# DESARROLLO DE MICROECONOMÍAS REGIONALES EN LA PRODUCCIÓN DE ACEITES ESENCIALES COSECHADOS EN SUELOS MINEROS

## Producto 10: Informe de factores del éxito del proceso de remediación

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# Resumen producto 10

Las investigaciones realizadas en ensayos de microcosmos sobre la eficiencia de la especie vegetal candidata (*Helianthus petiolaris*) en combinación con microorganismos rizosféricos en el proceso de remediación, comprometidas para el tercer año de proyecto como Producto 10, han sido publicadas en el formato de artículo científico en una de las revistas internacionales especializadas en fitorremediación de mayor impacto. A partir de estos estudios, profundizamos los conocimientos sobre la interacción entre *H. petiolaris* y sus microorganismos asociados en presencia de Pb y Cd, tanto en suelos artificialmente contaminados como en suelos crónicamente expuestos a metales, provenientes de un campo de tiro (dinámica planta-microorganismos-suelo). Pudimos evaluar además la supervivencia del *H petiolaris* a altas concentraciones de metal, y su capacidad para acumular metales en sus tejidos. Esto no solo constituye un insumo esencial para la identificación de los factores que determinan el éxito del proceso de remediación, sino la base científica necesaria para la toma racional de decisiones en la formulación de estrategias de remediación y el escalado a campo de futuros ensayos pilotos.

A continuación, se presenta el artículo publicado, como entregable del Producto 10 comprometido para esta actividad. Considerando que el artículo ha sido publicado en inglés y a fin de facilitar la difusión de nuestros resultados en el marco de FONTAGRO, incorporamos además su resumen en español.

# *Phytostabilization of Pb and Cd polluted soils using Helianthus petiolaris as pioneer aromatic plant species*

El área de suelos contaminados con metales pesados es cada vez mayor debido a la industrialización y la globalización. Las especies de plantas aromáticas pueden ser una alternativa adecuada para la valorización agrícola y la fitogestión de dichos suelos mediante la comercialización de aceites esenciales, evitando riesgos para la cadena alimenticia.
El potencial de cultivar *Helianthus petiolaris* en suelos contaminados con metales pesados se evaluó en experimentos en macetas, utilizando suelos artificialmente contaminados y en suelos de un campo de tiro. En términos de fitoestabilización, *H. petiolaris* podría crecer en suelos que contienen 1000 mg / kg de Pb2þ, 50 mg / kg de Cd2þ, acumulando más tres veces el contenido de Cd del suelo en las partes aéreas y trasladar cantidades significativas de Pb a las partes aéreas cuando crece en suelos contaminados con hasta 500 mg / kg de Pb.
Cuando se considera la fitoestabilización, la fitotoxicidad de los metales pesados depende en gran medida de las comunidades microbianas rizosféricas, ya sea mitigando la fitotoxicidad de los oligoelementos o promoviendo el crecimiento de las plantas a través de la producción de fitohormonas. Entonces, los efectos de los metales pesados sobre la diversidad de la comunidad bacteriana rizosférica se evaluó mediante análisis de ADN.

**Palabras Clave:** Fitoestabilización; fitomanagement, metales pesados, *Helianthus petiolaris*, bacteriana rizosférica, comunidades

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# Phytostabilization of Pb and Cd polluted soils using *Helianthus petiolaris* as pioneer aromatic plant species

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**ABSTRACT**

The area of soils polluted with heavy metals is increasing due to industrialization and globalization. Aromatic plant species can be a suitable alternative way for agricultural valorization and phytomanagement of such soils by the commercialization of essential oils avoiding risks for the food chain. The potential of growing *Helianthus petiolaris* in heavy metal polluted soils was assessed in pot experiments using spiked soils and soils from a shooting range. In terms of phytostabilization, *H. petiolaris* could grow in soils containing 1000 mg/kg $Pb^{2+}$, 50 mg/kg $Cd^{2+}$, accumulating more than three times the soil Cd content in the aerial parts and translocating significant amounts of Pb to the aerial parts when growing in soils polluted with up to 500 mg/kg Pb.

When phytostabilization is considered, phytotoxicity of heavy metals strongly depends on the rhizospheric microbial communities, either by mitigating trace element phytotoxicity or promoting plant growth via phytohormone production. So, the effects of heavy metals on the diversity of the rhizospheric bacterial community were assessed using DNA-fingerprinting.

**KEYWORDS**

Phytostabilization; phytomanagement; heavy metals; *Helianthus petiolaris*; rhizospheric bacterial communities

## Introduction

Pollution of the environment by heavy metals has become a serious problem around the world (Sessitsch *et al.* 2013) and is still increasing along with the ongoing industrialization and urbanization. Among other sources, the release of heavy metals through different applications in industry and agriculture is intensifying environmental and human health risks associated with exposure to these polluted soils and/or dust (Khan *et al.* 2010).

Currently, the conventional treatment methods for heavy metal polluted soils include *in situ* excavation and landfilling, soil washes, soil flushing, stabilization using physical and chemical methods, and electro-kinetic techniques (Wuana and Okieimen 2011). However, these methods have disadvantages like the high costs, they are labor-intensive and cause irreversible changes in soil properties and disturbance of native soil microflora (Vangronsveld *et al.* 2009).

