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Soil variables that determine lead accumulation in *Bidens pilosa* L. and *Tagetes minuta* L. growing in polluted soils

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ABSTRACT

Toxic metal soil pollution is one of the most serious environmental problems, with phytoextraction being an ecological and inexpensive potential solution to recuperate soil functionality. This emergent technology has produced good results for many elements, but phytoextraction has not been as successful for lead as for other pollutants. This research aimed to evaluate several factors that could be determinant on Pb uptake by the accumulator plants *Bidens pilosa* and *Tagetes minuta*. Topsoils (bulk and ryzospheric) and plants were collected around a former Pb smelter, and sequential extractions of Pb, Cu and Zn, organic matter content, electrical conductivity, pH, and texture were measured in soils. Also, bacterial diversity in soil was determined by the denaturing gradient gel electrophoresis (DGGE) technique. The Pb, Cu, and Zn concentrations in soil extractions and plants (leaves, stem, and root) were quantified by T-XRF in Synchrotron, Brazil. Our results demonstrated that Pb uptake from polluted soils and translocation to aerial tissues by two Asteraceae species are related not only to the traditional soil parameters, but even more importantly to the Zn and Cu concentrations in plant and soils. In addition, the soil bacterial biodiversity also affects the Pb uptake by plants. In this study, we propose the total translocation factor (TTF) to evaluate the phytoextraction efficiency, which is given by the ratio of total extraction of Pb in aerial organs and roots, instead of using the Pb concentrations alone.

1. Introduction

Toxic metal soil pollution is currently considered to be one of the most serious environmental problems due to its persistence and toxicity, and at many locations it is continuously worsening as a result of human activities (Rascio and Navari-Izzo, 2011). Soil conditions are affected negatively by toxic pollutants, with soil quality being diminished and subsequently impeding the economic development of agricultural areas (Becerril Soto et al., 2007). This requires complex and costly measures in order to be able to utilize soil which has been subject to a high residence time of metals with the added possibility of groundwater migration (Becerril Soto et al., 2007). In fact, the remediation of toxic metal polluted soils represents a technological challenge for both industries and government institutions, with phytoextraction being an alternative that contemplates soil conservation by harnessing the potential of plants to transform or eliminate the contaminants accumulating in their tissues (Alvarez and Illman, 2006). However, the implementation of this technology has certain difficulties because the complexity of hyperaccumulation is far from being understood with several aspects still awaiting explanation (Rascio and Navari-Izzo, 2011). It is important to explore the plants ability to absorb the metals, biomass production, the plant organs in which metals are accumulated, the bioavailability of metals in soil and the competition between different metal ions (Montes Botella, 2001). Also, a unique combination of these factors occurs at different sites implying that a specific phytoextraction prescription cannot be applied to every site, due to these site-specific conditions. Thus, it is of great importance to increase our understanding of accumulator-based remedial mechanisms, because this provides clues for optimizing the effectiveness of phytoextraction for specific agronomic practices.

Despite phytoextraction being a method that is currently being used in many parts of the world, studies related to this are scarce in Argentina, especially those using native species for toxic metal removal (Arreghini et al., 2006; Bonfranceschi et al., 2009; Flocco et al., 2002; Salazar and Pignata, 2014; Torri et al., 2009; Zubillaga et al., 2012).

Abbreviations: EC, electrical conductivity; OM%, organic matter percentage; SBD, soil bacterial diversity; DW, dry weight; TF, translocation factor; BCF, bioconcentration factor; TTF, Total Transfer Factor; BM, Biomass; Rhiz. (Bp, *Bidens pilosa* rhizosphere; Rhiz. (Tm), *Tagetes minuta* rhizosphere; DGGE, denaturing gradient gel electrophoresis.

