_2^- radical to H_2O_2 and O_2 alleviate toxicity (Scandalios, 1993; Mittler, 2002). In turn, H_2O_2 is further scavenged by peroxidases (GPOD, SPOD) and CAT (Siedlecka and Krupa 2002; Zhang *et al*. 2007); this was confirmed by the activities of GPOD and SPOD (Figure 3). The activities of these enzymes were compared among plants inoculated with different seed endophytic bacteria. The activities of SOD, GPOD and SPOD were higher in plants inoculated with *Sphingomonas* sp. (Table 2). This bacterial inoculum also increased the activity of CAT but only in plants from mine residues (Figure 3). The activity of peroxidases is one of the main defense mechanisms against the reactive properties of superoxide and H_2O_2 (Wang *et al.* 2004).). High CAT, GPOD and SPOD activities in plants inoculated with *Sphingomonas* sp. may be explained by the fact plants inoculated with this bacterial strain also contained the highest Zn and Pb shoot concentrations (Table 2). Besides the aforementioned antioxidative enzymes, inoculation of *Sphingomonas* sp. also increased the activity of GDH after inoculation to plants from mine residue seeds (Figure 3). The high SPOD, GPOD, SOD and CAT activity and high PTEs concentrations in plants might indicate that inoculation with *Sphingomonas* sp. resulted stressing for *C. pumila* plants.

Except *Sphingomonas* sp., for the other bacterial inocula the effect on the activity of antioxidative enzymes was variable and in some cases similar to values observed in non-inoculated plants. This

trend was opposite to the general findings: endophytes may protect host plant against PTEs phytotoxicity by increasing the antioxidative capacity of inoculated plants (Wang *et al.* 2004; Bonnet, Camares, and Veisseire 2000; Zhang *et al.* 2010; Mirzahosseini *et al*. 2014). In future studies, molecular techniques should be used to investigate the mechanisms involved in plant-endophytes interactions.

Some reports mention that endophytes possessing plant growth promotion traits and additionally exhibiting PTEs tolerance keep the same features when they are inoculated to their natural host but also non-host plants (Ma *et al.* 2011). However, in agreement to the findings of Long *et al*. (2008), the endophytic bacteria isolated from *C. pumila* seeds that presented plant growth promotion traits did not have general and predictable effects on the growth and wellness of their host plants. Even if the endophytes keep plant growth promoting features the effects can vary in host and non-host plant species (Long *et al.* 2008) or, as it is the case in our study, in the same plant species grown from seeds from different origin. We observed dissimilar responses to inoculation with endophytic bacteria in *C. pumila* grown from seeds from an unpolluted reference area and from the mine tailings, even at plant organ level. These differences could well be attributed to several factors such as seed quality or to the different environmental conditions under which the seeds developed (Long *et al.* 2008). Since the reference seeds were not exposed to PTEs, it is likely that they possessed a higher quality than those collected from mine residues. In addition, the effects may have been a consequence of the particular microflora to which the experimental plants were exposed before bacterial inoculation (Nejad and Johnson 2000). The specific endophytic community, including non-cultivable microorganisms (López *et al*. 2011), present in both types of seeds might originate from specific interactions affecting plant growth, responses to PTEs stress and differences in PTEs accumulation.

Conclusions

In the present research, we observed that the positive effects of seed endophytes inoculation varied in function of the bacterial inoculum used and the origin of the seeds. Despite that the same plant species was used, results regarding survival rate, biomass production and PTEs accumulation were different between plants from reference seeds and those grown from seeds collected on mine residues. In general, the latter ones showed higher survival rates and PTEs accumulation, but lower biomass production than plants from reference seeds. The environment from seeds were collected may affect seed characteristics, *e.g.* seed endophytic community, and thus affect the results of inoculation with seed endophytes.

All bacterial inocula, except *Bacillus* sp., diminished PTEs translocation to the aboveground plant tissues. *Methylobacterium* sp. promoted a higher percentage of survival in PTEs contaminated soil, while biomass production was stimulated by *Variovorax* consortium inoculation. The highest activities of antioxidative enzymes, and thus the highest capacity to combat PTEs-induced oxidative stress, was observed when *Sphingomonas* sp. was inoculated. However, the latter also reduced biomass production. It is recommended to test and apply consortia including outstanding endophytes to obtain synergistic effects of the different members of the consortia.

Results indicate practical applications of *C. pumila* and its seed endophytic bacteria to promote revegetation and remediation of PTEs-contaminated soils through the establishment of a vegetation cover. More knowledge about population dynamics and interrelations of PTEs tolerant endophytes (both fungi and bacteria), and interactions with their host in native metalliferous and accumulator plant species is required.

Acknowledgements

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Supplemental material

Supporting information shows survival rate and TF of *C. pumila* plants inoculated with endophytic bacteria.

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Table 1. Functional characteristics of seed endophytic bacteria tested on *Crotalaria pumila* plants.

Inoculum	Origin	IAA	OA	Sid	PO_4	Acetoin	Phytate	ACC	Maximum PTE tolerance
Arthrobacter sp. Cp20	Mine residues	+	+	+	+	+	-	+	Nt
Bacillus sp. Cp40	Non-contaminated site	+	+	-	+	+	-	+	Nt
Brachybacterium sp. Cp27	Mine residues	-	+	-	+	+	+	+	Zn (2.5 mM) Pb (0.4 mM) Cu (0.4 mM)
Methylobacterium sp. Cp3	Mine residues	+	-	-	+	-	-	+	Zn (5 mM) Cd (1.6 mM) Pb (0.4 mM)
Variovorax consortium	*Variovorax* sp. Cp29 mine residues	-	-	+	+	+	-	+	Zn (2.5 mM)
	Variovorax sp. Cp30 mine residues	-	+	+	+	+	+	+	Cd (1.6 mM) Cu (0.8 mM) Ni (3 mM) Pb (0.4 mM)
Sphingomonas sp. Cp1	Mine residues	+	-	-	+	-	-	+	Nt
Sphingomonas consortium	*Sphingomonas* sp. Cp21 mine residues	-	-	-	+	+	-	+	Zn (1 mM), Ni (3 mM Pb (0.4 mM)
	Sphingomonas sp. Cp23 mine residues	+	+	+	-	-	-	+	Zn (2.5 mM) Cd (0.4 mM)
	Sphingomonas sp. Cp4 mine residues	-	-	-	+	+	-	+	Zn (5 mM) Ni (5 mM) Cu (0.4 mM)

Isolates producing Indol Acetic Acid (IAA), organic acids (OA), siderophores (Sid); isolates mineralizing phytate (Phy) or solubilizing phosphate (PO_4); isolates with 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity; PTE: potentiallt toxic element;**N**t: non-metal tolerant.

Table 2. Effect of seed origin and bacterial inoculum on survival rate, biomass production, PTEs accumulation and antioxidative enzymes activity in *Crotalaria pumila* plants.

Variable		Seed origin		Inoculum							
		Mine residues	Control	Arthr	Bac	Brch	Meth	Sphin	SphinC	VarC	NonI
Survival rate (%)		82[a]	62b	73ab	76ab	69ab	81a	75ab	75ab	64ab	62b
Fresh weight (mg)	Shoot	781.9a	802.8a	698.5c	854abc	842.6abc	789.2bc	370.3d	941.6ab	1026.7a	816.7bc
	Root	554.8a	548.7a	505.5bc	444.3cd	503.5bcd	716.3ab	263.0d	645.1abc	750.2a	586.7abc
Dry weight (mg)	Shoot	133.3a	139.3a	129.1b	134.2b	157.2ab	149.1ab	60.5c	137.2b	184.8a	138.4b
	Root	78.6b	99.3[a]	56.7c	72.4c	63.8c	107.7b	46.1c	103.1b	143.1a	118.0ab
Pb (mg kg^{-1})	Shoot	4.8a	4.1b	4.5b	4.4bc	3.9bc	4.2bc	6.8a	4.6b	3.3c	3.8bc
	Root	107.1a	92.7[a]	107.3ab	96.1ab	140.3a	96.2ab	89.6ab	110.1ab	76.2b	83.0ab
	TF	0.05a	0.05[a]	0.05b	0.05b	0.03b	0.05b	0.08a	0.04b	0.05b	0.05b
Cd (mg kg^{-1})	Shoot	3.9a	2.6b	3.2ab	3.7a	3.1 ab	3.7a	2.8b	3.5 ab	3.0ab	3.3ab
	Root	12.9a	8.1b	13.8a	7.6 ab	8.9 ab	12.1ab	11.1ab	13.3a	11.1ab	6.3b
	TF	0.4a	0.3[a]	0.2b	0.5ab	0.4b	0.3b	0.3b	0.3b	0.3b	0.7a
Zn (mg kg^{-1})	Shoot	257.4a	246.9a	201.9b	239.9ab	240.9ab	237.2ab	291.5a	263.8 ab	266.8ab	275.3ab
	Root	114.4a	97.2b	105.4abc	71.9c	147.8ab	95.7bc	114.4abc	154.3 a	91.8c	65.1c
	TF	1.4a	1.5[a]	1.2c	1.8ab	1.1c	1.3bc	1.6abc	1.2c	1.5bc	2.1a
SOD (mU mg^{-1} of fresh weight)	Shoot	1752.6a	1747.3a	1766.1a	1725.7a	1679.2a	1711.3a	1746.0a	1604.4a	1964.5a	1802.6a
	Root	217.7a	220.1a	250.2a	219.7a	239.6a	198.2a	207.9a	227.2a	203.5a	204.8a
GPOD (mU mg^{-1} of fresh weight)	Shoot	9757.1a	10690.2a	9947.0abc	11212ab	8000bc	10905abc	13271a	8358bc	12902a	7195c
	Root	9629.1b	12429.8a	13551.0a	8683a	12597a	9431a	10039a	11763a	10883a	11289a
SPOD (mU mg^{-1} of fresh weight)	Shoot	6317.5a	6635.1a	7240.5a	6807.5a	5442.5a	6275.3a	7682.5a	7038.2a	7263.4a	4060.9a
	Root	2549.6b	3548.0a	4207.3a	2377.8b	4347.2a	2639.7ab	2100.9b	3408.4ab	2697.0ab	2612.0ab
GR (mU mg^{-1} of fresh weight)	Shoot	731.6a	838.7a	642.2a	939.9a	742.3a	741.5a	698.4a	678.4a	899.1a	939.4a
	Root	276.2a	256.8a	267.9ab	202.5b	278.6ab	238.1ab	340.8a	317.8a	207.8b	278.7ab
CAT (mU mg^{-1} of fresh weight)	Shoot	528.5a	440.2[a]	666.3ab	453.3abc	419.9bc	314.6c	712.9a	397.9bc	496.4abc	413.7bc
	Root	117.8a	121.3[a]	127.9ab	75.6b	129.2a	110.8ab	140.3a	135.3a	115.1ab	122.2ab
GDH (mU mg^{-1} of fresh weight)	Shoot	475.0a	303.4b	228.4b	285.8b	275.4b	239.4b	1102.9a	280.6b	416.1b	285.3b
	Root	228.7a	226.3[a]	179.4a	180.9a	198.2a	208.1a	248.6a	312.4a	223.0a	269.3a

In each studied factor, average (n=6) with the same letter in each row indicate significant difference according to two-ways ANOVA and Tukey's test ($p<0.05$); Arthr: *Arthrobacter* sp.; Bac: *Bacillus* sp.; Brch: *Brachybacterium* sp.; Meth: *Methylobacterium* sp.; Sphin: *Sphingomonas* sp.; SphinC: *Sphingomonas* consortium; VarC: *Variovorax* consortium; NonI: non-inoculated plants; TF: translocation factor.