Phytostabilization refers to the use of plants and their associated microorganisms as a green alternative to mitigate the toxic effects of pollutants in the environment (Greipsson 2011). In contrast with conventional methods, plants can take up, degrade or sequestrate pollutants without deteriorating the topsoil, preserving its functionality and fertility. Indeed, plants may improve soil structure and fertility, due to input of organic matter (Mench *et al.* 2009). Also, the establishment of a vegetation cover on polluted soils helps to avoid wind and water erosion and trace element leaching (Vangronsveld *et al.* 1995a; Vangronsveld, Van Assche, *et al.* 1995b; Vangronsveld *et al.* 1996; Ruttens *et al.* 2006).

The ideal plant species to remediate a heavy metal polluted soil should be a high biomass producing crop that can both tolerate and accumulate the pollutants of interest (Ebbs and Kochian 1997). Several studies reported that commercial crops as sunflower (*Helianthus annuus* L.), mustard (*Brassica juncea* L.), alfalfa (*Medicago sativa* L.), ricinus (*Ricinus communis* L.) can phytoextract Cd and Pb (Zhi-Xin *et al.* 2007). However, later studies showed that the accumulation of heavy metals in some crops inhibits plant growth, biomass, grain yield, and crop quality (Ramzani *et al.* 2016).

A promising approach for economic valorization and phytomanagement of polluted soils is the use of aromatic plants for phytostabilization; these are non-food crops thus the risk of food chain contamination is minimal. In this way, fast-growing aromatic plants can be used for both phytostabilization and production of high-value essential oils without the risks of trace element cross-contamination of the product (Croes *et al.* 2015; Pandey *et al.* 2015, 2019).

*Helianthus petiolaris* Nutt. is an annual plant species native to Argentina, considered the wild ancestor of *H. annuus* (Poverene *et al.* 2004). This species grows in xeric, sandy soils in the center of South America with a flowering period from December to March (Rieseberg *et al.*

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Supplemental data for this article can be accessed here.

**Table 2.** Effect of Cd and Pb on the dry weights and lengths of roots and shoots of *H. petiolaris* after a growth period of 90 days on trace element spiked soils.

| | | Seedlings dry weight (mg) | | Length (cm) | | |
|---|---|---|---|---|---|---|
| Element | Conc. (mg/kg) | Root | Aerial part | Root | Aerial part | Tolerance index |
| Cd | 0 | 0.22 ± 0.1a | 1.30 ± 0.2a | 2.8 ± 0.9b | 2.6 ± 0.3b | 1.0 |
| | 1 | 0.21 ± 0.0a | 1.47 ± 0.2a | 1.1 ± 0.5a | 1.9 ± 0.5ab | 1.1 |
| | 3 | 0.21 ± 0.1a | 1.22 ± 0.1a | 0.9 ± 0.1a | 1.5 ± 0.3a | 0.9 |
| | 5 | 0.21 ± 0.1a | 1.59 ± 0.08a | 0.7 ± 0.2a | 1.5 ± 0.4a | 1.2 |
| | 10 | 0.10 ± 0.0a | 1.52 ± 0.1a | 0.5 ± 0.1a | 1.1 ± 0.2a | 1.1 |
| | 20 | 0.11 ± 0.0a | 1.51 ± 0.0a | 0.4 ± 0.1a | 1.1 ± 0.1a | 1.1 |
| Pb | 0 | 0.26 ± 0.1a | 1.45 ± 0.1ab | 2.8 ± 0.8b | 2.7 ± 0.7b | 1.0 |
| | 50 | 0.27 ± 0.1a | 1.36 ± 0.2ab | 2.6 ± 1.1b | 3.2 ± 0.4b | 1.0 |
| | 100 | 0.24 ± 0.1a | 1.36 ± 0.2ab | 2.3 ± 1.0ab | 2.2 ± 0.3ab | 0.9 |
| | 250 | 0.25 ± 0.1a | 1.27 ± 0.2a | 2.0 ± 0.3ab | 2.5 ± 0.4ab | 0.9 |
| | 500 | 0.32 ± 0.1a | 1.38 ± 0.1ab | 1.9 ± 0.2ab | 1.8 ± 0.3ab | 1.0 |
| | 1000 | 0.13 ± 0.0a | 1.18 ± 0.2b | 0.4 ± 0.1a | 1.3 ± 0.5a | 0.8 |

$n = 50$. Values are presented as mean ± standard deviation. Values in a column followed by the same letter(s) are not significantly different at $p \leq 0.05$ by ANOVA and Tukey test.

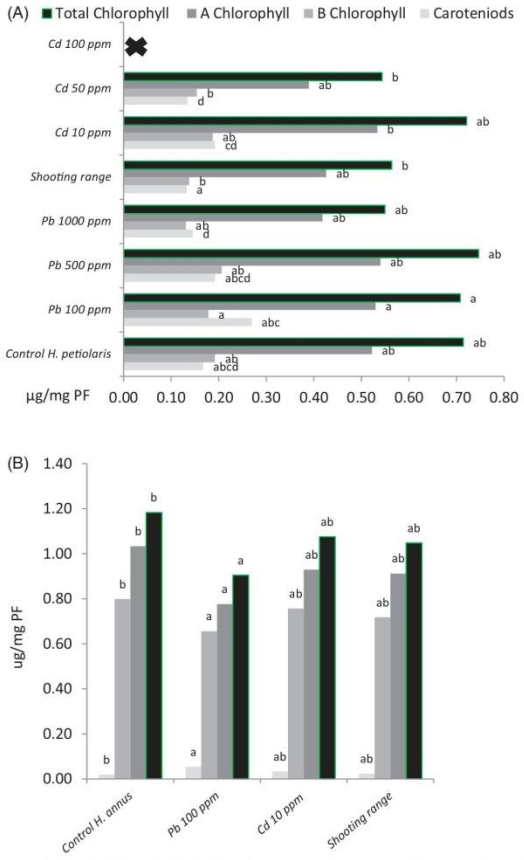


**Figure 1.** Total chlorophyll, A, B and carotenoids content of *H. petiolaris* (A) and *H. annuus* (B) in Cd and Pb-spiked soil and Pb polluted shooting range soil. $n = 5$, values in a bar followed by the same letter(s) are not significantly different at $p \leq 0.05$ by ANOVA and Tukey test. Black cross represent died plants.