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In a previous investigation (Salazar and Pignata, 2014), we ran a screening of native species for phytoextraction in a Pb polluted area and found that *Tagetes minuta* L. and *Bidens pilosa* L. accumulated relatively high concentrations of Pb in their leaves (380.5 μg g^{-1}DW and 100.6 μg g^{-1}DW, respectively) when growing near a former Pb smelter. However, Pb accumulation in these species was not clearly related to the Pb concentration (pseudototal, extracted with pure HNO_3 and HCl, and extractable with HCl 0.5 M) in soils and was not consistently high. In this context, the bibliography provides extensive information about toxic metal uptake and translocation mechanisms (Ahmed et al., 2012), but not in the case of Pb. Moreover, as Pb uptake does not occur in most plants (Pourrut et al., 2011), this is the main reason for failure of phytoextraction of this pollutant. Thus, it is important to study species that have shown Pb uptake potential, even if they are not hyperaccumulators. Schreck et al. (2012) showed that foliar uptake of Pb occurs in some species when they are exposed to atmospheric industrial fallout, with different uptake rates occurring for each species. In addition, Kabata (2011) affirmed that 95% of the Pb present in leaf tissues came from foliar uptake. Pb from soil is adsorbed on the root surfaces, bound to root mucilage compounds (Glinski and Lipiec, 1990) or to the polysaccharides of rhizodermic cell surface, and finally penetrates the root system passively. However, Pourrut et al. (2011) reviewed the latest advances in this field and reported that at the molecular level, the mechanisms through which Pb penetrates the roots have not yet been explained. These authors suggested the possible following input pathways of Pb into the roots: i) Pb may use non-selective channels or transporters, mainly related to the ordinary uptake of Ca^{2+}, ii) Pb uptake could also be facilitated by families of Cation Diffusion Facilitator (CDF), ZRT/IRT-like Protein (ZIP) or the Natural resistance-associated macrophage proteins (Nramps) associated with the transport of Cu, Zn, Cd and Mn; iii) Pb may enter the cell as a pectin-Pb complex during internalization of low-methyl esterified pectins from the cell wall.

The aim of the present study was to determine the reasons for the variability in Pb uptake and translocation observed in our previous results and to contribute new evidence about Pb uptake mechanisms. With this purpose in minds, we analyzed the influence that several soil variables have on Pb accumulation by *T. minuta* and *B. pilosa*, paying special attention to Cu and Zn availability in soils and their concentrations in plants.

2. Material and methods

2.1. Study area and species description

The study area was located in Bouwer, which is 18 km south of Córdoba City, Argentina (Fig. 1). A detailed description of the study area was given by Salazar and Pignata (2014), who reported Pb pollution around a former battery recycling smelter and a variable accumulation pattern in wild plants.

In the present study, six sampling sites (Fig. 1) were chosen in order to evaluate the Pb concentration gradient in soils and the presence of the two species of interest *Tagetes minuta* L. and *Bidens pilosa* L.

Both species belong to the compositae family, and are herbaceous plants with an annual life cycle. *Tagetes minuta* is native to South America (including Argentina) and is nowadays present in the whole world. According to Sérsic et al. (2006) it grows to between 0.5 and 1.8 m in height and is especially adapted to disturbed habitats. This species is used in popular medicine (Tereschuk et al., 1997), and its bioactive components can be extracted for pesticide production (Vasudevan et al., 1997), and the oil is used as an established flavour and perfumery raw material (Soule, 1993). *Bidens pilosa* is widely distributed, growing in tropical and subtropical Asia, America and other continents. According to Sérsic et al. (2006) it grows to between 0.3 and 1.5 m in height, but during this study taller individuals were found. It has been reported as a Cd and Pb accumulator (Sun et al., 2009; Salazar and Pignata, 2014), but in contrast with other metal accumulators, it has a stronger tolerance to adverse environments, grows faster and has a greater biomass (Sun et al., 2009). The leaf extract of this species prevents and attenuates blood hypertension (Dimo et al., 2002).

2.2. Sampling procedure

Plant and topsoil samples were collected at the sampling sites following a systematic sampling, for which each sampling site consisted of a 9 m^2 square. Within each sampling site three composite samples were collected for each species, and a stainless steel shovel was employed to remove plants from their roots conserving their rhizospheric soil. Three specimens growing at no more than 10 cm distance from each other were combined to obtain one composite sample. The stem of each plant was cut at the base to separate the rhizosphere samples, which were then kept in sterile plastic bags. All aerial plant samples were kept in paper bags. At each sampling site, three composite samples of bulk topsoil were collected using a composite blast hole sampler, and these samples were also kept in sterile plastic bags. All work tools and operator hands were cleaned using water and ethanol 70% after each sampling.

Once in the laboratory, the soil sterile bags were opened under sterile conditions to obtain 1 g of soil in duplicate for each sample with the aim of running a denaturing gradient gel electrophoresis (DGGE) study of the soil microorganism community to determine soil bacterial diversity. These samples were kept in a 1.5 mL eppendorf tube at −80 °C until being analyzed.

Roots and rhizospheric soil were separated by shaking them in plastic boxes and sieving. Roots, as well as separated stems and leaves, were washed with tap water first and then three times with ultrapure water (roots were also sonicated between tap and ultrapure water washing) and then oven-dried at 40 °C to dry weight (DW).