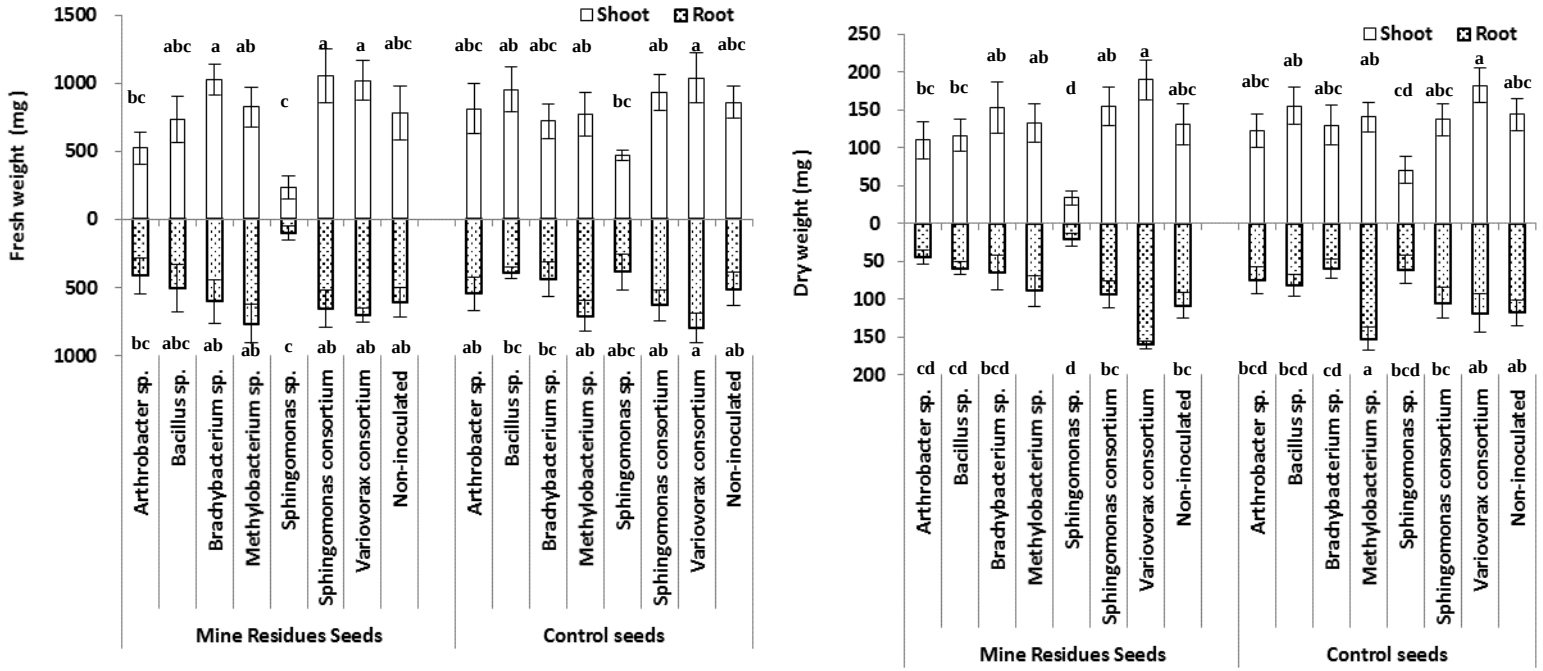

Figure 1. Fresh and dry weight of *Crotalaria pumila* plants inoculated with seed endophytic bacteria. Average of n=6 ± standard deviation; comparison among all treatments using Tukey´s test ($p<0.05$), bars with the same letter indicate no significant differences in root or in shoot biomass.

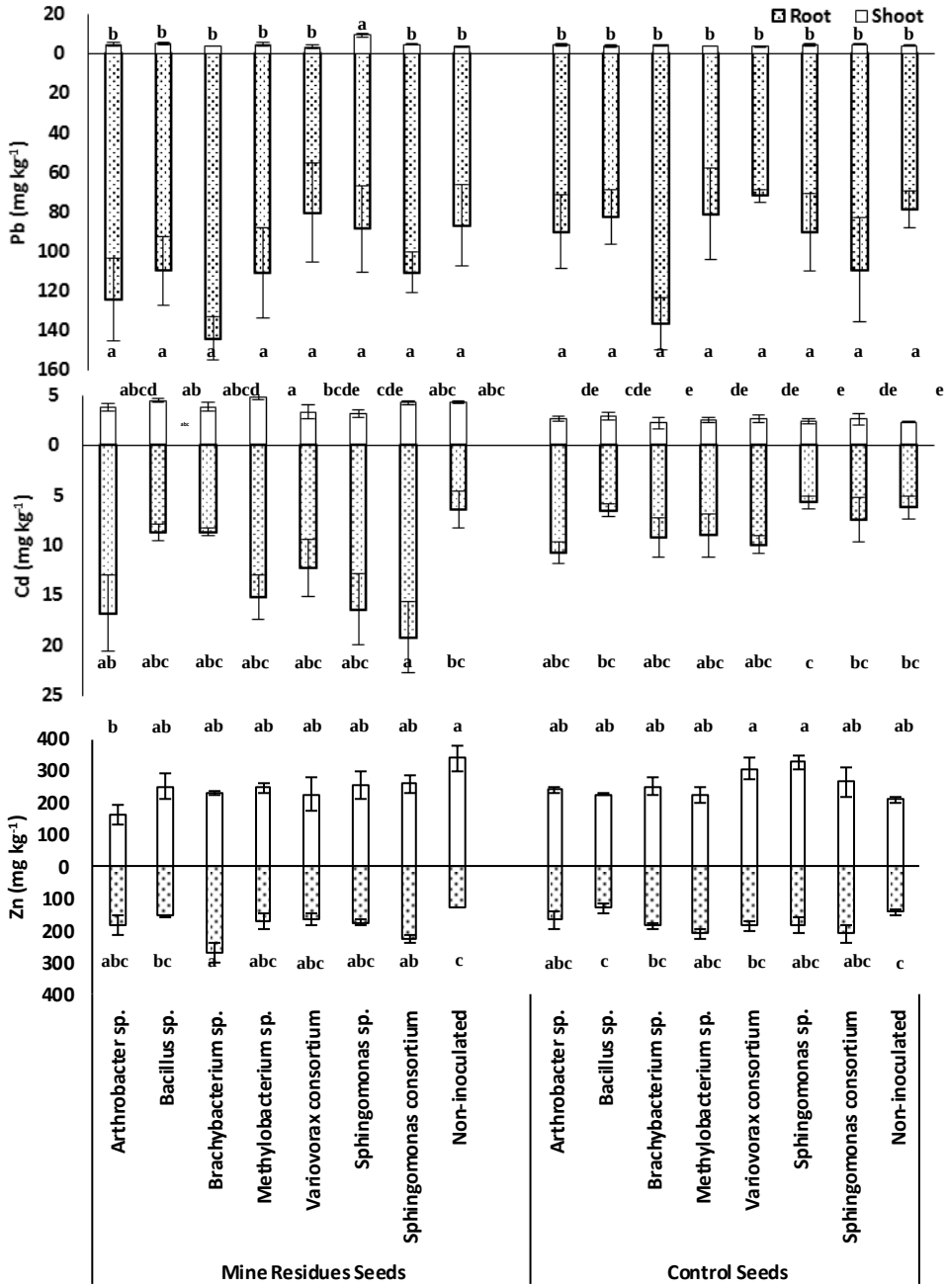

Figure 2. Concentration of Pb, Cd and Zn in roots and shoot of *Crotalaria pumila* inoculated with seed endophytic bacteria. Average of n=6 ± standard deviation; comparison among all treatments using Tukey´s test ($p<0.05$), bars with the same letter indicate no significant differences in root or in shoot.

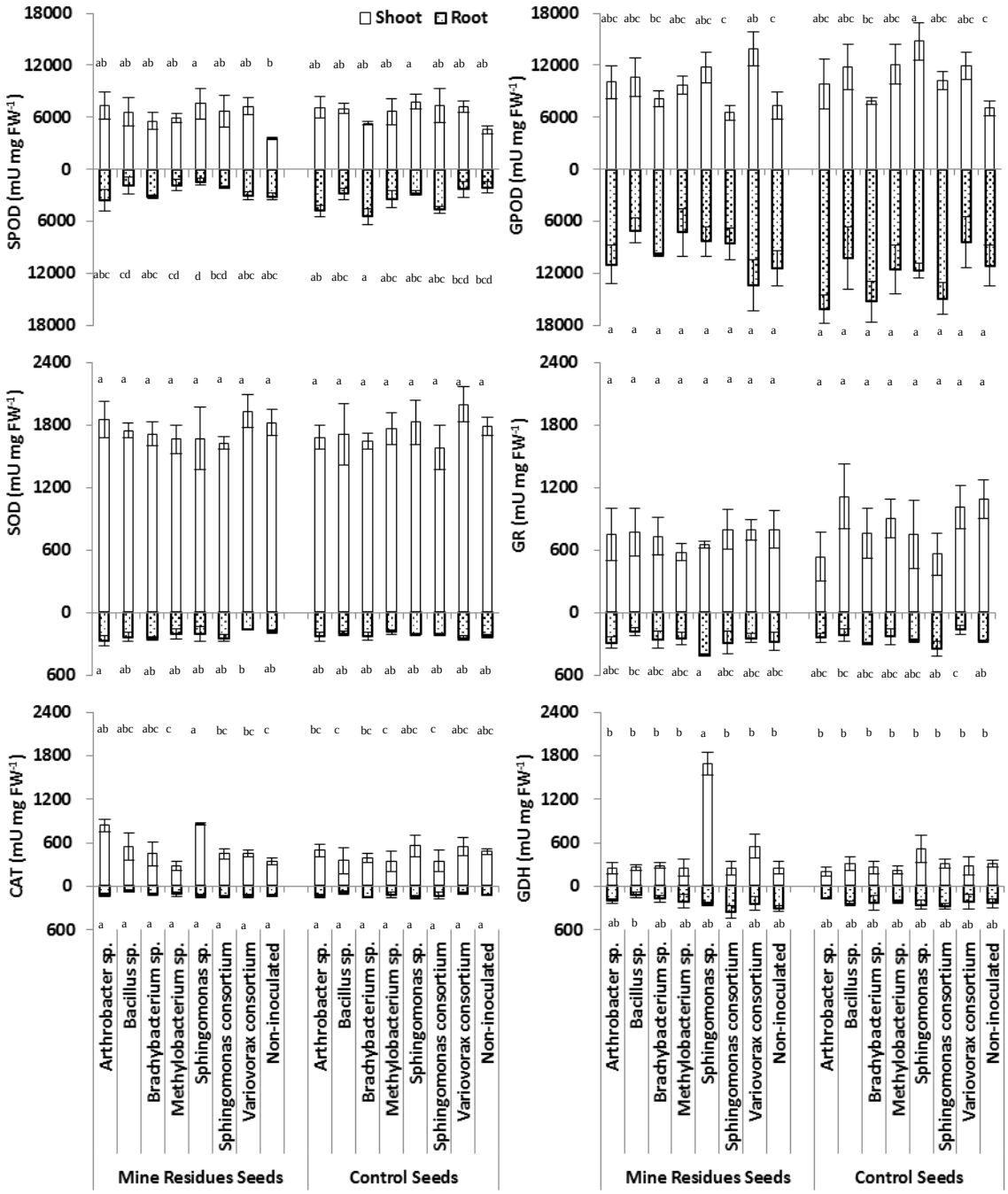

Figure 3. Antioxidative enzymes activity of root and shoot of *Crotalaria pumila* growing on PTEd-containing soil and inoculated with seed endophytic bacteria. Average of n=6 ± standard deviation; comparison among all treatments using Tukey´s test ($p<0.05$), bars with the same letter indicate no significant differences in root or in shoot.

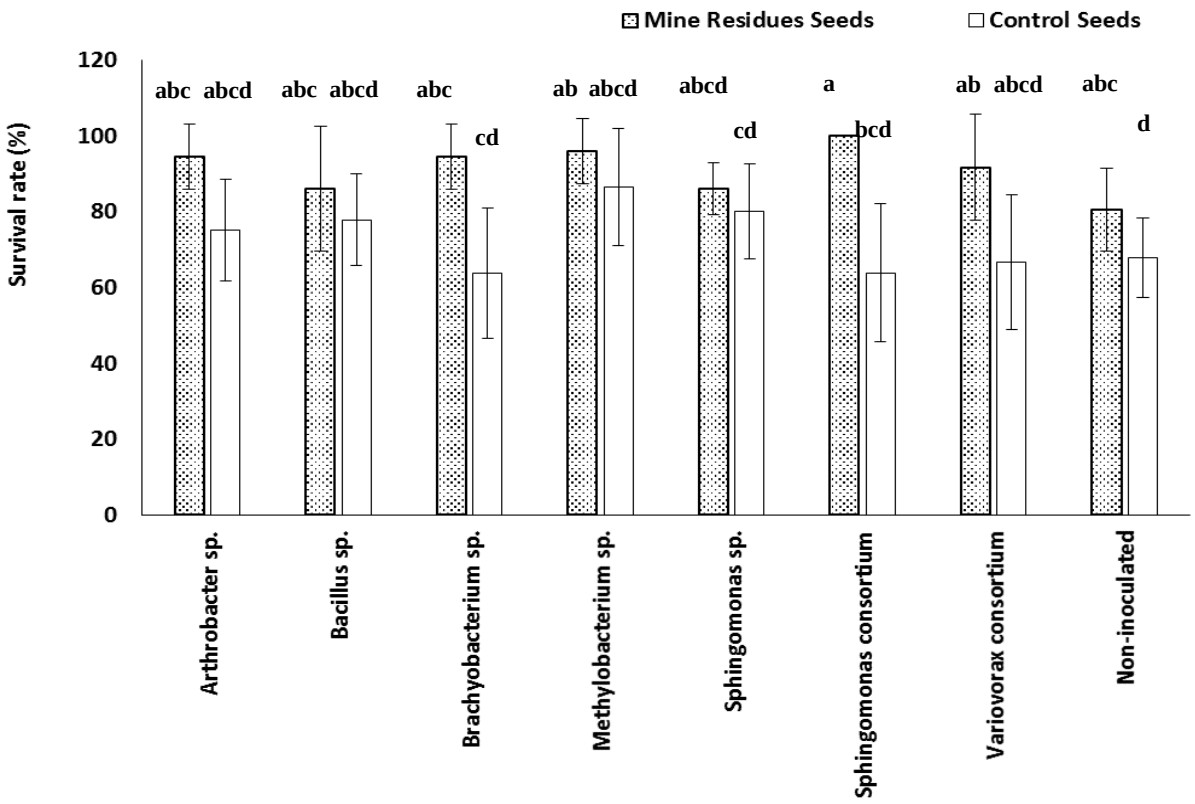

Figure S1. Percentage of survival of inoculated *Crotalaria pumila* plantlets after 10 days of transplanting in a PTEs containing-soil. Average of n=6 $\pm$ standard deviation; comparison among all treatments using Tukey´s test ($p<0.05$), bars with the same letter indicate no significant differences.