At the lowest concentrations tested total chlorophyll decreased by 24% in Pb exposed plants, 10% in Cd exposed plants and 11% in plants growing in the shooting range soil, while at these concentrations the chlorophyll contents in *H. petiolaris* did not change.

When growing at 100 mg/kg Pb, both species showed significantly enhanced carotenoid contents. The other exposure concentrations did not cause significant changes to the carotenoid content.

Plantlets transplanted to the pots containing 100 mg/kg Cd, withered after 32 days (Table 3). Weight and length of shoots were lower compared to the control for all treatments; this effect was more pronounced for Cd in comparison to Pb. Plant dry weight decreased in stems (50%) and roots (35%) when the plants were grown on soil spiked with 50 mg/kg Cd. However, there were no statistically significant differences with the control plants, except for those plants which withered.

Instead, our control species *H. annuus* showed statistically significant lower stem length and weight for all exposure concentrations. The reduction in weight was notably high for Cd exposure, followed by Pb.

### *Trace element concentrations in plant tissue*

Trace element concentrations in plant tissues were assessed by MIP-OES. Roots of *H. petiolaris* plants growing in soils spiked with 10 mg/kg Cd contained 25 mg/kg Cd dry weight and up to 149 mg/kg Cd dry weight when growing in soils with 50 mg/kg Cd. Although they started to wither 32 days after transplanting, the Cd concentration in the shoots of plants growing at 100 mg/kg Cd was more than three times higher than in the soil (BAF for Cd = 3.30). On the other hand, *H. annuus* accumulated less Cd in the root compared with *H. petiolaris* (BAF = 3.87 *versus* 2.14, respectively) and translocated less of this element to the shoot (TF = 1.43 *versus* 0.98). Translocation of Cd to the flower tissue was also found in *H. annuus*.

On the soils polluted with Pb, *H. petiolaris* accumulated a high concentration in roots, which decreased with the increment of the Pb concentration in the soil. The concentration of Pb in the shoots is considerably higher for plants grown on the soil with 500 mg/kg Pb (TF = 2.69 and 2.45). However, *H. annuus* translocated more Pb to the shoots even on soils with 100 mg/kg Pb (BAF = 0.12 *versus* 0.01 of *H. petiolaris* 100 mg/kg Pb) (Table 3). *Helianthus annuus* and *H. petiolaris* were taking up more Cd compared to Pb.

transplanted to pots, containing 1.5 kg of soil. After a week of greenhouse acclimatization, plants were thinned to 1 plant per pot.

Pigment content as total chlorophyll, chlorophyll A, chlorophyll B, and carotenoids was measured along the experiment according to Lichtenthaler and Buschmann (2001). After 3 months the plants were harvested and biomass production (fresh and dry weight, root and shoot length) were determined.

### Trace element accumulation in plant tissues

At the end of the experiment, plants were harvested and divided into roots, shoots (stem and leaves), and flowers in order to assess concentrations of Cd and Pb in the different parts. Plant material was first washed with milli-Q water and dried at 60 °C for 72 h and subsequently ground to powder in a mortar with liquid nitrogen. 250 mg of homogenized plant material were acid digested by different procedures depending on the trace element. For Pb, digestion was performed with 1 ml 65% $HNO_3$ and 1 ml 30% $H_2O_2$; for Cd, an ultrasonic bath (30 min at 110 °C) with 5 ml HCl 8% was used (Neher *et al.* 2018). The samples were analyzed by MIP-OES (Agilent MP 4100). Certified reference material RM-Agro E1001a (*Brachiaria brizantha* cv Marandu) from the EMBRAPA Pecuária Sudeste (São Carlos, SP, Brazil) was included in each batch for quality control.

The bioaccumulation factors (BAF) were calculated by dividing the total contents of heavy metals in the plant tissues by the total contents of heavy metals in the soil (Abbas and Abdelhafez 2013). The shoot-root Translocation Factor (TF), defined as the ratio between the concentration of the trace element in plant shoots and the concentration in roots, was calculated according to Gupta *et al.* (2008).

### DNA-fingerprinting profiles

The effects of each treatment on the microbial diversity of bulk soil, rhizoplane (soil immediately surrounding the root) and root endophytic compartment (microorganisms within the root) were investigated. From each pot, five subsamples (5 g of bulk soil and 0.5 g of root per subsample) were collected at a depth of 0–10 cm. The subsamples were mixed in the laboratory to obtain a composite sample. DNA extraction from endophytes was performed by using the Invisorb Spin Plant Mini kit (Stratec Biomedical AG), rhizoplane and soil DNA extractions were done by using the MOBIO PowerSoil Isolation kit (MOBIO). The ITS region was amplified by PCR using the ITSF (5′-GTCGTAACAAGG TAGCCGTA-3′) and ITSReub (5′-GCCAAGGCATCCACC-3′) primers as described previously by Cardinale *et al.* (2004).