Rhizospheric and bulk soil samples were oven-dried at 40 °C for 24 h. All samples were sieved to <2 mm (using a polyethylene sieve) and stored in darkness until analytical procedures were carried out (Bäckström et al., 2004).

2.3. Physico-chemical and microbiological analyses

2.3.1. Electrical conductivity, pH, percentage of organic matter and texture in topsoils

The topsoil pH and electrical conductivity (EC) were measured in 1:5 soil:water suspension triplicates at room temperature (Bäckström et al., 2004). In order to calculate the dry weight (DW), samples were oven-dried for 4 h at 105 °C to constant weight (Al-Khashman and Shawabkeh, 2006), and the organic matter percentage (OM%) was determined according to Gallardo et al. (1987) by the combustion of the samples at 700 °C for 2 h. This temperature was reached by applying increments of 5 °C per minute.

The texture was determined by laser-diffraction size analysis according to Gaiero et al. (2013) using a Horiba LA-950 particle size analyzer after eliminating the presence of organic matter by using 30% *v*/v H_2O_2 (analytical grade). The precision (reproducibility) of the laser diffraction particle sizer was tested by using mixtures of glass beads (NIST Traceable polydisperse particle standard PS202/3–30 μm and PS215/10–100 μm, Whitehouse Scientific W). For both runs (PS202, $n = 6$ and PS215, $n = 5$), the median (D50) was within 3% of the certified nominal value, and the percentiles D10 and D90 were within 5% of the nominal values for the standards.

2.3.2. Pb, Cu and Zn quantification in topsoils

With the aim of analyzing different fractions of Pb, Cu and Zn in the topsoils, a sequential extraction was performed according to Tessier et al. (1979) after sieving to <64 μm. The studied fractions were: Fraction 1 “Exchangeable”; Fraction 2 “Bound to Carbonates”; Fraction 3 “Bound to Iron and Manganese Oxides”; Fraction 4 “Bound to Organic