Table S1. Translocation factors of Pb, Cd and Zn in *Crotalaria pumila* inoculated with seed endophytic bacteria

Seed origin	Inoculum	Pb	Cd	Zn
Mine residues	*Arthrobacter* sp.	0.04 b	0.26 b	0.88 c
	Bacillus sp.	0.05 b	0.52 ab	1.64 bc
	Brachybacterium sp.	0.04 b	0.44 b	0.86 c
	Methylobacterium sp.	0.04 b	0.37 b	1.47 bc
	Sphingomonas sp.	0.10 a	0.21 b	1.49 b
	Sphingomonas consortium	0.04 b	0.23 b	1.16 bc
	Variovorax consortium	0.04 b	0.37 b	1.37 bc
	Non-inoculated	0.04 b	1.05 a	2.64 a
Non-contaminated	*Arthrobacter* sp.	0.05 b	0.25 b	1.48 bc
	Bacillus sp.	0.04 b	0.46 b	1.85 ab
	Brachybacterium sp.	0.03 b	0.26 b	1.36 bc
	Methylobacterium sp.	0.06 b	0.31 b	1.08 bc
	Sphingomonas sp.	0.05 b	0.46 b	1.77 bc
	Sphingomonas consortium	0.05 b	0.36 b	1.26 bc
	Variovorax consortium	0.05 b	0.27 b	1.66 bc
	Non-inoculated	0.05 b	0.38 b	1.53 bc

CHAPTER 6. ENDOPHYTIC COLONIZATION OF *Crotalaria pumila* BY SEED ENDOPHYTIC mCherry-LABELED *Methylobacterium* sp.

Abstract

Methylobacterium sp. Cp3 is an endophytic bacterial strain isolated from *Crotalaria pumila* Ort. seeds growing on potentially toxic elements (PTEs) contaminated mine residues. Previous research showed that this strain produces indole-3-acetic acid, solubilizes phosphate, shows 1-aminocyclopropane-1-carboxylate deaminase activity and is tolerant to Zn, Cd and Pb. To better understand plant colonization abilities of this strain, *Methylobacterium* sp. Cp3 was transformed with a plasmid harboring the mCherry construct (pMP7604). *Crotalaria pumila* seeds were inoculated with the fluorescent transformed strain using a gnotobiotic system, with or without Cd and Zn addition. Plant colonization was studied using confocal laser scanning microscopy. mCherry-tagged *Methylobacterium* sp. Cp3 colonized not only the main root and root hair surfaces, but also cortex cells. Labeled *Methylobacterium* sp. Cp3 colonized the xylem vessels in root and shoots of *Crotalaria* only in the presence of PTEs. The transformed strain was re-isolated from surface-sterilized inoculated plants, confirming its endophytic colonization abilities and survival *in planta*. Results validate that *Methylobacterium* sp. Cp3 is a true endophyte which can play an important role in plant growth-promotion and fitness in PTEs contaminated conditions. The observations support the hypothesis that seed endophytes can enter root cells, move through the xylem and reach different organs (*e.g.* seeds) and by consequence can be transferred to successive plant generations.

Keywords: plant growth-promoting endophyte, mCherry protein, colonization, xylem

Introduction

Endophytic bacteria generally are reported to bring several benefits to their host plant, such as promoting plant growth and yield, assisting to withstand stress conditions and helping remove contaminants (Siciliano *et al*. 2001; Arshad *et al.* 2007; Hardoim *et al.* 2008). Therefore, phytoremediation of potentially toxic elements (PTEs) contaminated soils assisted with endophytic bacteria has been proposed for successful results (Rajkumar *et al.* 2009; Weyens *et al.* 2009; Ma *et al.* 2011). The success of endophytes application at large scale requires both an efficient colonization of the plant tissues and the preservation of the desirable properties inside the plant (Ribeiro-Torres *et al.* 2013). To evaluate bacterial colonization, the accurate identification of the inoculum and also the spatial tracking of the bacteria in plant tissues are necessary (Errampalli *et al*. 1999; Compant *et al.* 2005). Thus, techniques using living tissues are essential in this kind of studies. A common method of tracking bacteria is the introduction of a label such as the green fluorescent protein (Valdivia *et al.* 1998), or other stable fluorescent labels like mCherry (Lagendijk *et al.* 2010). Microscopic tools, which allow the detection of fluorescent-labeled strains, together with the use of gnotobiotic conditions are useful tools to visualize bacterial cell colonization in plant tissues (Compant *et al.* 2010). Different studies demonstrated plant tissue colonization by endophytic bacteria (Compant *et al.* 2008; Jonhston-Monje and Raizada 2011; Anand and Chanway 2013; Zhang *et al.* 2013). However, reports about endophytic colonization in the presence of PTEs are scarce.

Although the importance of plant-associated micro-organisms for plant growth and health got more and more recognized, the potential benefits of seed-associated micro-organisms, and especially seed endophytic bacteria, still is underestimated. Nevertheless, as seed endophytes are already present at the moment of germination and in the very early stages of plant development,

these associations could be beneficial for germination and establishment of seedlings in harsh conditions. Moreover, bacteria possessing beneficial traits can be selected by the host plants and transferred via the seed to benefit the next generation (Truyens *et al*, 2013, 2014, 2015). From a collection of seed bacterial endophytes isolated from *Crotalaria pumila* plants growing on PTEs-containing mine residues, five strains were selected to be transformed with a stable plasmid harboring the mCherry construct (pMP7604). The selected strains were tested for their plant growth promotion capacities on *C. pumila* in a PTEs-containing soil (Sánchez-López et al. 2015c; submitted). Some strains increased plant survival rate, other bacteria enhanced biomass production or affected PTEs translocation from root to shoot (Sánchez-López et al. 2015c; submitted). The ability of mCherry-labeled seed endophytes to colonize *C. pumila* plants in the presence of PTEs was investigated.

Materials and methods

Bacterial strains, culture conditions and plasmids

The bacterial strains and plasmids used in this work are presented in Table 1. In an earlier study 32 seed endophytic bacteria were isolated from *C. pumila* seeds (Sánchez-López et al., 2015b; submitted) and were tested for their plant growth promotion abilities in a pot experiment (Sánchez-López *et al*. 2015c). Based on these results six bacterial strains were selected: *Methylobacterium* sp. Cp3, *Variovorax* sp. Cp29, *Variovorax* sp. Cp30, *Brachyobacterium* sp. Cp27, *Sphingomonas* sp. Cp1 and *Caulobacter* sp. CP32. The first one induced the highest survival rate of *C. pumila* on PTEs-contaminated soil, while *Variovorax* sp. CP29 and CP30 increased plant biomass production. *Sphingomonas* sp. Cp1 and *Brachybacterium* sp. C27 affected the activities of antioxidative enzymes.

Due to the fact that the used plasmid harboring the mCherry construct (pMP7604) was designed for Gram-negative bacteria, it is tetracycline resistant (Lajendijk *et al.* 2010), and Cd tolerance (4 mM) was used as a pressure factor too, the selected isolates own these characteristics.

Five bacteria selected for transconjugation were grown at 27 °C in 869 liquid medium pH 7 (Mergeay *et al.* 1985), containing per liter: 10 g tryptone, 5 g yeast extract, 5 g NaCl, 1 g D-glucose and 0.35 g $CaCl_2 \cdot 2H_2O$. 0.5 mL of tetracycline per liter of medium was added when required (10 mg tetracycline mL^{-1} of methanol). Bacteria were grown until they reached OD_{600} =0.7.

Donor *Escherichia coli* DH5α containing the environmental stable mCherry-plasmid pMP7604 was grown in 869 liquid medium supplemented with 0.5 mL of tetracycline per liter of medium (10 mg tetracycline mL^{-1} of methanol). The helper strain *E. coli* H2013 was cultured in the same medium without addition of antibiotic. Donor and helper cultures were grown at 27 °C at 120 rpm until they reached OD_{600} 0.3, which was approximately 10^7 cells of both donor and helper.

Transformation of seed endophytic bacteria

Bacterial strains were transformed through triple conjugation in ratio of 1:1:10 formed by helper strain, donor strain and recipient strains either *Methylobacterium* sp. Cp3, *Variovorax* sp. Cp29, *Variovorax* sp. Cp30, *Brachyobacterium* sp. Cp27, *Sphingomonas* sp. Cp1 or *Caulobacter* sp. Cp32. The culture was at darkness overnight at 27 °C. Subsequently, the culture was recovered in 4 mL of 10 mM $MgSO_4$; 100 µL aliquots were plated on 284 selective medium containing 0.4 mM Cd and tetracycline. 10^{-3} dilutions from $MgSO_4$ solution were plated as well. After 5 days, bacterial grown was verified; ideally, transconjugants were the only colonies that

could grow on the selective medium. Colonies were analyzed for expression of mCherry using fluorescence microscopy (Nikon Eclipse 80i, Nikon Instruments Inc., NY, USA). Experiment were performed in triplicate.

***Crotalaria pumila* colonization assays**

Crotalaria pumila seeds were collected from PTEs contaminated mine residues (Sánchez-Lopez *et al*. 2015a). In order to increase and homogenize germination rate seeds were submerged for 30 min in H_2SO_4 (Linding-Cisneros and Lara-Cabrera 2004), subsequently 8 times rinsed with sterile distilled water and surface sterilized. The protocol for surface sterilization consisted of 1 min immersion in ethanol 70%, 1 min in NaClO 0.1%, and 8 rinses with sterile distilled water. To verify the effectiveness of the surface sterilization protocol 100 µL aliquots of the last rinsing water were plated in 869 1/10 diluted medium (Mergeay *et al.* 1985) and incubated one week at 27° C in darkness. Once seeds were surface sterilized they were put in sterile Petri dishes at 27° C in darkness overnight.

Methylobacterium sp. was the only recipient strain where transformation was successful. *Methylobacterium* pMP7604-labeled inoculum was grown in 1/10 869 liquid medium. Subsequently, the culture was washed with $MgSO_4$ 10 mM, centrifuged at 2000 rpm for 10 min and resuspended in sterile distilled water. 1 mL of inoculum (10^7 cfu mL^{-1}) was smeared on the medium before placing seeds.

After 72 h seedlings were sufficiently developed, and were placed on vertical agar plates (VAPs) with 50-fold dilution of Gamborg´s B5 medium (Zhang and Forde 1998) with or without addition of PTEs (0.2 mM Cd ($CdSO_4$) and 0.4 mM Zn ($ZnSO_4$)). Composition per liter of this medium was: 0.5 mM KNO_3, 0.02 mM $MgSO_4 \cdot 7H_2O$, 0.02 mM $CaCl_2 \cdot 6H_2O$, 0.022 mM NaH_2PO_4, 0.94 µM $MnSO_4 \cdot H_2O$, 0.02 mM $(NH_4)_2SO_4$, 0.97 nM H_3BO_3, 0.14 nM $ZnSO_4 \cdot 7H_2O$,

2 nM $CuSO_4 \cdot 5H_2O$, 20.6 nM $Na_2SO_4 \cdot H_2O$, 2.6 nM $CoSO_4 \cdot H_2O$, 3.6 µM $FeCl_3$ and 2.56 mM MES.

Two days later a second inoculation was performed (1 mL of 10^7 cfu mL^{-1} solution); inoculum was added to the roots and around them. Two further inoculations were done at day 6 and 9. Plants were kept in VAPs for in total 10 days. Sections of the root, root hairs, and stem were hand cut and screened at 20x or 40x for fluorescence expression using a confocal laser scanning microscope (Ultra VIEW VoX, Perkin Elmer). Images were obtained using a using excitation 568–585 nm long pass emission for mCherry. Background fluorescence from the plant tissue was checked in advance.