After amplification, samples were loaded onto Agilent DNA 1000 Chips and analyzed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Expert Software (Agilent Technologies) was used to digitalize the ARISA fingerprints, resulting in electropherograms in ASCII formats that were processed using the StatFingerprints package (Michelland *et al.* 2009).

### Statistical analysis

The data from the *in vitro* experiments were analyzed by using analysis of variance (ANOVA). When ANOVA indicated a treatment effect, the Least Significant Difference (LSD) test was applied to make comparisons between the means at $p < 0.05$. Biomass parameters obtained in the greenhouse assays were analyzed by nonmetric multidimensional scaling (NMDS) using Euclidean dissimilarity matrix. Fingerprinting information was subjected to correspondence analysis (PCA), a principal component analysis related ordination technique based on chi-square distances, illustrating correlations between compartments in the 2.13.0 version of the R project (The R Foundation for Statistical Computing, Vienna, Austria).

## Results

### MIC of Cd and Pb on agar-water

Since no information was available on how to pretreat seeds of *H. petiolaris* (wild species), a novel seed treatment protocol was developed. Using the described protocol over 70% of germination was achieved, in comparison with the 15% germination rate for non-treated seeds.

When plants were exposed to Cd, there was a significant inhibition of root and shoot length (60 and 63%, respectively); however, no statistically significant differences in the root or shoot weight were found compared to the control (Table 2).

In plants growing at different concentrations of Pb, roots and aerial parts were significantly shorter only when plants were grown at the highest concentration tested (1000 mg/kg), which resulted in a 86% reduction of root length and a 51% reduction of the length of the shoot in comparison to the control ($p < 0.01$). Also, the weight of the aerial parts showed a 19% reduction at 1000 mg/kg compared to the control. The tolerance index indicates that *H. petiolaris* tolerates up to 20 mg/kg Cd and 1000 mg/kg Pb in *in vitro* conditions.

### Trace element tolerance of H. petiolaris and H. annuus in Cd and Pb-spiked soil and Pb polluted shooting range soil

To estimate the effects of Cd and Pb on plant growth the profile of photosynthetic pigments was examined. Results show that neither the total chlorophyll, nor levels of chlorophyll A and B of *H. petiolaris* showed significant differences with the control at the pollution limits established by US-EPA (10 mg/kg Cd and 100 mg/kg Pb) or even at 500 mg/kg Pb, (Figure 1). However, these pigment contents were significantly lower at concentrations of 50 mg/kg Cd or 1000 mg/kg Pb in the soil. Pigment contents in *H. annuus* showed more sensitive to heavy metals than in *H. petiolaris*.

Table 1. Physico-chemical parameters of the soil used for spiking (S1–S2) and the weathered shooting range soils (SR1–SR2).

| Soil sample | pH | EC (dS/m) | Organic matter (%) | Clay | Limo | Sand | Texture | Pb (mg/kg) |
|---|---|---|---|---|---|---|---|---|
| S1 | 7.1 | 0.45 | 1.6 | 4.0 | 13.0 | 83.0 | Sandy Franc | nd |
| S2 | 7.4 | 0.20 | 1.9 | 2.0 | 13.0 | 85.0 | Sandy Franc | nd |
| SR1 | 7.4 | 0.18 | 0.5 | 2.0 | 8.0 | 90.0 | Sandy | 416.5 ± 102.1 |
| SR2 | 7.7 | 2.71 | 1.8 | 3.0 | 23.0 | 74.0 | Sandy Franc | 427.8 ± 135.54 |

nd; not detected; EC: Electric conductivity. SRM2711 – Montana soil and RM-Agro E2002a – Solo arenoso (EMBRAPA Pecuária Sudeste, São Carlos, SP, Brazil) were used as soil certified reference material. Recoveries: 80–120%.

2003). In addition, this species has potential for biotechnological applications, since its essential oils can be used for pest control in stored grains (Saran *et al.* 2019). The combination of such features and the background of trace element tolerance of closely related species as *H. annuus* and *Helianthus tuberosus* (Montalban *et al.* 2017; Zhao *et al.* 2019) make this aromatic plant an outstanding candidate for designing phytoremediation strategies.

The process of phytostabilization strongly depends on the activity of belowground microbial communities that are able to decompose soil organic matter (OM) and stabilize soil structure (Porazinska *et al.* 2003). Soil microorganisms indeed are intimately involved in element biogeochemistry with a whole variety of processes determining the mobility of heavy metals, and consequently their bioavailability (Gadd 2004). The balance between immobilization and mobilization depends on the microorganisms involved, physicochemical and environmental conditions. Heavy metal mobilization can be the result of, for example, complexation by metabolites like humic and fulvic acids and siderophores. Immobilization can be due to sorption to biomass or secreted exopolymers, intracellular sequestration or organic and inorganic precipitates like oxalates, phosphates or sulfides.

In this context, we explored the possibilities to use *Helianthus petiolaris* Nutt. as a pioneer species to phytostabilize heavy metals polluted areas and investigated the effects of heavy metals on its development and on the belowground microbial communities.

## Materials and methods

### *Minimum inhibitory concentration (MIC) of Cd and Pb on agar-water*

Seeds of *H. petiolaris* (accession nos. LMH00006 and LMH00009) were scarified and surface sterilized by washing them in ethanol 70% (v/v) for 10 min, sodium hypochlorite 15% (v/v) for 5 min and subsequently 5 times rinsing with sterile distilled water. Once sterile, the seeds were incubated for 20 days and soaked in sterile water at 4 °C to break the dormancy. Finally, to promote the germination process, they were incubated for 24 h in 400 mg/kg gibberellic acid in dark. Before seeding, an additional surface treatment with fludioxonil/metalaxyl-M (Maxim Semillero®) was performed to remove any natural infection of its cuticle with the pathogenic fungus *Alternaria* sp.