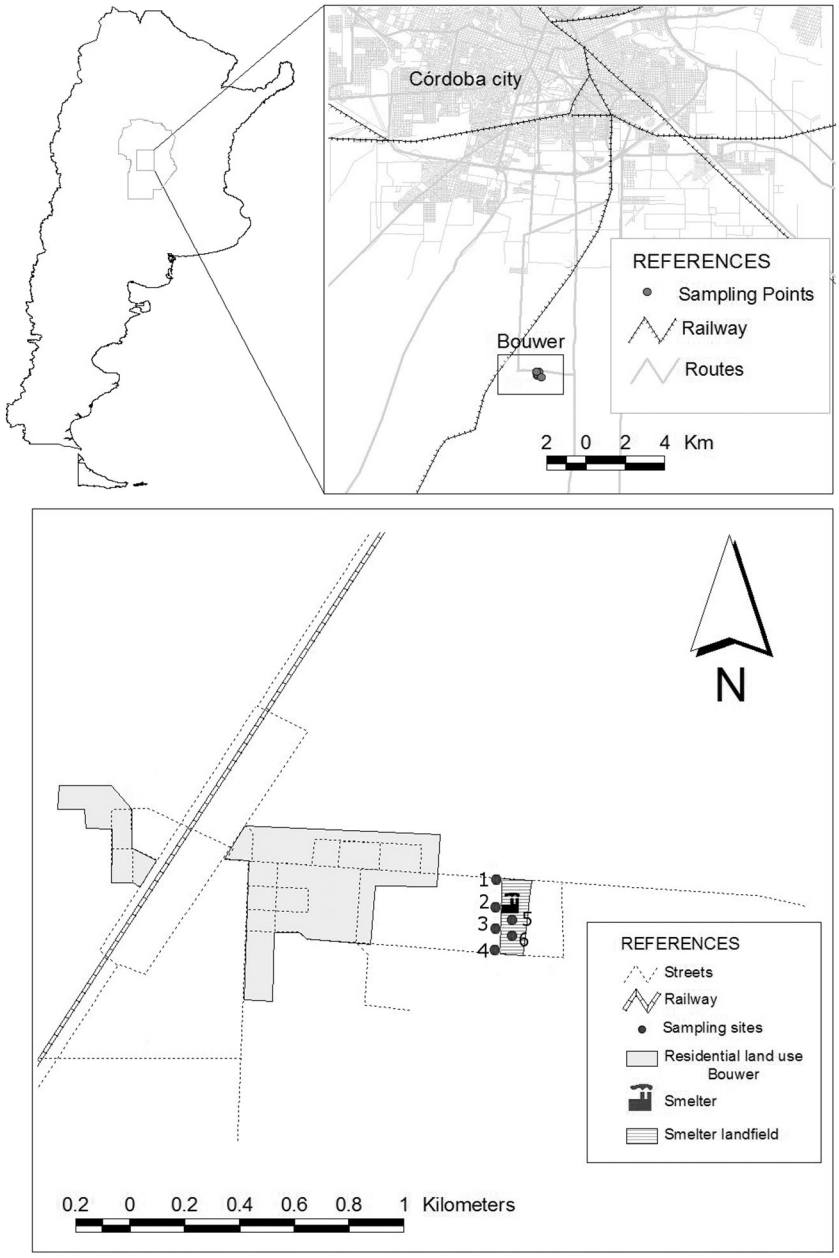

Fig. 1. Topsoil and vegetation sampling area in the surroundings of a lead smelter plant located at Bouwer in Córdoba (Argentina).

Matter"; and Fraction 5 "Residual" (with the last one performed according to Ketterer et al. (2001)).

These five extractions were used for multi-elemental analysis by Total Reflection X-Ray Fluorescence (TXRF) using Synchrotron Radiation as described below for plant samples.

2.3.3. Soil bacterial diversity (SBD)

Soil bacterial diversity was determined using denaturing gradient gel electrophoresis (DGGE). This is a molecular fingerprint technique widely used for microbial ecology and provides information on the microbial diversity in response to environmental triggers (Smalla et al., 2007). For this purpose, the total DNA was extracted from 1 g of soil using the Omega Bio-TEK E.Z.N.A. Soil DNA Kit, with this extract stored at −20 °C. The polymerase chain reaction (PCR) was performed using GoTaq DNA Polymerase kit reagent (Promega/Madison, USA), and the PCR amplification of 16S rDNA was carried out using the bacterial universal primers 341F-GC: (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G CCTACGGGAGGCAGCAG-3′; melting temperature = 92.8 ° C) and 907R: (5′-CCGTCAATTCCTTTRAGTTT −3′; melting temperature = 55.3 ° C). The 16S rRNA fragment was obtained for subsequent DGGE analysis as previously described (Demergasso et al., 2005).

The thermocycling program used for the amplification was: Step 1: 1 cycle of 4 min at 96 ° C. Step 2: a) 10 cycles of 30 s at 94 ° C, 45 s at 62 ° C and 1 min at 72 ° C; b) 25 cycles of 30 s at 94 ° C, 45 s at 57 ° C and 1 min at 72 ° C. Step 3: 1 cycle of 10 min at 72 ° C.

Bacterial PCR products were run at 60 ° C, 100 V for 16 h in denaturing gradients ranging from 30 to 70%, where 100% denaturant is defined as 7 M urea and 40% (*v*/v) formamide (Muyzer et al., 1993). Gels were stained with SYBR-Gold visualized on a UV transilluminator and photographed, which were then analyzed using Image Tool software (University of Texas, Health Science Center/San Antonio, USA). Band counting was used as a measurement of soil bacterial diversity (SBD).

2.3.4. Pb, Cu and Zn concentration in plants

The concentrations of Pb, Cu and Zn in plant tissues were determined according to Wannaz et al. (2011). Roots, stems and leaves of *Tagetes minuta* and *Bidens pilosa* plants from each sampling site were dried to constant weight in an oven at 50 ± 2 °C, and a 1 g dry weight sample of these materials was used for multi-elemental analysis by Total Reflection X-Ray Fluorescence (TXRF) using Synchrotron Radiation. Plant material was ground and reduced to ashes at 450 °C for 4 h, which were then digested with 3 mL of analytical commercial 20% HNO_3 at 25 ± 2 °C. The solid residue was separated by centrifugation, and the volume adjusted to 5 mL with Milli-Q water. Then, 10 ppm of a Ga solution was added as an internal standard. Aliquots of 5 μL were taken from this solution and dried on an acrylic support. Standard solutions with known concentrations of Cu^{2+}, Pb^{2+} and Zn^{2+} (with Ga as an internal standard) were prepared for the calibration of the system. Samples were measured for 200 s, using the total reflection setup mounted at the X-ray fluorescence beamline of the National Synchrotron Light Laboratory (LNLS), Campinas, SP, Brazil. A polychromatic beam approximately 5 mm wide and 0.1 mm high was used for excitation. For the X-ray detection, a Si (Li) detector was used with an energy resolution of 165 eV at 5.9 keV.