In order to verify the endophytic colonization, mCherry tagged bacterial cells were isolated from surface sterilized roots and stem sections. Three 1 cm long segments of both roots and stems, from seedlings grown on VAPs with and without PTEs were surface sterilized as follows: 30 sec immersion in 70% (v/v) ethanol solution, then 1 min in 1% active chloride solution supplemented with Tween 80 (1 droplet per 100 mL solution), and rinsed 8 times with sterile distilled water. 100 µL of the last rinsing water were plated on 1/10 diluted 869 medium to verify the effectiveness of the sterilization protocol. Subsequently, tissues were crushed using a sterile mortar and pestle containing 3 mL of 10 mM sterile $MgSO_4$, and then crushed. 100 µL aliquots of the slurry and from the 1/10 and 1/100 dilutions were plated on 1/10 869 medium supplemented with 0.5 mL of tetracycline per liter of medium (10 mg tetracycline mL^{-1} of methanol). The plates were incubated at 28 °C for 2 days, after which the numbers of cfu were determined.

Results

After the mixed cultures of donor, helper and recipient bacterial growth was observed for *Methylobacterium* sp. Cp3, *Variovorax* sp. Cp29, *Sphingomonas* sp. Cp1 and *Caulobacter* sp. Cp32 However, when mCherry expression was verified results were only positive when *Methylobacterium* sp. Cp3 was the recipient. Therefore, pMP7604-tagged *Methylobacterium* was used for plant colonization assays. The colony morphologies of *Methylobacterium* sp. wild type strain and the pMP7604-tagged strain were identical, but they clearly distinguished by the pink color of the pMP7604 labeled strain.

Endophytic colonization

mCherry expressing cells were clearly visible on surfaces of roots and root hairs of *C. pumila* plants grown with and without PTEs in the growth medium (Figure 1). At 20 x and 40x magnification, mCherry-tagged cells were localized intracellularly in root cortex cells of plants growing in media supplemented or not with PTEs (Figure 2; Figure 3). The root hairs were often colonized by bacterial colonies forming a kind of biofilm (Figure 1), while inside root tissue colonization by single cells was observed (Figure 2; Figure 3).

Methylobacterium CP1 pMP7604-tagged cells were also observed colonizing the xylem vessels in root and shoots of *C. pumila*, but only when plants were growing in the presence of Cd or Zn (Figure 4). Stem or root xylem colonization was not observed in plants growing in medium without PTEs. To check the presence of *Methylobacterium* sp. CP1 pMP7604-tagged cells inside vascular plant tissue different Z planes were observed (Figure 5). Orthogonal merged images were also obtained. *Methylobacterium* sp. CP1 pMP7604-tagged cells were detected inside the xylem tissue of *C. pumila* stem (Figure 6).

During microscopical analysis, higher amounts of tagged cells were observed in roots than in stems. This was confirmed after isolating tagged cells from surface sterile plant tissues (Figure 7). The amount of cfu of *Methylobacterium* sp. CP1 pMP7604-tagged cells in roots was $3.2x10^6$, compared to $0.17x10^6$ observed in stems of *C. pumila* growing in medium supplemented with Cd and Zn.

No *Methylobacterium* sp. CP1 pMP7604-tagged cells were isolated from surface sterilized stems of *C. pumila* that were not exposed to PTEs. From seedlings grown in this condition, *Methylobacterium* sp. CP1 pMP7604-tagged were isolated only from roots. The amounts of mCherry-labeled *Methylobacterium* sp. CP1 cfu isolated from roots were similar for plants with and without PTEs exposure (Figure 7).

Discussion

In this study labeling of seed endophytes was successful for one out of six bacterial endophytes. Also Jonhsnton-Monje and Raizada (2011) found that positive *gfp* tagging was not possible for all endophytes under study, with 11 positive out of 124 isolates attempted. This illustrates that fluorescence tagging is a powerful technique, there still exist methodology limitations. This could be overcome with other methods, *in situ* hybridization for instance (Lo Piccolo *et al.* 2010; Compant *et al.* 2011; Lopez, Bashan and Bacilio 2011). Nevertheless, it was possible to obtain interesting data with the mCherry labeled *Methylobacterium* sp. CP1.

Bacteria belonging to the genus *Methylobacterium* have been reported as endophytes of different plant species growing on PTEs containing substrates; they were isolated from different tissues but mainly from stems and leaves (Idris *et al.* 2004; Dourado *et al.* 2015; Chen *et al.* 2014). In the present work colonization of pMP7604 tagged *Methylobacterium* sp. Cp3 inside

plant tissues was observed. At both experimental conditions, *i.e.* with and without PTEs exposure, the tagged strain was detected intracellularly in root cortex cells (Figure 2, 3). The ability of different *Methylobacterium* species to colonize inner plant tissues of *Cataranthus ruseus* has been shown previously (Gai *et al.* 2009; Filho *et al*. 2012). However, in the latter studies, *Methylobacterium* was used as a model endophyte for the behavior of pathogenic bacteria and the presence of PTEs was not an experimental factor.

As mentioned above, *Methylobacterium* sp. Cp3 was isolated from seeds of a plant growing on PTEs containing mine residues (Sánchez-López *et al*. 2015b, submitted). Nevertheless, in the present work inoculation of pMP7604-tagged *Methylobacterium* was not performed on seeds but to explore its ability to colonize and move through plant tissues inoculation was done at root level. Several rhizosphere derived bacteria do not only colonize the rhizosphere and root surface but can also enter plant roots, spread systemically and colonize internal tissues; many of them were shown to induce plant growth-promoting effects (Sessitsch, Reiter, and Berg 2004; Compant *et al.* 2005, 2008; Johnston-Monje and Raizada 2011).

Earlier results showed that *Methylobacterium* sp. Cp3 possesses plant growth promoting traits like Indol Acetic Acid production, phosphate solubilization and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, traits that have the potential to enhance survival rate of *C. pumila* growing on PTEs contaminated soil (Sánchez-López *et al*. 2015c, submitted). Further, this strain is tolerant to Zn (5 mM) and Cd (1.6 mM) (Sánchez-López *et al*. 2015b), and induced higher Cd shoot concentrations in *C. pumila* when growing on PTEs containing soil (Sánchez-López *et al*. 2015c). By consequence, *Methylobacterium* sp. Cp3 may produce benefits to its host *C. pumila* when this is exposed to PTEs.

It has been suggested that endophytes move throughout the plant up to the aerial plant parts; colonization of vascular tissue to allow systemic spreading into plant shoots (Reinhold-Hurek and Hurek 2011), being the most likely route via the xylem (Compant *et al.* 2008). Observations from the present work indicate that after entering the root tissues pMP7604-tagged *Methylobacterium* sp. Cp3 colonize the root xylem vessels and afterwards migrate towards aboveground plant parts. However, this could be observed only when plants were growing in the presence of Cd and Zn (Fig. 4, 5, 6). These results support the hypothesis of enrichment of certain endophytes in function of the presence of contaminants as mentioned previously by Siciliano *et al*. (2001), Truyens *et al.* (2013, 2014) and Croes *et al.* (2015). Endophytic colonization is the result of a complex communication between microorganisms and their host plant (Kogel *et al.* 2006; Hardoim *et al*. 2008); however, depending on the environmental conditions, plants may control colonization of endophytes in order to receive their benefits. In order to track the delivery of endophytes benefits *in planta*, for future studies we suggest the development and use of other techniques such polymerase chain reaction using specific primers, analyzing expression of a reporter gene (Yoon *et al*. 2015) or visualization of this kind of gene by *in vivo* detection of bioluminescence (Cantag and Bachmann 2002).

In several investigations the inner colonization of root tissues (including cortex and xylem) by plant growth promoting bacteria, and the eventual migration of these bacteria toward aboveground plant part were reported. In these studies, bacteria were isolated from rhizosphere (Compant *et al.* 2005, 2008) and also some endophytes from stems (de Procopió *et al*. 2011; Anand and Chanway 2013; Filho *et al.* 2012) and seeds (Verma *et al*. 2004; Ferreira *et al.* 2008; Johnston-Monje and Raizada 2011) were used. However, these studies did not take into consideration the presence of PTEs.

Using *gfp* transformation, Zhang *et al*. (2013) demonstrated that the root endophytes *Burkholderia, Sphingomonas* and *Variovorax* colonized the root surface of their host plant, *Sedum alfredii*, when growing on a PTEs containing substrate. Only *Burkholderia* and *Variovorax* were observed inside the root cortex also in the presence of PTEs. However, bacterial colonization of the plant vascular system was not reported.

To our knowledge the present research is the first report where a seed endophyte was observed colonizing the inner root and stem xylem, suggesting that the presence of PTEs promotes endophyte migration to the aboveground plant parts. Still more research taking into consideration other seed endophytes and plant species is required. Schulz and Boyle (2006) mentioned that endophytic bacteria are those isolated from plant tissue whose surface has been disinfected and that are able to re-colonize internal tissues of disinfected seedlings. According to these criteria *Methylobacterium* sp. Cp3 isolated from *C. pumila* seeds is a real endophyte.

These observations together with the fact that *Methylobacterium* sp. Cp3 was isolated from seeds support the hypothesis that seed endophytes can enter root cells, move through the xylem and reach different aerial plant parts including seeds, allowing them to be transferred to successive plant generations. As a next step in this research it is suggested to track endophytic bacteria along plant life cycle until production of new seeds. Since effective colonization by the seed endophyte *Methylobacterium* sp. Cp3 was demonstrated, these results are promising for novel applications of endophyte-stimulated phytoremediation of PTEs-contaminated soils.

Conclusions

The ability of the seed endophyte *Methylobacterium* sp. Cp3 to colonize tissues of its host plant host was studied using mCherry labeling. In absence of PTEs, mCherry-tagged

Methylobacterium was shown to colonize *C. pumila* root cortex cells. However, in the presence of Cd and Zn mCherry-tagged bacterial colonization was observed not only in root cortex, but also in root and stem xylem. Our results confirm that *Methylobacterium* sp. Cp3 is a real endophyte. One of the requirements for successful application of endophytes at large scale phytoremediation is the effective colonization of plant tissues in PTEs contaminated environments. Taking into account plant growth promotion features and endophytic colonization abilities of this strain, *Methylobacterium* sp. Cp3 has potential to be used for endophyte-assisted phytoremediation of PTEs-contaminated soils. Additionally, these results indicate that seed endophytes can enter root cells, move through the xylem and reach different organs (*e.g.* shoot and seeds) and that they thus can be transferred to successive plant generations.

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Table 1. Bacterial strains and plasmids.

Bacterial strains	Characteristics	Source
Recipients		
Methylobacterium sp. Cp3 *Variovorax* sp. Cp29 *Variovorax* sp. Cp30 *Brachybacterium* sp. Cp27 *Sphingomonas* sp. Cp1 *Caulobacter* sp. Cp32	Seed endophytic bacteria isolated from *Crotalaria pumila* growing on PTEs-containing mine residues.	Sánchez-López *et al*. (2015b; submmited)
Plasmids		
Escherichia coli H2013	Helper plasmid	Lajendik *et al*. (2010)
Escherichia coli pMP7604	Donor of plasmid harboring mCherry construct	Lajendik *et al*. (2010)