The tolerance of *H. petiolaris* to different concentrations of Cd and Pb was examined. Agar-water medium was chosen to perform *in vitro* assays (Merini *et al.* 2011). For these assays, 25 sterilized seeds were sown in flasks containing 50 ml of 1% (w/v) water–agar, pH 7, supplemented with $Cd^{2+}$ and $Pb^{2+}$ separately in concentrations of 1, 3, 5, 10, and 20 mg/kg Cd and 50, 100, 250, 500, 1000 mg/kg Pb ($CdCl_2$, $Pb(NO_3)_2$, respectively, no other nutrient was added. The trace element concentrations were selected based on the limits established by the Environmental Protection Agency (US EPA 1992) for polluted soils. Three replicates of each concentration level were aseptically sown, sealed with plastic film and incubated in a growth chamber with controlled temperature (25 °C day/19 °C night), photoperiod (16/8), 400 µM/cm$^2$/s PAR. After 32 days, germination rate, roots and stems lengths, weight, and Tolerance index (Ti = weight trace element exposed plant/weight control plant) (Tong *et al.* 2009) were determined.

### *Performance of* H. petiolaris *and* H. annuus *in Cd and Pb-spiked soil and long-term Pb polluted shooting range soil*

One-month-old seedlings of *H. petiolaris* were transplanted to one and a half liter pots containing soils spiked with 0, 10, 50, and 100 mg/kg Cd, and 0, 100, 500, and 1000 mg/kg Pb. For this purpose, a concrete mixer was used to prepare 20 kg of soil polluted with 100 mg/kg Cd or 1000 mg/kg Pb (10 min at 15 rpm), using $CdCl_2$ and $Pb(NO_3)_2$ reagents. From these highest pollution levels, the lower pollution levels were prepared by serial dilutions with unpolluted soil. All artificially polluted soils were aged for 60 days before starting the experiment.

In parallel, a long-term weathered soil sample containing 400 mg/kg Pb, collected from an abandoned shooting range (36°37′22.696″ S; 64°15′25.811″ W), was included. The physico-chemical parameters of the soils were processed in the Soil Laboratory of EEA-INTA, Anguil, La Pampa, Argentina and metal concentrations were determined using ICP-OES at the Department of Analytical Chemistry, National University of La Pampa, Argentina (Table 1).

In order to compare the performance of *H. petiolaris*, a wild species with a commonly cultivated plant species, a parallel set of pots was established using one-month-old seedlings of *H. annuus* into pots filled with either soil polluted with 10 mg/kg Cd, 100 mg/kg Pb, or shooting range soil.

Pre-germination of *H. petiolaris* and *H. annuus* (used as positive control) was done in a plant growth chamber by sowing them in germination trays filled with potting soil and controlled conditions of temperature (25 °C day/19 °C night), light (400 µM/cm$^2$/seg PAR), humidity (60%) and watering (500 ml per day). After 25 days each substrate unit (containing 1–3 seedlings in its third true leaf stage) was

**Table 3.** Plant growth parameters and trace element concentrations in tissues of plants grown in Cd and Pb-spiked soils and long-term Pb polluted shooting range soil after 90 days of growth.

| | Dry weight biomass (gr) | | | | Metal Concentration (mg/kg) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Element Conc. Soil (mg/kg) | Root | Stems | Leaves | Steam lengths (cm) | Root | Aerial Part | Flower | TF | BAF |
| *H. petiolaris* | | | | | | | | | |
| 0 | 0.14 ± 0.04ab | 1.09 ± 0.22a | 1.04 ± 0.09b | 61.6 ± 12.2e | nd | nd | nd | – | – |
| 10 Cd | 0.20 ± 0.09a | 1.32 ± 0.24a | 1.38 ± 0.11ab | 52.2 ± 4.8de | 27.17 ± 5.32 | 38.78 ± 20.15 | nf | 1.43 | 3.87 |
| 50 Cd | 0.14 ± 0.08ab | 0.51 ± 0.21b | 0.63 ± 0.21c | 35 ± 9.6b | 149.01 ± 26.83 | 121.87 ± 37.73 | nf | 0.82 | 2.43 |
| 100 Cd | 0.04 ± 0.03b | 0.13 ± 0.05c | 0.17 ± 0.08d | 19 ± 7.6a | ner | 329.83 ± 96.68 | nf | – | 3.30 |
| 100 Pb | 0.23 ± 0.04a | 1.32 ± 0.29a | 1.66 ± 0.39a | 56.6 ± 3.2de | 5.15 ± 7.81 | 1.16 ± 0.24 | nd | 0.22 | 0.01 |
| 500 Pb | 0.16 ± 0.13ab | 0.92 ± 0.35ab | 1.16 ± 0.49b | 48.5 ± 7.0b | 4.06 ± 0.91 | 2.72 ± 0.93 | nd | 0.66 | 0.01 |
| 1000 Pb | 0.21 ± 0.06a | 1.11 ± 0.13a | 1.17 ± 0.39b | 51.4 ± 9.8cd | 2.59 ± 2.20 | 7.03 ± 5.89 | nf | 2.69 | 0.01 |
| Shooting range | 0.16 ± 0.05ab | 1.03 ± 0.29ab | 1.13 ± 0.34b | 59.6 ± 7.1e | 1.46 ± 0.45 | 3.59 ± 0.81 | nf | 2.45 | 0.01 |
| *H. annuus* | | | | | | | | | |
| 0 | 0.48 ± 0.26ab | 2.14 ± 0.26a | 1.35 ± 0.18[a] | 96.2 ± 11.4d | nd | nd | nd | – | – |
| 10 Cd | 0.44 ± 0.15ab | 1.84 ± 0.38ab | 1.30 ± 0.11a | 76.8 ± 7.3bc | 30.56 ± 13.14 | 21.38 ± 5.99 | 8.41 ± 1.31 | 0.98 | 2.14 |
| 100 Pb | 0.74 ± 0.62a | 1.96 ± 0.49a | 1.29 ± 0.41a | 72.7 ± 2.1a | 0.92 ± 0.87 | 12.69 ± 10.78 | nd | 0.14 | 0.12 |
| Shooting range | 0.47 ± 0.17ab | 1.49 ± 0.08b | 1.21 ± 0.13a | 69.8 ± 6.2ab | 0.60 ± 0.53 | 6.17 ± 5.89 | nd | 0.01 | 0.01 |