As a quality control, blanks and samples of the standard reference materials Oriental Tobacco Leaves (CTA-OTL-1, ICTJ) and CRM 281 (ryegrass, European Commission/BCR) were prepared in the same way and were run after five determinations to calibrate the instrument. The results were found to be within ±2% of the certified value. The coefficient of variation of replicate analysis was calculated for different determinations and the variations were found to be less than 10%.

2.4. Data analyses

2.4.1. Statistical analyses

The Shapiro–Wilks test for normality was applied, and as non-normal distributed elements were not found a log-transformation was not necessary. Heteroscedasticity was encountered in almost all cases, so this was included in the model using Infostat/E (Universidad Nacional de Córdoba/Córdoba Argentina) coupled to R (The R Foundation for statistical computing) to perform an Analysis of Variance (ANOVA). Whenever the ANOVA indicated significant effects ($p < 0.05$), a pairwise comparison of means was undertaken using the Tukey test. Multiple lineal regressions were performed to evaluate the variables that determine Pb accumulation in plants. Multivariate analyses (principal components) and multiple lineal regressions were performed in order to relate the studied variables using Infostat/E.

2.4.2. Translocation and bioconcentration factors

In this study, the translocation factor (TF) and bioconcentration factor (BCF) were calculated using the ratio of Pb concentration in roots and shoots [$TF = C_{shoot}/C_{root}$] and the ratio of Pb concentration (psuedo-total) in soils and shoots [$BCF = C_{shoot}/C_{soil}$], respectively (Bu-Olayan and Thomas, 2009). Additionally a modified BCF was calculated using the Pb soil concentration in fraction 1 in order to include the bioavailability of the pollutant in soil. To obtain the Pb concentration in shoots, the values found for leaves and for stems were multiplied by the total biomass of each organ, which were then added in order to determine the total mass of Pb in aerial organs. Finally, this result was divided by the total mass of the aerial organs (stems plus leaves).

3. Results

3.1. Soil

The results found for pH, OM%, EC and texture are shown in Table 1. A bifactorial ANOVA in cells was performed by crossing the factor categories (sites and soil compartments), with the aim of enhancing the statistical power and reducing type II errors.

In general, all the studied sites presented pH values lower than 7, with the most acidic soil being found at site 5 in the bulk soil and *T. minuta* rhizosphere. In contrast, the least acidic soils were those collected at sites 1, 2 and 6, while the remaining ones presented intermediate pH values.

The results corresponding to the OM% showed significant differences, with sites 4 and 6 showing the highest values for bulk and rhizospheric soils. Moreover, on comparing all the sites together, *B. pilosa* rhizosphere revealed a generally higher OM% than the other two compartments (Bulk and Rhiz *T. minuta*).

Regarding the EC values, these were around 100 ± 40 μs cm^{-1} in most of the samples, with a few exceptions such as the bulk soils at sites 4 and 6, which presented low values, and the *T. minuta* rhizospheric soil at site 5, which presented high EC values.

The texture results revealed a general high dominance of more than 70%, of the silt fraction (2 to 50 μm). Despite statistically significant differences, there was no clear trend among sites or compartments.

Table 1
Mean values ± standard deviation and ANOVA results for pH, organic matter percentage (OM%), electrical conductivity (EC) and texture (clay, silt and sand percentage content) corresponding to topsoil compartments at different sites.