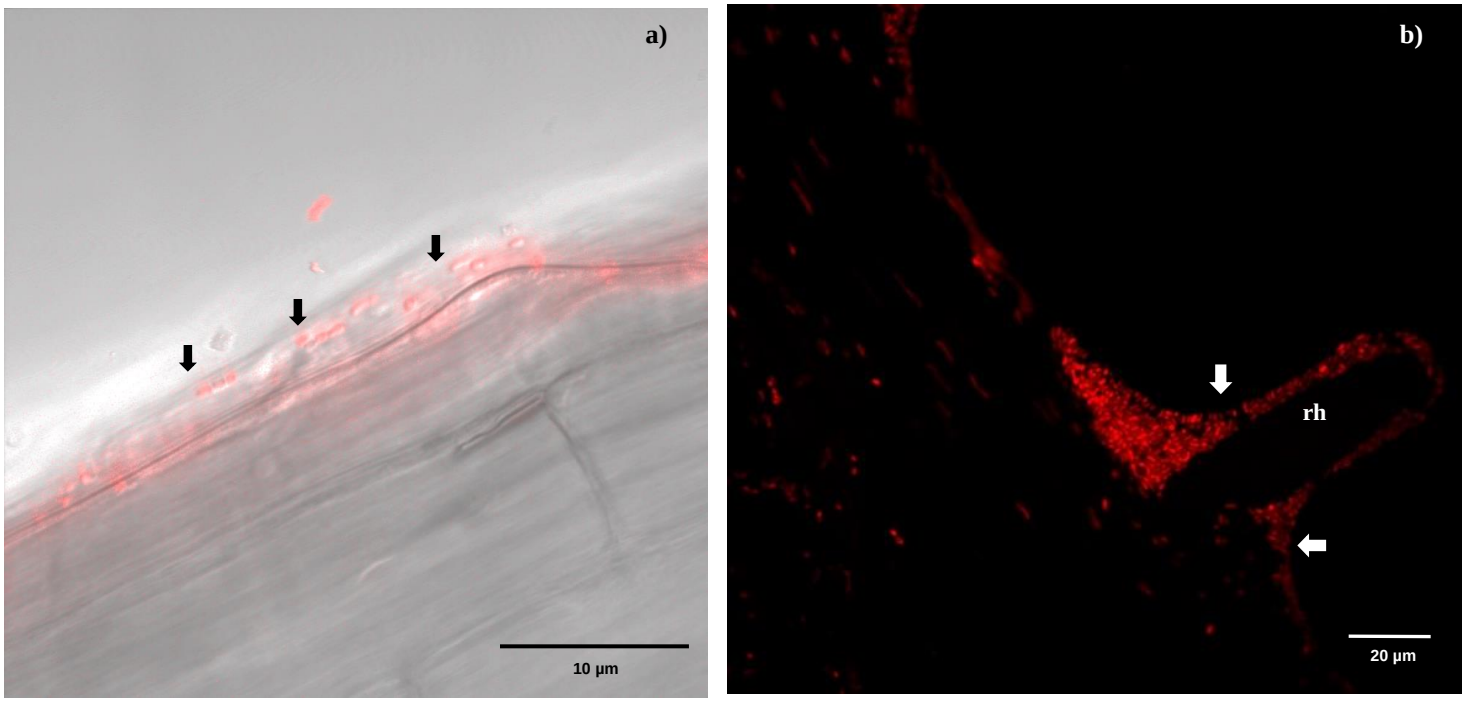

Figure 1. Confocal laser scanning microscopy image of mCherry-tagged *Methylobacterium* forming a film (arrows) on root hairs surfaces of *Crotalaria pumila* (a). mCherry-tagged *Methylobacterium* cells (arrows) on root hair (rh) surface of *Crotalaria pumila* in medium supplemented with Zn and Cd observed using confocal laser scanning microscopy (b).

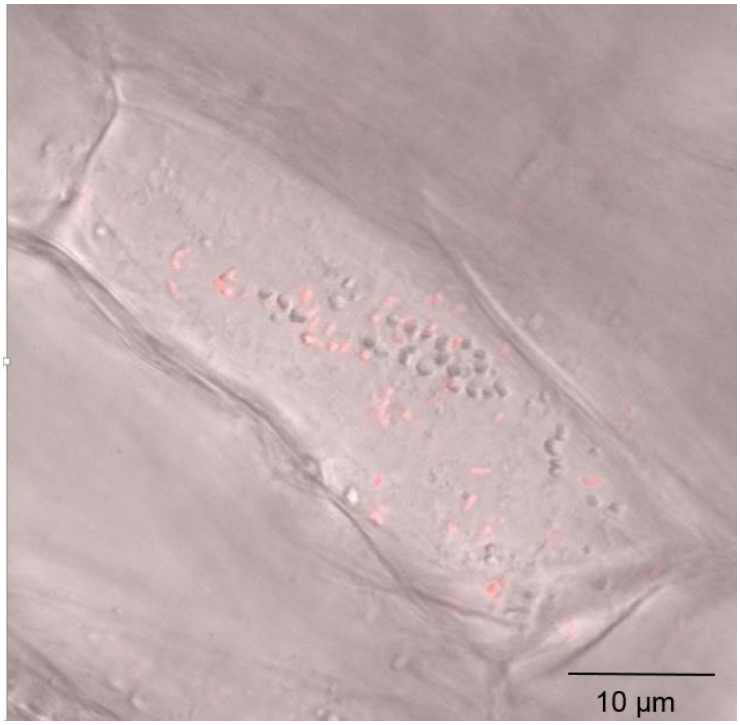

Figure 2. mCherry tagged *Methylobacterium* sp. Cp3 localized intracellularly in root cortex of *Crotalaria pumila* without PTEs addition in growth medium. Image obtained by confocal laser scanning microscopy.

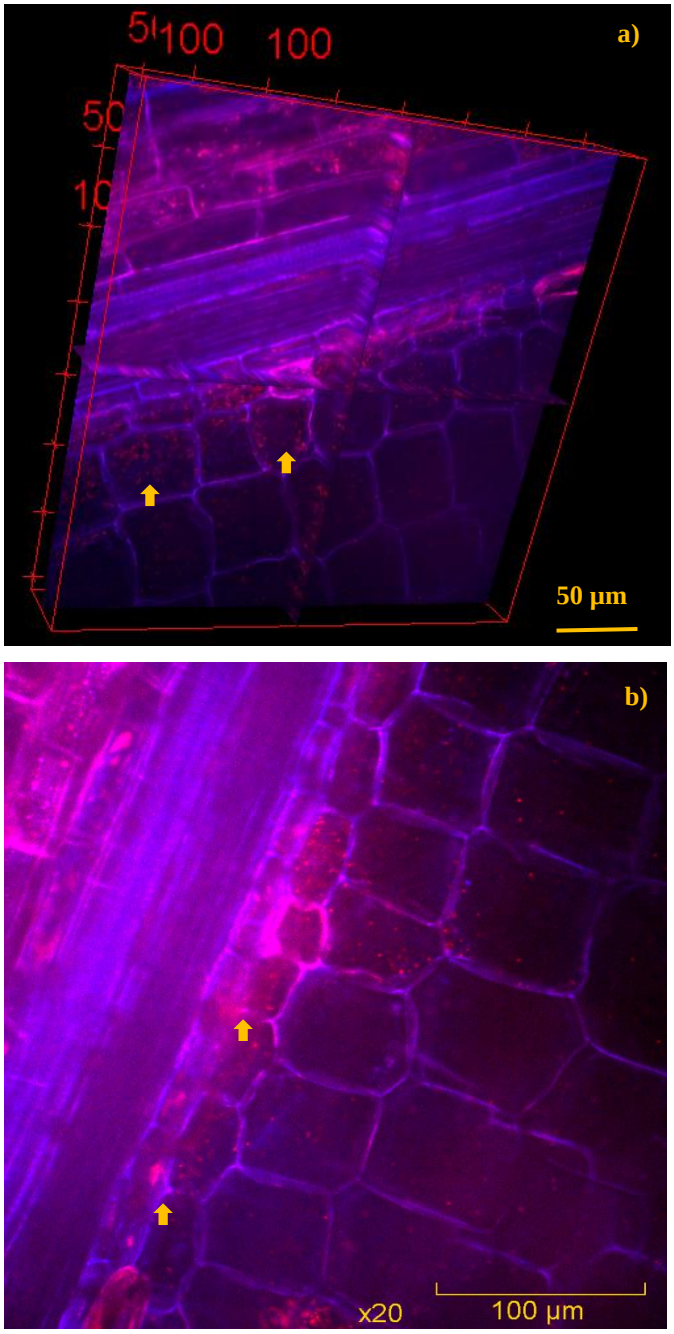

Figure 3. 3D projection of mCherry tagged *Methylobacterium* (arrows) localized intracellularly in root cortex (a) and visualization within root vascular system of *Crotalaria pumila* growing in medium supplemented with Zn and Cd (b). Image obtained by confocal laser scanning microscopy.

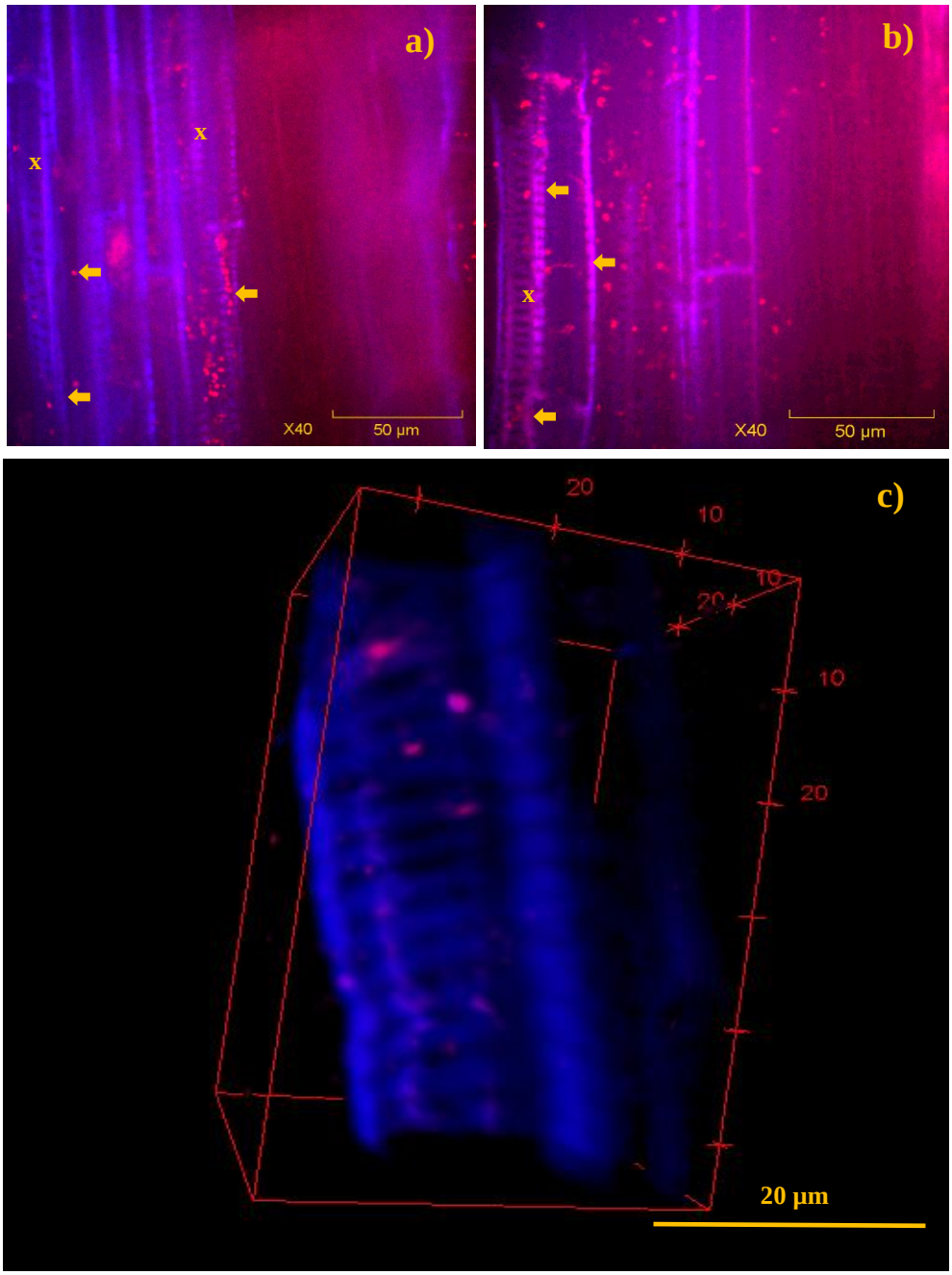

Figure 4. Visualization of mCherry-tagged *Methylobacterium* in xylem (x) of root (a) and stem (b) of *Crotalaria pumila* growing in medium supplemented with Zn and Cd. 3D projection of mCherry-tagged *Methylobacterium* in stem xylem (c). Images obtained by confocal laser scanning microscopy.