$n = 5$; values are presented as mean ± standard deviation. Values in a column followed by the same letter(s) are not significantly different at $p \leq 0.05$ by ANOVA and Tukey test. nd: not detected; nf: not flower; ner: not enough root. Limits of detection (LD) Cd = 0.6 mg/kg, Pb = 53.651 mg/kg. Limits of quantification (LQ) Cd = 2.0 mg/kg, LD Pb = 17.705 mg/kg.

### *DNA-fingerprinting profiles*

The microbial diversities of bulk soil, rhizoplane and root endophytic compartments of *H. petiolaris* and *H. annuus* were investigated for plants growing on unpolluted and polluted soils. A complete linkage algorithm was used to perform a cluster analysis of Bray–Curtis dissimilarity matrices inferred from the fingerprints profiles.

As shown in Figures 2 and 3, microbial diversity and richness were higher in the rhizosphere and the root endosphere of both, *H. petiolaris* and *H. annuus*, than in the bulk soil. Electropherogram profiles with peaks ranging from 0 bp to 1200 bp are presented in Supplementary Figures S1 and S2. Due to the high sensitivity of the automated sequencer, the Bioanalyser software allowed us to detect between 10 and 25 peaks in the electropherograms (bands in the heatmaps) per profile. The structure of the profiles, characterized by the number and length distribution of major bands (peaks of highest relative fluorescence intensity), varied between compartment and soil type. The Principal Component Analysis (PCA) of these data indicates that the bacterial communities associated with the plants growing in soils polluted with different concentrations of Cd and Pb were correlated with those of the control soil without pollution. There were more differences between repetitions for the same treatment than between treatments. Only in the soil from the shooting range (originating from a different location) bacterial communities were not related to those present in the other soils. At the assayed concentrations, the soil as a matrix had a larger influence on microbial communities than the pollution.

## Discussion

*H. petiolaris* tolerance to Cd and Pb was evaluated *in vitro* by exposing it to different concentrations of each trace element. As commonly accepted, plant biomass can indirectly indicate the tolerance of plants to toxic metals. When plants were exposed to Cd, roots and shoots length were significantly lower (Table 2). Growth impairment is typical for Cd toxicity (Pál *et al.* 2006). Weight decreases of roots and shoots were commonly reported (Tran and Popova 2013), as well as foliar chlorosis and necrosis (Das *et al.* 1997). Such effects were also reported for other species belonging to the same family as *H. annuus* and *H. tuberosus* (Chen *et al.* 2011; Rivelli *et al.* 2012). When exposed in an agar medium, *H. petiolaris* was able to tolerate and grow in presence of up to 20 mg/kg Cd.

When exposed to Pb, a significant decrease in weight was only observed at the highest concentration tested (1000 mg/kg Pb) (Table 2). Fresh weight was reported to decrease progressively with increasing Pb concentrations in hydroponically grown alfalfa plants (Ghelich *et al.* 2014) and in other species such as *Brassica napus*, *Sesbania grandiflora*, and *Hydrocotyle vulgaris* (Bilal Shakoor *et al.* 2014; Malar *et al.* 2014; Yang and Ye 2015). Growth inhibition induced by Pb may be related to a disturbance of the water balance caused by $Pb^{2+}$ ions (Ekmekçi *et al.* 2009) or disturbed mineral nutrition (Fahr *et al.* 2015).

Once the *in vitro* MIC of trace element was established, trace element tolerance of *H. petiolaris* was assessed in Cd and Pb spiked soils and a long-term Pb polluted soil from a shooting range. Total chlorophyll, chlorophyll A and B concentrations in leaves of *H. petiolaris* increased when the plant was exposed to moderate concentrations of heavy metals and decreased significantly when the concentrations the soil reached 50 mg/kg Cd or 1000 mg/kg Pb, respectively (Figure 1). Also Atefeh *et al.* (2015) reported that *Robinia pseudoacacia* responded to Cd and Pb by increasing its content of chlorophyll at low heavy metals exposure concentrations. Similar results were described by Tripathi and Gautam (2007) and Seyyednejad *et al.* (2009) with different plant species. High Cd concentrations in leaf tissues have been suggested to indirectly influence the chlorophyll content via metabolic disruption and premature senescence (Vassilev *et al.* 1997). A similar effect was observed with Pb, inhibiting chlorophyll biosynthesis by impairing the uptake of elements essential for photosynthetic pigments, such as

In the future, inoculation experiments will be performed to evaluate the potential of promising strains to enhance phytoextraction efficiency of *H. petiolaris*.