Site	Soil compartment	pH (***)	OM% (***)	EC (μs cm^{-1}) (***)	Clay (%) (***)	Silt (%) (***)	Sand (%) (***)
1	Bulk	6.8 ± 0.2 a	14 ± 1 c	83 ± 18 b	1.2 ± 0.6 c	81 ± 2 c	18 ± 2 d
	Rhiz (*B. pilosa*)	7.0 ± 0.2 a	18.1 ± 0.3 b	113 ± 17 b	2.6 ± 0.3 c	75.1 ± 0.2 d	22.3 ± 0.5 c
	Rhiz (*T. minuta*)	6.8 ± 0.2 a	14 ± 1 c	80 ± 17 b	2.3 ± 0.3 c	74.8 ± 0.2 d	22.9 ± 0.5 c
2	Bulk	6.7 ± 0.2 a	16 ± 2 b	62 ± 18 c	2.0 ± 0.4 c	76.5 ± 0.7 c	21.6 ± 0.5 c
	Rhiz (*B. pilosa*)	6.8 ± 0.2 a	23 ± 3 a	157 ± 17 b	1.5 ± 0.5 c	80.1 ± 0.7 c	18.5 ± 0.5 d
	Rhiz (*T. minuta*)	6.8 ± 0.2 a	17.5 ± 0.3 b	89 ± 18 b	1.8 ± 0.4 c	75 ± 2 d	23 ± 2 c
3	Bulk	5.8 ± 0.2 b	11.0 ± 0.2 c	104 ± 43 b	1.48 ± 0.02 c	72.0 ± 0.2 e	26.5 ± 0.5 b
	Rhiz (*B. pilosa*)	6.17 ± 0.07 b	14 ± 1 c	82 ± 17 b	1.93 ± 0.02 c	72.12 ± 0.03 e	25.95 ± 0.01 b
	Rhiz (*T. minuta*)	5.96 ± 0.07 b	12 ± 1 c	72 ± 18 c	1.62 ± 0.02 c	65.5 ± 0.7 f	32.9 ± 0.5 a
4	Bulk	6.13 ± 0.07 b	2.6 ± 0.6 a	46 ± 2 d	2.0 ± 0.4 c	76.8 ± 0.7 c	21.2 ± 0.5 c
	Rhiz (*B. pilosa*)	6.18 ± 0.07 b	37 ± 9 a	141 ± 18 b	2.3 ± 0.3 c	84.6 ± 0.2 b	13.2 ± 0.5 e
	Rhiz (*T. minuta*)	6.39 ± 0.07 a	29 ± 5 a	103 ± 2 b	1.7 ± 0.5 c	87.9 ± 0.7 a	10.4 ± 0.5 f
5	Bulk	4.8 ± 0.2 d	14 ± 1 c	117 ± 2 b	1.3 ± 0.6 c	87 ± 2 a	12 ± 2 e
	Rhiz (*B. pilosa*)	6.23 ± 0.07 b	26 ± 4 a	95 ± 17 b	1.8 ± 0.4 c	78.6 ± 0.2 c	19.6 ± 0.5 d
	Rhiz (*T. minuta*)	5.62 ± 0.07 c	17.6 ± 0.3 b	510 ± 18 a	2.8 ± 0.3 b	78 ± 2 c	19 ± 2 d
6	Bulk	6.46 ± 0.07 a	17 ± 2 b	40 ± 2 d	1.12 ± 0.6 c	80.7 ± 0.7 c	18.2 ± 0.5 d
	Rhiz (*B. pilosa*)	6.57 ± 0.07 a	22 ± 3 a	57 ± 2 c	1.7 ± 0.5 c	78 ± 2 c	20 ± 2 d
	Rhiz (*T. minuta*)	6.41 ± 0.07 a	28.8 ± 0.8 a	69 ± 2 c	4.67 ± 0.09 a	85.2 ± 0.2 b	10.2 ± 0.5 f
N		54	54	54	54	54	54

ANOVA references: Values in each column followed by the same letter do not differ significantly at $p < 0.05$, those followed by different letters differ significantly: significance levels: ns, not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Abbreviations: Rhiz: rhizospheric soil. Texture: particles size: Sand: 2–0.05 mm; Silt: 0.05–0.002 mm; Clay: <0.002 mm.

Table 2
Pseudototal Pb concentrations in soil compartments (Bulk and *Bidens pilosa* and *Tagetes minuta* rhizospheres) and their percentages in each fraction of the sequential extraction around a Pb smelter in Córdoba, Argentina. ANOVA results comparing concentrations among sites and compartments.