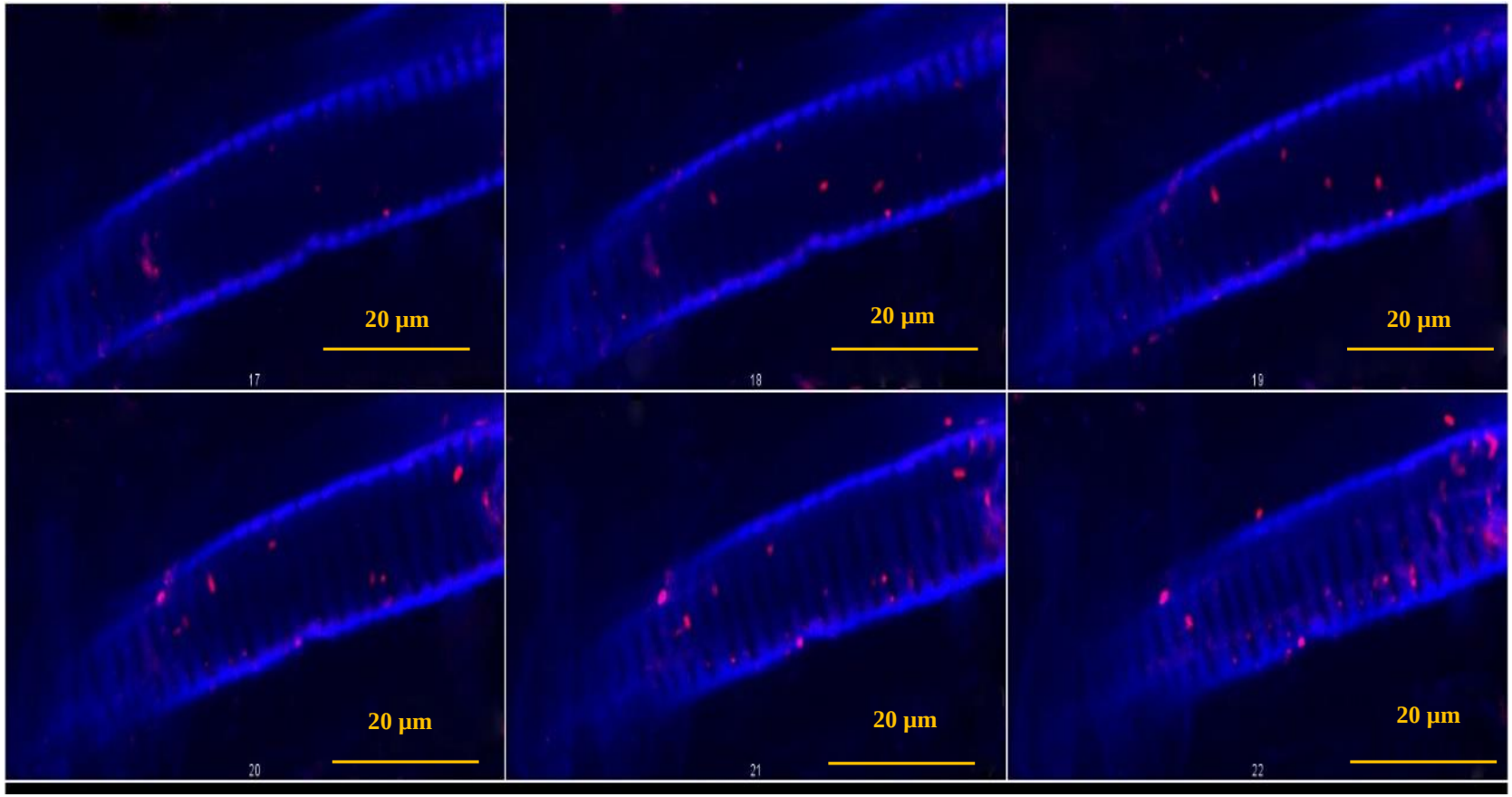

Figure 5. Montage of different consecutive Z planes where mCherry –tagged *Methylobacterium* is observed inside stem xylem of *Crotalaria pumila* growing on medium supplemented with Zn and Cd. Z planes allow corroborating the presence of mCherry –tagged *Methylobacterium* at different depths inside stem xylem.

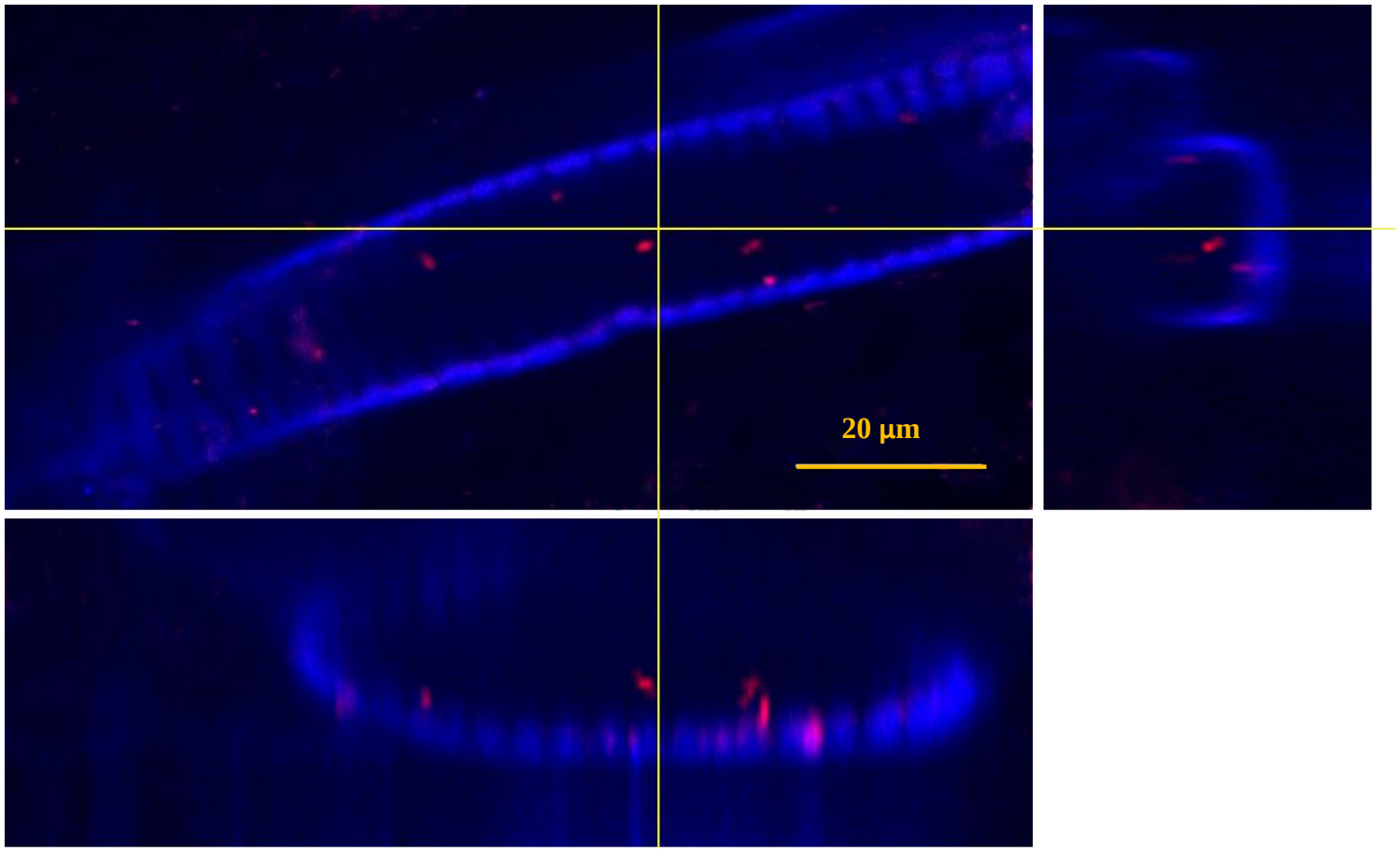

Figure 6. Orthogonal merged image of *Crotalia pumila* stem xylem, mCherry-tagged *Methylobacterium* is observed inside xylem tissue (arrows).

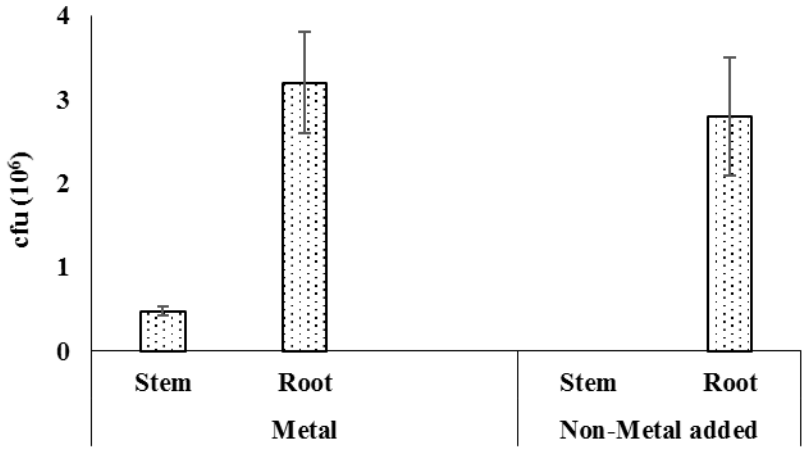

Figure 7. Colonies forming units (cfu) of mCherry-labeled *Methylobacterium* isolated from surface sterilized roots and stems of *Crotalaria pumila.*

GENERAL DISCUSSION AND CONCLUSIONS

As a consequence of concern about environmental issues and public health problems caused by potentially toxic elements (PTEs) contamination different remediation technologies have been proposed. Among these procedures, the use of plants - phytoremedation- has received particular attention in the last years (Ali, Khan, and Sajad 2013; Adki, Jadhav, and Bapat. 2014; Mani and Kumar 2014), this is apparently due to the relative low costs and environment impact of application. However, the success of phytoremediation at field conditions depends on several factors including the type and concentration of contaminants; the ability of plants to establish on polluted soil and eventually to absorb or stabilize PTEs (Vangronsveld *et al*. 2009; Mench *et al.* 2010). PTEs polluted soils usually contain low concentrations of nutrients and organic matter; this fact together with the presence of toxic contaminants results in restrictive conditions for plant establishment and further growth. The use of plant associated microorganisms, or assisted phytoremediation, has been proposed to overcome such limitations (Ullah *et al*. 2015). Most of the research regarding plant-associated microorganisms has been focused on rhizospheric bacteria. However, due to the closer interaction between plants and endophytic bacteria, in recent years the function and potential use of such microorganisms on effective PTEs phytoremediation has been addressed (Weyens *et al*. 2009; Wani *et al.* 2015). Taking into account diversity and concentration of contaminants; responses of plants and their associated microorganisms; and the specific environmental conditions present on contaminated sites we realized that all mentioned factors result in a complex unique system. Therefore, more research is needed to better understand, and eventually take advantage of the contaminant-plant-microorganisms interactions in PTEs polluted soils.

Based on the available information, the present dissertation was performed to gather knowledge about the responses and interaction of plants-endophytic bacteria-PTEs in a mining area located in a semi-arid environment, Zimapan, Mexico. To address this general objective a set of studies were performed. Firstly, chemical and mineralogical characterization of nine deposits of mine residues were described. The effect of handle of mine residues on PTEs release from tailing heaps was investigated. Secondly, pioneers wild plants, observed growing on two mine tailings, were identified for their abilities to either accumulate PTEs in shoots or stabilize these elements into their roots or in the rhizosphere. These two mine tailings were characterized by clearly different chemical and mineralogical characteristics representing different degree of oxidation. Thirdly, besides accumulation and stabilization of PTEs, the function of plants as physical barriers against atmospheric dispersion of particles bearing PTEs from bare mine tailings was documented. Identification of plants with potential to retain particles and contribution of their leaf structures was also investigated. Fourthly, during sampling of mine residues and plants, a plant species, *Crotalaria pumila*, was observed to complete its life cycle and produce viable seeds while growing on mine residues. Therefore, this species was selected to study the role of plant-associated microorganisms during the establishment of *C. pumila* on mine residues containing PTEs. Seed endophytic bacteria from three consecutive generations of *C. pumila* were isolated, identified and characterized for their plant growth promotion features. A comparison about composition and function of these bacteria with the endophytic community of *C. pumila* seeds from a non-contaminated site was also made. Fifthly, in a pot experiment, selected bacterial isolates (with plant growth promotion traits) were tested for their beneficial effects on plant growth and PTEs uptake/stabilization by *C. pumila* plants. Finally, the endophytic plant

colonization ability of selected bacterial strains was studied through labeling with a fluorescent protein.

The first results showed that among the nine studied mine tailings, several of them presented chemical features and mineral composition indicating risk for PTEs releasing and spreading into the environment. These tailings were considered as oxidized. However, other mine tailings did not present current oxidation advances, although, due to their mineralogical composition, oxidation and PTEs release will most likely appear. Differences in chemical characteristics and mineralogical composition were observed among the nine tailing heaps (Chapter 1 Table 1, 2). The characteristics of the residues also varied between the slope and plateau of the same tailing heap. While slope residues were oxidized and had high proportion of extractable PTEs, residues from plateau remain in the reduced state and the proportion of extractable PTEs was low (Chapter 1 Figure 2, 3). Normally, these differences can be attributed to the original characteristics of mine residues and the exposure time (Romero *et al*. 2008; Moreno-Tovar, Barbason and Coreño-Alonso 2009). However, obtained results showed that the percentage of DTPA-extractable PTEs, as an indicator of potential release of such elements from mine tailings, was affected by other factors too. Tailings heaps were constructed as stair-step impoundments within a canyon (Chapter 1 Figure 1) with as a consequence differences in wetting-drying cycles. Even in the same tailing heap the oxygenation may be different, due to the fact that particles were separated by size during the construction. Although the mine residues originated from the same mine, even residues from the same pile (slope and plateau) showed different chemical and mineralogical characteristics (Chapter 1 Table 1, 2). The addition of a fresh residue layer over the oxidized tailing heap certainly cause these differences. Thus, the propounded hypothesis: the percentage of extractable PTEs, regarded as a result of chemical characteristics and mineralogical composition of mine

residues is affected by the management of the mine tailings; was accepted. Results indicated a unique phytoremediation strategy cannot be applied for all mine tailings originating from the same mine. Instead, each tailing heap or the plateau and slope of the same pile requires each a specific phytoremediation plan.