Supplementary Figure S1 – DNA-fingerprinting electropherograms profiles of *H. petiolaris* plants and Supplementary Figure S2 – DNA-fingerprinting electropherograms profiles of *H. annuus* plants – can be found online in the publisher's website.

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Ca, Mg, and K (Piotrowska *et al.* 2009). Although there were no statistically significant differences, our findings agree with these reports, where the photosynthetic pigment profile is affected by low concentrations of heavy metals in soils and plant tissues and inhibited when the concentrations rise in both compartments.

Similarly, the carotenoids content has been shown to increase the tolerance to oxidative stress caused by heavy metals as a detoxification mechanism (Holm-Hansen *et al.* 1954). Both *Helianthus* species growing at 100 mg/kg Pb showed significantly enhanced carotenoid contents (Figure 1). This profile of carotenoids *versus* heavy metals concentrations has been described before by Ralph and Burchett (1998) in *Halophila ovalis* and Rehman Hakeem *et al.* (2019) in *Fagopyrum kashmirianum.* In the latter species the carotenoid levels were higher from the lowest concentration of Pb and did not increase when exposed to higher Pb concentrations.

*H. petiolaris* plants were harvested after 90 days and dry weights and lengths of roots and shoots were determined. Weight and shoot length decreased compared to the control for all treatments; this effect was more pronounced for Cd in comparison to Pb (Table 3). These results agree with several other studies, demonstrating that Cd is toxic for plants, reducing the biomass of different species (Anwer *et al.* 2012; Paschalidis *et al.* 2013).

In case of *H. annuus*, however, stem length and weight decreased for all treatments. Weight reduction was quite high for Cd exposure, followed by Pb (Table 3). A recent report (Alaboudi *et al.* 2018) is in accordance with our results, showing that Cd exposure caused a significantly higher reduction in biomass compared to Pb exposure in sunflower.

*H. annuus* and *H. petiolaris* were taking up more Cd than Pb (Table 3). This agrees with studies of Lee *et al.* (2013) and Forte and Mutiti (2017); they reported that *H. annuus* is less efficient for taking up Pb in its tissues compared to other heavy metals. It was also reported that a higher percentage of Pb gets restricted within the roots in the cell wall complex while only a limited fraction of it is transported to the aerial parts of the plants (Inoue *et al.* 2013; Kiran *et al.* 2017). Plants that, when growing on native soils, concentrate >100 mg/kg (0.01%) of Cd in the aerial parts without showing phytotoxicity symptoms and are considered as Cd hyperaccumulators (Verbruggen *et al.* 2009). *Arabidopsis halleri*, *Sedum alfredii*, *Thlaspi (now Noccaea) caerulescens*, *Thlaspi praecox*, *Solanum nigrum* (Solanaceae) are some of the species that have been reported to be Cd hyperaccumulators (Sun *et al.* 2006). Considering that the US-EPA legislation mentions that soils polluted with 400 mg/kg of Pb and 10 mg/kg of Cd must be remediated, *H. petiolaris* obviously tolerates these threshold concentrations in greenhouse experiments. Although it is necessary to test this species in field experiments, these observations and the fact that it is a native species able to grow in poor and disturbed soils, make it a promising candidate for phytostabilization of heavy metal polluted soils, especially where commercial food or fodder crops production cannot be considered.

Heavy metal phytoremediation strongly depends on the activities of belowground microbial communities (Thijs *et al.* 2017). Therefore, the influence of the different heavy metal treatments on the microbial diversity in bulk soil, rhizoplane and roots were investigated. Although DNA-fingerprinting patterns cannot reveal the taxonomic composition of the communities due to the overlapping of size classes among unrelated populations (Ranjard *et al.* 2000), some conclusions can be drawn about the diversity and distribution of the communities. Microbial diversity and richness were higher in the rhizosphere and the root endosphere of *H. petiolaris* and *H. annuus* than in the bulk soil (Figures 2 and 3). Growth and activity of soil microorganisms are primarily limited by organic carbon. Since soil organic matter is poorly decomposable in contrast with the easily decomposable root exudates, the microbial density/diversity in the rhizosphere is higher than in bulk soil (Soderberg and Bååth 1998; Demoling *et al.* 2007).

At the tested heavy metal concentrations, the soil as a matrix had a greater influence on the microbial community than the pollutants. The fact that one of the replicates often diverges to some extent most likely reflects soil sampling heterogeneity (Perelman *et al.* 2018).

## Conclusions

Although it is necessary to confirm the tolerance of this species in the field, our results indicate that *H. petiolaris* should be able to grow in soils with up to 50 mg/kg of Cd and up to 1000 mg/kg of Pb. Cadmium affected the growth of *H. petiolaris* in concentrations above 100 mg/kg, which is probably correlated with the high uptake of this metal, which reaches 3 times the soil concentration in the aerial parts. Although the Pb shoot concentration was not as high as found in *H. annuus*, *H. petiolaris* has a high tolerance to this metal, especially considering that phytotoxicity was not observed at Pb concentrations up to 1000 mg/kg. In addition, *H. petiolaris* seemed to adapt its photosynthetic pigments contents in order to cope with the presence of both heavy metals in its leaf cells. Comparing to the commercial crop species *H. annuus*, *H. petiolaris* seems to be less affected in ist pigment expression and biomass production when it grows in polluted soils containing high metal concentrations. The tested heavy metal concentrations did not have a huge influence on microbial communities.