Site	Compartment	Pb Pst *** (mg kg^{-1})	Pb F1 *** (%)	Pb F2 *** (%)	Pb F3 *** (%)	Pb F4 *** (%)	Pb F5 *** (%)
1	Bulk	471 ± 67 d	25 ± 9 b	23 ± 5 a	39 ± 6 b	11 ± 2 b	4.2 ± 0.7 d
	Rhiz (*B. pilosa*)	1638 ± 117 b	1.0 ± 0.1 e	5 ± 1 c	76 ± 6 a	14 ± 3 b	2.7 ± 0.5 d
	Rhiz (*T. minuta*)	493 ± 100 d	5 ± 1 d	10 ± 2 b	64 ± 6 a	15 ± 3 b	7 ± 1 c
2	Bulk	2686 ± 203 a	11 ± 3 c	26 ± 6 a	33 ± 5 b	28 ± 4 a	2.3 ± 0.4 d
	Rhiz (*B. pilosa*)	2834 ± 225 a	2.7 ± 0.5 e	34 ± 8 a	32 ± 6 b	24 ± 4 a	7 ± 1 c
	Rhiz (*T. minuta*)	4667 ± 858 a	11 ± 3 c	16 ± 4 b	39 ± 6 b	31 ± 5 a	1.8 ± 0.3 e
3	Bulk	1379 ± 109 b	27 ± 10 b	23 ± 5 a	39 ± 5 b	11 ± 2 b	2.5 ± 0.4 d
	Rhiz (*B. pilosa*)	1040 ± 104 c	11 ± 3 c	19 ± 5 b	49 ± 6 b	17 ± 3 b	3.9 ± 0.6 d
	Rhiz (*T. minuta*)	845 ± 102 c	19 ± 7 b	24 ± 5 a	39 ± 5 b	14 ± 3 b	6.1 ± 0.9 c
4	Bulk	685 ± 101 c	6 ± 1 d	12 ± 3 b	48 ± 6 b	29 ± 4 a	5.1 ± 0.8 c
	Rhiz (*B. pilosa*)	796 ± 101 c	2.0 ± 0.3 e	12 ± 3 b	44 ± 5 b	39 ± 5 a	3.3 ± 0.6 d
	Rhiz (*T. minuta*)	374 ± 52 d	9 ± 3 c	17 ± 4 b	50 ± 6 b	13 ± 3 b	6.1 ± 0.9 b
5	Bulk	6989 ± 638 a	35 ± 15 a	44 ± 11 a	13 ± 5 d	7 ± 2 c	0.8 ± 0.2 f
	Rhiz (*B. pilosa*)	4429 ± 728 a	12 ± 4 c	29 ± 7 a	38 ± 6 b	19 ± 3 b	1.4 ± 0.2 e
	Rhiz (*T. minuta*)	4125 ± 585 a	10 ± 3 c	39 ± 10 a	41 ± 6 b	7 ± 2 c	3.3 ± 0.5 d
6	Bulk	858 ± 102 c	4.1 ± 0.8 d	11 ± 1 b	24 ± 5 c	12 ± 3 b	49 ± 8 a
	Rhiz (*B. pilosa*)	839 ± 101 c	2.4 ± 0.4 e	5 ± 1 c	26 ± 5 c	37 ± 5 a	30 ± 5 a
	Rhiz (*T. minuta*)	702 ± 101 c	8 ± 2 c	15 ± 4 b	14 ± 5 d	21 ± 4 a	42 ± 7 a
N		54	54	54	54	54	54

ANOVA references: Interaction was found between Pb pseudototal concentrations and the five fractions. Values in each column followed by the same letter do not differ significantly at $p < 0.05$, those followed by different letters differ significantly: significance levels: ns, not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Abbreviations: Pst: pseudototal. F1, Fraction 1 "Exchangeable"; F2, Fraction 2 "Bound to Carbonates"; F3, Fraction 3 "Bound to Iron and Manganese Oxides"; F4, Fraction 4 "Bound to Organic Matter"; and F5, Fraction 5 "Residual".

Table 2 presents the Pb pseudototal concentration in soils and its distribution in the five studied fractions, while Table 3 and Table 4 present these results for Zn and Cu. The metal concentrations for Zn and Cu were within the levels allowed by Argentinean legislation for agricultural, residential and industrial land use (Argentinean Legislation, 1993). Fraction 3 (bound to Fe and Mn oxides) was in general the soil fraction containing the most Zn; while Cu was mainly contained in fractions 3 and 4 (bound to organic matter). The spatial distribution (among sites and compartments) of Cu and Zn was homogeneous, with no significant differences among samples. Concerning Pb concentrations, all the sites exceeded the legal threshold for agricultural land use (375 μg g^{-1}) and most sites also surpassed the legal threshold for residential land use (500 μg g^{-1}), while several sites (1, 2, 3, 5) exceeded the legal threshold for industrial land use (1000 μg g^{-1}).

The ANOVA results for fraction 1 indicated a significantly higher percentage of Pb in soil collected at site 5 in the bulk compartment than in the rest of the samples. In addition, the Pb percentage in fraction 2 presented higher values at site 5 for the three compartments. It is important to note that the elements contained in fractions 1 and 2 are potentially available for living organisms, especially plants. Therefore, it is important that sites 2, 3 and 5 had between 25 and 50% of their Pb content in these fractions. Concerning the Pb content in fraction 1 at these sites, when statistical differences were recorded among bulk and rhizospheric soils, the higher percentage corresponded to the bulk compartment soil, thus indicating possible root incorporation by plants. Most of the Pb contained in soil was concentrated in fraction 3 at most of the studied sites. Fraction 4 also revealed significantly higher Pb percentages at sites 2, 4 and 6, but with no differences found among soil compartments. Finally, the Pb percentage in fraction 5 was relatively variable but with low values at all the studied sites except for site 6.