The second set of results shows that despite the variety and high concentrations of PTEs in mine residues, wild flora colonized these sites. Such plants are already adapted to the limiting conditions present in mine tailings (González and González-Chávez 2006; Mendez and Maier 2008) and to the semi-arid environmental conditions in Zimapan. Consequently, such plant species are candidates to be used for phytoremediation purposes. A total of 12 plant species were identified at two mine tailings with different chemical characteristics.

Two types of phytoremediation of PTEs can be distinguished: phytoextraction and phytostabilization (Arthur et al., 2005; Bolan et al. 2014). Generally, a comparison between the bioconcentration factor and the translocation factor allows to decide if plants accumulate PTEs in their shoots or exclude them (Peuke and Rennenberg 2005; Yoon *et al.* 2006). However, there exists no general consensus on how to calculate such factors. It is therefore required to specify and validate which methodology is the best for the calculation of bioconcentration and translocation factors. Among the different factors interfering in the calculation of bioconcentration factors, a very important question is which soil PTEs concentration should be taken into account, the rhizospheric one or that of the bulk substrate, the total or extractable concentrations. Obtained results showed differences between rhizospheric and bulk substrate conditions (Chapter 2 Table 1). For instance, one mine tailing was oxidized and had very acidic pH (1.7); whilst, the pH of rhizospheres of plants established on such residues varied between 5.8 and 6.8. For this reason, the importance of rhizospheric processes in phytoremediation is

recognized, but at the same time their complexity and heterogeneity is accepted. This fact, together with the specific chemical characteristics present at two mine tailings may explain that the same species growing on the two study sites presented different behavior regarding PTEs accumulation. For example, *Dalea bicolor* was behaving as a Zn accumulator at one site; while, the same species acted as Zn excluder at the other site (Chapter 2 Figure1, Table 2). Nevertheless, taking into account PTEs concentrations in shoots, roots and rhizospheres of the studied plant species it was possible to identify some candidate species for phytoremediation purposes as mentioned in the hypothesis. The hypothesis "among native flora colonizing mine residues there are plant species appropriate for phytoextraction or phytoestabilization of PTEs" was accepted. *Pteridium* sp. showed suitable for Zn and Cd phytostabilization; while *Aster gymnocephalus* might be a potential phytoextractor for Zn, Cd, Pb and Cu, *Gnaphalium* sp. for Cu and *Crotalaria pumila* for Zn. However, it is necessary to keep in mind the limitations of phytoextraction.

Phytoextraction can only be used for low to moderately contaminated soils; its applicability is limited to rooting depth, which is dependent among differences of root system of plant species. It is necessary to use fast-growing plant species that are able to accumulate high PTEs concentrations in harvestable plant tissues and that at the same time produce a high biomass. Moreover an efficient processing of harvested plant material is necessary (Robinson, Anderson, and Dickinson. 2015). It is obvious that phytoextraction is not a feasible technology for mine tailings. In contrast, phytostabilization should be used to reduce the mobility, bioavailability and spreading of pollutants in the environment. The basis of phytostabilization is to decrease PTEs bioavailability by fixing elements in the rhizosphere or in roots (Vangronsveld *et al*. 2009). Therefore, phytostabilization does not depend on plant biomass production, neither on PTEs

accumulation in shoots, and, since it aimed to be a low maintenance vegetation cover, it does not require plant processing after harvest.

There was not a high diversity of plant species found on mine residues; however, studied plants involved annual species, shrubs, perennial herbs, and creeping perennial species, which allowed the establishment of a vegetation cover on bare mine tailings along the year. This information is very relevant since the study site is located in a semi-arid area, and due to this the mine residues are dry for the major part of the year. By consequence, aerial dispersion of particles containing PTEs from the bare mine tailings is very common. Besides the fact that the vegetation cover is stabilizing the tailings, the surfaces of the plants represent an important sink for diverse pollutants in terrestrial environments (Nowak, Crane, and Stevens 2006; Terzaghi *et al*. 2013). To our knowledge studies regarding plants as sinks of particles containing PTEs has not been carried out before in mining areas. The stated hypothesis "plant species colonizing mine residues can retain PTEs containing particles on their leaf surfaces" was accepted and demonstrated by the obtained information. Results from this thesis showed that on mine tailings vegetation participates to reduce PTEs dispersion not only by stabilizing the tailings and accumulation in roots and shoots, but also by retention of particles on aerial plant parts. It was proposed to call this principle phytobarriers. *Brickellia veronicifolia* was the species that contributes the most in particles retention (Chapter 3 Figure 2), followed by *Viguiera dentata* and *Dichondra argentea*. Unwashed leaf samples of *B. veronicifolia* showed up to 3 times higher amount of particles than washed leaves. The magnitude of PTEs held on leaves surfaces varied according the element, plant species and site of sampling. But it was remarkable that particles retained on leaf surfaces represented a concentration of Mn up to 24 times higher than inside plant tissue; and 14 times in the case of Zn (Chapter 3 Table 1). Depending on leaf morphology all plant species participated

at different extents in particle retention. Thus, the second hypothesis proposed on this chapter was accepted: the amounts of retained particles on leaves differ among plant species. In general it can be stated that, plants with higher amounts or longer/more branched trichomes retained the highest amounts of particles (Chapter 3 Figure 4). Glands and stomata were observed retaining particles too (Chapter 3 Figure 4). Besides the leaf characteristics themselves, it was remarkable that fungal mycelium also participated in retention of particles containing PTEs (Chapter 3 Figure 5). The participation of phyllospheric fungi in capturing particles containing PTEs was documented for the first time. As a consequence, a new research field regarding plant- microbe interaction in phytoremediation was opened.

Studies searching for plants suitable for phytoextraction mainly use concentrations of PTEs in shoots (together with biomass production per hectare) to decide about eventual applicability of a certain species to extract PTEs from contaminated soils. Information arising from this thesis shows that the application of an adequate washing protocol is crucial in this kind of analysis. In most of the works the plant samples were washed only with distilled or deionized water. Our data demonstrate that this is not sufficient. Particles containing PTEs on leaf surfaces significantly influenced the measured concentrations of these elements in aerial tissue (Chapter 3 Table 1). For instance, *Gnaphalium* sp. (544 mg kg^{-1}) exceeded the Cu hyperaccumulation level of 300 mg kg^{-1} (van der Ent *et al.* 2013) while, in the same plant species but washed following a thorough protocol the Cu concentration was below this level (274 mg kg^{-1}). The results illustrate that an inadequate washing protocol does not eliminate all leaf surface retained PTEs, leading to an overestimation of PTEs concentrations in plant tissues results and an incorrect estimation of the PTEs accumulating or hyperaccumulating capacities of plants.

Besides the functions of plants themselves during phytoremediation, it is important to take into account the benefits that associated microorganisms generate to their host plants to better understand how these plants overcome the adverse environmental factors commonly found on polluted sites. Another aspect considered in this thesis was the study of endophytic bacteria of a plant species growing on mine tailing containing multiple PTEs. *Crotalaria pumila* was chosen because during three consecutive years, it was observed to complete its life cycle and produce viable seeds when growing on the mine tailings. Moreover, among the studied plant species *C. pumila* was the most abundant and concentrations of Zn, Cd, Pb, and Cu in shoots were above concentrations that are normally toxic for most plant species (Vamerali, Bandiera, and Mosca 2010). Endophytic bacteria from three consecutive generations of *C. pumila* seeds were isolated, identified and biochemically characterized for their plant growth promotion traits. These results were compared to endophytes originating from *C. pumila* reference seeds (collected from non-polluted site).

Results validated the two following hypotheses: i) the composition of seed endophytic bacterial community of *C. pumila* growing on mine tailings is different from that of plants of the same species growing in non-contaminated site; ii) functional traits of endophytic bacteria of *C. pumila* are different between seeds collected on mine tailings and seeds from non-contaminated site. Eleven isolates classified in 7 genera were identified from reference seeds (Chapter 4 Figure 1). Phosphate solubilization, organic acids and indol acetic acid production were common traits in these isolates (Chapter 4 Table 1). In total, 33 strains belonging to 13 bacterial genera were isolated from mine residue seeds. Two strains of *Methylobacterium* and one of *Sphingomonas* were present in the three generations of cultivable endophytic communities (Chapter 4 Figure 1). Phytate mineralization, 1-aminocyclopropane-1-carboxylate deaminase activity, phosphate

solubilization and PTEs tolerance were common functional traits of mine residue seed isolates (Chapter 4 Table 1). Undoubtedly, improving plant nutrition by solubilization of phosphate and reducing levels of the stress hormone ethylene are very important factors for plant establishment in limiting or stressful conditions (Truyens *et al*. 2014). These traits together with the PTEs tolerance of the seed endophytes (Chapter 4 Table 2) may explain the success of *C. pumila* to germinate and to complete its life cycle on a multi-PTE contaminated substrate, tolerating high PTEs concentrations and the inherent environmental restrictions of a semi-arid climate. Obtained data suggest that selected endophytic bacteria are transferred via seeds to eventually bring benefits to their plant host. These findings support both hypotheses: i) seeds of *C. pumila* growing on mine tailings contain cultivable endophytic bacteria that are transferred transgenerationally; ii) seed endophytic bacteria present functional characteristics that contribute to the establishment and growth of *C. pumila* plants on mine tailings containing PTEs.

The capability of seed endophytes to promote plant establishment and growth on Zn- and Cd-containing soil was tested on two batches of *C. pumila* seeds, one batch collected from plants growing on mine residues and the other one from a non-contaminated site. Ten bacterial isolates were selected based on their *in vitro* plant growth promoting characteristics and PTEs tolerance. Results indicated differential responses to endophyte inoculation in the same plant host species originating from polluted or non-polluted conditions. Regarding bacterial inocula, *Methylobacterium* sp. triggered the highest survival rate and a *Variovorax* consortium the highest biomass production (Chapter 5 Table 2). However, inoculation with *Sphingomonas* sp. apparently resulted to be stressing for plants and had negative effects on biomass production (Chapter 5 Figure 3, Table 2). Also PTEs accumulation and translocation from roots to shoots were affected by seed endophytes; all inocula except *Bacillus* sp. diminished PTEs translocation compared to

non-inoculated plants (Chapter 5 Figure 2). Because of the variable trends the stated hypotheses were not accepted: i) selected seed endophytic bacteria stimulate biomass production of *C. pumila* arising from two different seed batches (collected from mine residues or non-contaminated site) when the plants are growing on PTEs polluted soil; ii) studied seed endophytic bacteria affect translocation of PTEs of *C. pumila* arising from two different seed batches (collected from mine residues or non-contaminated site) when the plants are growing on PTEs polluted soil; iii) inoculation with seed endophytic bacteria promotes activity of antioxidative enzymes of *C. pumila* arising from two different seed batches (collected from mine residues or non-contaminated site) in the presence of PTEs. Due to the miscellaneous plant response, it is suggested to test endophytic bacteria consortia to possibly observe their synergistic effect. Still, results indicate practical applications of *C. pumila* and its seed endophytic bacteria to promote revegetation and remediation (phytoestabilization) of PTEs-contaminated soils through the establishment of vegetation cover.

The success of endophytes at large scale phytoremediation requires both an efficient colonization of the plant tissues and the maintenance of the beneficial properties inside the plant (Ribeiro-Torres *et al.* 2013). To evaluate these aspects live cell techniques, like stable fluorescent labels together with microscopical tools, are useful (Valdivia, Cormack, and Falkow 1998). Zhang *et al*. (2013) studied endophytic colonization in the presence of PTEs; these authors observed bacterial colonization only in root cortex cells, endophytic bacteria inside the plant vascular system were not reported.

To study the colonization ability of seed endophytes of *C. pumila* in the presence of PTEs five endophytic isolates, previously tested for their plant growth promotion abilities (above paragraphs), were transformed with a plasmid harboring the mCherry fluorescent construct

(pMP7604). Transconjugation was successful only for *Methylobacterium* sp. Labeled bacterial cells colonized the main root and root hair surfaces (Chapter 6 Figure 1), and also cortex cells (Chapter 6 Figure 2, 3). Thus, the hypothesis "seed borne *Methylobacterium* sp. colonizes the inner root tissue of *C. pumila* in presence of PTEs" was accepted. Colonization of the xylem vessels in root and shoots of *C. pumila* was observed only in the presence of Cd and Zn (Chapter 6 Figure 4, 5, 6). Consequently, the following hypotheses were confirmed: i) seed endophyte *Methylobacterium* colonizes the transport system of its host plant when growing in the presence of PTEs; ii) labeled *Methylobacterium* migrates from roots to shoots of *C. pumila* in the presence of PTEs. Previous works reporting colonization of the plant vascular system by endophytic bacteria have not taken into account exposure to PTEs (Johnston-Monje and Raizada 2011; Anand and Chanway 2013). To our knowledge the present research is the first report where a seed endophyte was observed colonizing the inner root and stem xylem. These observations validate that labeled *Methylobacterium* sp. is a real endophyte. Moreover, our results support the theory that seed endophytes can enter root cells, move through the xylem and reach different organs (*e.g.* seeds) and thus they can be transferred to successive plant generations. These results show potential field application of endophytes for phytoremediation of PTEs contaminated soils.