Furthermore, since *H. petiolaris* an aromatic plant species, essential oils and added value by-products obtained from its biomass can be commercialized, with the benefits of reclaiming polluted lands for production.

These features and the fact that it is a species able to grow in poor and disturbed soils, make it a promising candidate to phytostabilize heavy metals polluted soils, especially when they are not suitable for producing other commercial crops.

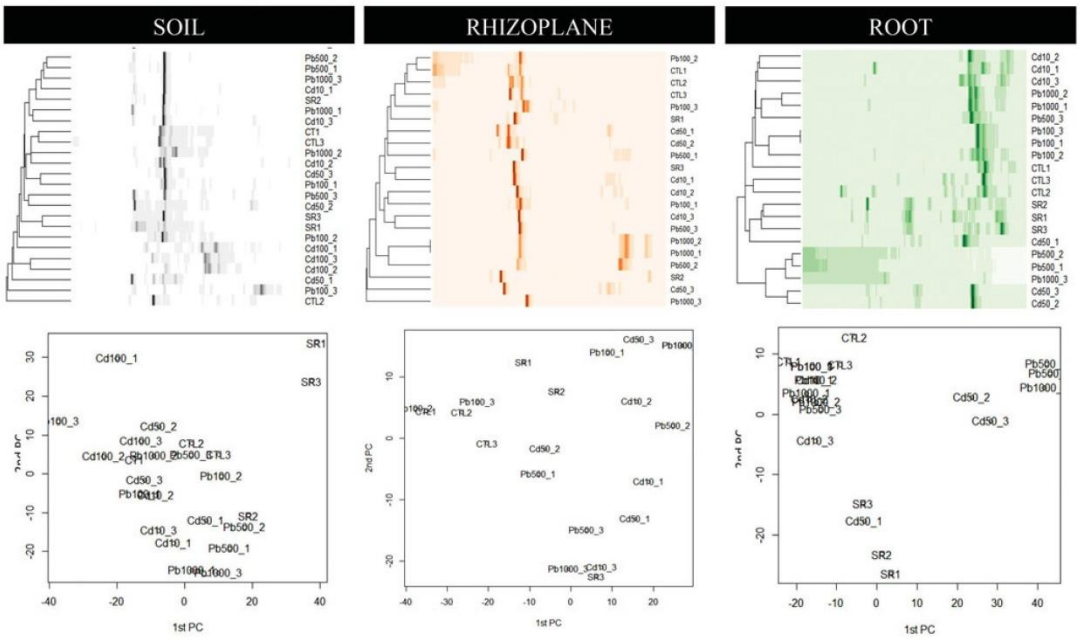


**Figure 2.** *Helianthus petiolaris* heatmap and PCA distribution of the microorganisms in bulk soil, rhizospheric, and endophytic compartments. A complete linkage algorithm was used to perform a cluster analysis of Bray–Curtis dissimilarity matrices inferred from ARISA values.

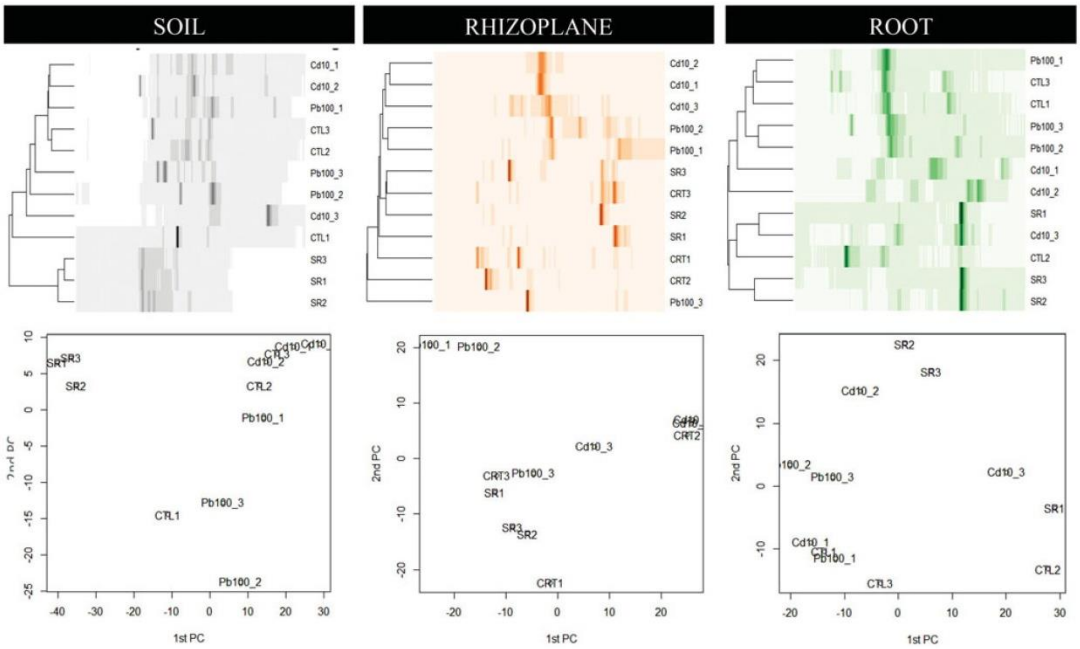


**Figure 3.** *Helianthus annuus* heatmap and PCA distribution of the microorganisms in bulk soil, rhizospheric, and endophytic compartments. A complete linkage algorithm was used to perform a cluster analysis of Bray–Curtis dissimilarity matrices inferred from ARISA values.

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