In conclusion, the results from this thesis have helped to provide a better understanding about PTEs-plants-microorganisms interactions, and its eventual application for phytoremediation. The main outcomes were:

1) Due to the chemical and mineralogical differences observed among tailing heaps from the same mine, and even between the slope and plateau of the same mine tailing, it is not possible to propose a general phytoremediation formula. For mitigation and remediation plans it is important take into account the specific chemical and mineralogical

characteristics of the site that needs to be remediated leading to different PTEs exposure of plants.

2) The specific construction approach of mine tailings, management of the mine residues and the location of the pile within the valley in the studied area affect the potential risks of PTEs release into the environment and thus needs of phytoremediation.
3) Despite the low diversity of plant species, the native and wild flora colonizing the mine residues provide us some plant species that are adapted to multi-PTE containing mine residues in the Mexican semi-arid region of Zimapan, Hidalgo.
4) The identified flora involved annual species, shrubs, perennial herbs, and one creeping perennial plant; this allows the establishment of a plant cover that diminishes dispersion of PTEs from bare mine tailings throughout the year. Assisted establishment of higher plant cover should be performed to enhance natural attenuation occurring on these PTEs polluted sites.
5) After further investigations, some plants might be considered for phytoextraction purposes of PTEs-contaminated substrates. Such species are *Aster gymnocephalus* for Zn, Cd, Pb and Cu; and *Crotalaria pumila* for Zn.
6) *Pteridium* sp. is a plant species suitable for phytostabilization of PTEs.
7) Some plant species (*Dalea bicolor, Viguiera dentata, Brickellia veronicifolia*) can change their PTEs accumulation behavior according to environmental and rhizosphere characteristics and specific PTEs. At one site they act as accumulators, while in a different site they are excluders of certain element.

8) Rhizospheric processes are important for PTEs phytoremediation. Each plant species and the diverse PTEs formed a complex system with characteristics different from the bulk substrate.

9) Plants can participate in reducing PTEs dispersion in the environment in three ways: a) accumulation of PTEs in aboveground tissues (phytoextraction), b) immobilization of PTEs in root or rhizosphere (phytostabilization), and c) retention of particles containing PTEs on leaves surfaces (phytobarriers). This is the first report concerning the functioning of plants as a physical barrier (phytobarriers) to avoid aerial dispersion of PTEs-containing particles arising from bare mine residues. These results are the basis for further studies regarding vegetation covers as sinks for atmospherically dispersed PTEs arising from mine residues.

10) *Brickellia veronicifolia* is the species that retains the highest amount of PTEs containing particles on its leaves. Particles were retained mainly in the gully formed by the veins.

11) The leaf structures that contributed most to particle retention in the majority of the studied plant species were the trichomes, although other structures such as veins, epidermal glands and stomata also contributed to retention of PTEs containing particles.

12) Mycelium of epiphytic fungi also participated in retention of PTEs containing particles. These results are innovative in scope of plant-microorganisms interaction for PTEs phytoremediation.

13) Inadequate washing protocols lead to an overestimation of PTEs concentrations in plant tissues and thus to an incorrect estimation of the PTEs accumulating or hyperaccumulating capacities of a certain plant species.

14) This is the first time that seed endophytic bacteria present in a natural multi-PTE contaminated (Zn, Cd, Pb, Cu, Ni, Co) system were isolated, identified and characterized according their plant growth promotion and PTEs tolerance traits.

15) Differences in community composition of cultivable endophytes were observed between *Crotalaria pumila* seeds from a non-contaminated site and those from mine residues. Thirty-three strains belonging to 13 bacterial genera were isolated from mine residue seeds. Whilst, eleven isolates classified in seven genera were identified in seeds from non-contaminated site. This is the first study of seed endophytes of a plant colonizing mine residues.

16) Differences in functional traits of cultivable endophytes were observed between *C. pumila* seeds from a non-contaminated site and those from mine residues. Endophytic bacteria present in seeds bring benefits to their host plant for germination, stress alleviation, subsequent growth and proliferation of *C. pumila* on PTEs containing-mine residues.

17) Two isolates of *Methylobacterium* and one of *Sphingomonas* were observed in three consecutive generations of *C. pumila* seeds collected from PTEs-containing mine residues. Functional traits of these bacterial strains suggest vertical transmission of seed endophytes is not a random process; instead, the selected seed endophytes possess characteristics that are beneficial during germination and growth of *C. pumila* in PTEs containing mine residues.

18) *Crotalaria pumila* plants from the distinct seed batches (mine residues and non-contaminated site) responded differently to seed endophyte inoculation especially regarding survival rate and PTEs accumulation.

19) Different endophytic inocula induce distinct results when inoculated to *C. pumila*. *Methylobacterium* sp. promoted the highest survival rate of *C. pumila* when growing on Cd and Zn-containing substrate. The *Variovorax* consortium induced the highest plant biomass production. Inoculation with *Sphingomonas* sp. had negative effects on biomass production.

20) Using transconjugation with a plasmid harboring the fluorescent mCherry construct, it was possible to observe colonization of xylem vessels of root and stem of *C. pumila* in presence of Zn and Cd. The observations support the model that endophytes can enter root cells, move through the xylem and reach different organs, including seeds, and thus they can be transferred to successive plant generations. This evidence indicated that seed endophytes have potential for field application in phytoremediation of PTEs-contaminated soils. This is the first report of a seed endophyte colonizing the plant vascular system in both root and shoot, in the presence of Zn and Cd.

In general, this new information represents an important step towards a better understanding of PTEs-soil-plant-microorganisms interactions regarding phytoremediation purposes. The identification of PTEs hyperaccumulator plant species used to be main goal in PTEs phytoremediation studies. However, it was demonstrated that all plant species and their associated microorganisms are participating in remediation of PTEs contaminated sites in different ways.

We mentioned the necessity for specific phytoremediation strategy according to the specific characteristics of a polluted site, as well as the prospects that native flora and its associated microorganism offer to improve the phytoremediation process. It is suggested that before moving to genetic modification of organisms, the native flora and its associated microorganisms should

be studied in more detail. There is a wide field to explore the diversity of the plants themselves, and also their associated microorganisms to increase the knowledge of the complex interactions among them in multi PTE contaminated environments.

Up to now, most of the work performed on assisted phytoremediation focused on rhizospheric microorganisms. Certainly, endophytic microorganisms, both fungi and bacteria, also offer possibilities for their eventual application in the field. However, more knowledge about population dynamics and interrelations of PTEs tolerant endophytes and their host, native metalliferous and accumulator plant species, is required. New technologies including omics tools and bioinformatics approaches will allow to understand, in an integrated way, interaction dynamics among plants, microorganisms and PTEs. Eventually, this information will be useful to optimize phytoremediation of PTEs-polluted sites assisted by microorganisms.

Taking into account the chemical and mineralogical characteristics of mine residues it is possible to identify those PTEs with mobility risk. Subsequently, preventive measures using vegetation cover can be applied to avoid PTEs dispersion and their adverse effects on the environment and public health.

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RESEARCH OFFSPRINGS

Published scientific papers

- Sánchez-López AS, González-Chávez MCA, Carrillo-González R, Vangronsveld J, Díaz-Garduño M. 2015. Wild flora of mine tailings: perspectives for use in phytoremediation of potentially toxic elements in a semi-arid region in Mexico. International Journal of Phytoremediation 17(5): 476-484, DOI: 10.1080/15226514.2014.922922
- Sánchez-López AS, Carrillo-González R, González-Chávez MCA, Rosas-Saito GH, Vangronsveld J. 2015. Phytobarriers: Plants capture particles containing potentially toxic elements originating from mine tailings in semiarid regions. Environmental Pollution 205: 33-42. DOI: http://dx.doi.org/10.1016/j.envpol.2015.05.010

Participation in congresses

- Sánchez-López A, Carrillo-González R, Gutiérrez-Castorena MC, González-Chávez MC, Loeppert RH. 2011. Caracterización mineralógica de presas de jal y sus implicaciones ambientales. Poster. VI Congreso de la Sociedad Iberoamericana de Física y Química Ambiental (SiFyQA), Cancun, Mexico, 25-29 April 2011.
- Sánchez-López A, Carrillo-González R, González-Chávez, MC, Loeppert RH. 2011. Oxidation of tailings: chemical characterization and factors affecting it. Poster. 11th International Conference on the Biogeochemistry of Trace Elements. Florence, Italy, 3-7 July 2011.
- Sánchez-López AS, Carrillo-González R, González-Chávez MC, Loeppert R, Díaz-Garduño M, Velázquez-Aradillas JC. 2011. Contaminación de metales en la rizósfera de plantas silvestres que crecen en áreas mineras abandonadas en Zimapan, Hgo. Oral presentation.

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- Sánchez-López A. 2011. Implicaciones ambientales del intemperismo de residuos de mina. Oral presentation. Tercer Ciclo de Conferencias y Talleres del Cuerpo Académico Contaminación Ambiental, Benemérita Universidad Autónoma de Puebla. Puebla, Mexico, 3-4 November 2011.
- Sánchez-López A, González-Chávez MC, Carrillo-González R. 2012. Particle deposition containing metals on vegetation growing around a tailing mine in Hidalgo, Mexico. Poster. Urban Environmental Pollution: Creating healthy, liveable cities. Amsterdam, The Netherlands, 17-20 June 2012
- Sánchez-López A, González-Chávez MC, Carrillo-González R. Plants growing on mine tailings avoid dispersion and contribute to stabilization of potentially toxic elements. Oral presentation. 9th International Phytotechnologies Society Conference. Hasselt, Belgium, 11-14 September 2012.
- Sánchez-López AS, González-Chávez MCA, Carrillo-González R, Cruz-Díaz J, Díaz-Garduño M, Rodríguez-Justino L. 2012. Comportamiento de algunas plantas silvestres expuestas a residuos de mina. Oral presentation. XXXVII Congreso Nacional de la Ciencia del Suelo. Zacatecas, Mexico, 11-16 November 2012.
- Sánchez-López AS. 2013. Función de las plantas en le remediación de sitios contaminados con metales pesados. Oral presentation. Minisimposio Funcionalidad de organismos en las tecnologías ambientales. Colegio de Postgraduados Campus Montecillo. Montecillo, Mexico, 11 February 2013.

- Sánchez-López A, González-Chávez MCA, Carrillo-González R. 2013. Absorber, inmovilizar o atrapar: la función de las plantas en la remediación de sitios contaminados por elementos potencialmente tóxicos. Oral presentation. Reunión Anual Unión Geofísica Mexicana A.C. Puerto Vallarta, Mexico, 3-8 November 2013.
- Sánchez-López A, Vangronsveld J, Weyens N, González-Chávez MCA, Carrillo-González R. Transgenerational characterization of seed endophytic bacteria of a plant growing on mine residues. Oral presentation. 11th International Phytotechnologies Conference. Heraklion, Greece, 30 September to 3 October 2014.
- Sánchez-López AS, González-Chávez MCA, Carrillo-González R. 2014. Vías de remediación vegetal de sitios contaminados por elementos potencialmente tóxicos. Oral presentation. XX Congreso Latinoaméricano y XVI Congreso Peruano de la Ciencia del Suelo. Cusco, Peru, 9-15 November 2014.
- Sánchez-López A, Thijs S, Vangronsveld J, Weyens N, Pintelon I, Timmermans JP, González-Chávez C, Carrillo-González R. 2015. Visualization of mCherry-labeled seed endophytic *Methylobacterium* in *Crotalaria pumila* seedlings. Poster. 13th International Phytotechnologies Society Conference. Manhattan, USA, 27-30 September 2015.

Participation in courses

- Seminario-Taller: Nuevas herramientas moleculares y sus aplicaciones en la investigación: pesaje en el laboratorio y buenas prácticas de pipeteo. Bio-Scientia. Colegio de Postgraduados Campus Montecillo, 20 June 2013.
- Curso: Análisis espacial de la contaminación de suelos urbanos con metales pesados. Reunión Anual Unión Geofísica Mexicana A.C. Puerto Vallarta, México, 3-8 November 2013.

Academic distinctions

- 1st. Place Student Platform Competition. Plants growing on mine tailings avoid dispersion and contribute to stabilization of potentially toxic elements. 9th International Phytotechnologies Society Conference. University of Hasselt, Belgium, 11-14 September 2012.
- BOF-BILA grant from Hasselt University. Research project: Transgenerational characterization of seed endophytic bacteria of *Crotalaria pumila* Ort. growing on mine tailings in a semi-arid region.