The bacterial diversity of soils is presented in Fig. 2, where it can be observed that the amount of lines was similar at the six studied sites for

Table 3
Pseudototal Zn concentrations in soil compartments (Bulk and *Bidens pilosa* and *Tagetes minuta* rhizospheres) and their percentages in each fraction of the sequential extraction around a Zn smelter in Córdoba, Argentina. ANOVA results comparing concentrations among sites and compartments.

Site	Compartment	Zn Pst * (mg kg^{-1})	Zn F1 ** (%)	Zn F2 *** (%)	Zn F3 *** (%)	Zn F4 * (%)	Zn F5 *** (%)
1	Bulk	38.7 ± 6.1 a	15 ± 4 a	12 ± 3 b	37 ± 7 a	20 ± 4 b	15 ± 2 a
	Rhiz (*B. pilosa*)	72 ± 16 a	17 ± 5 a	9 ± 3 b	29 ± 6 b	38 ± 8 a	6 ± 1 b
	Rhiz (*T. minuta*)	30 ± 5 b	8 ± 1 a	16 ± 4 b	36 ± 7 a	30 ± 6 a	9 ± 2 b
2	Bulk	64 ± 13 a	7 ± 1 a	6 ± 2 b	60 ± 10 a	21 ± 5 b	6 ± 1 b
	Rhiz (*B. pilosa*)	100 ± 38 a	5 ± 1 b	13 ± 3 b	56 ± 9 a	17 ± 4 b	10 ± 2 b
	Rhiz (*T. minuta*)	39 ± 6 a	8 ± 1 a	7 ± 2 b	32 ± 7 b	33 ± 7 a	20 ± 3 a
3	Bulk	50 ± 8 a	6 ± 1 a	16 ± 3 b	49 ± 9 a	22 ± 5 b	8 ± 2 b
	Rhiz (*B. pilosa*)	42 ± 7 a	26 ± 17 a	7 ± 3 b	20 ± 6 b	20 ± 4 b	27 ± 5 a
	Rhiz (*T. minuta*)	54 ± 9 a	10 ± 2 a	35 ± 5 a	24 ± 6 b	12 ± 3 b	19 ± 3 a
4	Bulk	45 ± 7 a	15 ± 4 a	9 ± 3 b	43 ± 8 a	18 ± 4 b	15 ± 2 a
	Rhiz (*B. pilosa*)	30 ± 5 b	20 ± 7 a	11.3 ± 0.1 b	29 ± 6 b	32 ± 7 a	8 ± 2 b
	Rhiz (*T. minuta*)	48 ± 8 a	8 ± 1 a	10 ± 4 b	53 ± 9 a	15 ± 4 b	15 ± 2 a
5	Bulk	32 ± 5 b	8 ± 1 a	6 ± 1 b	55 ± 4 a	24 ± 5 b	7 ± 2 b
	Rhiz (*B. pilosa*)	56 ± 10a	11 ± 2 a	10 ± 3 b	28 ± 6 b	41 ± 9 a	10 ± 2 b
	Rhiz (*T. minuta*)	52 ± 9 a	11 ± 2 a	6 ± 1 b	55 ± 9 a	16 ± 4 b	13 ± 2 a
6	Bulk	45 ± 7 a	11 ± 2 a	25 ± 3 a	26 ± 6 b	22 ± 5 b	17 ± 3 a
	Rhiz (*B. pilosa*)	40 ± 6 a	8 ± 1 a	6 ± 2 b	24 ± 6 b	50 ± 13 a	12 ± 2 a
	Rhiz (*T. minuta*)	34 ± 5 b	12 ± 3 a	12 ± 4 b	19 ± 6 b	30 ± 6 a	27 ± 5 a
N		54	54	54	54	54	54

ANOVA references: Interaction was found between Zn pseudototal concentrations and the five fractions. Values in each column followed by the same letter do not differ significantly at $p < 0.05$, those followed by different letters differ significantly: significance levels: ns, not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Abbreviations: Pst: pseudototal. F1, Fraction 1 "Exchangeable"; F2, Fraction 2 "Bound to Carbonates"; F3, Fraction 3 "Bound to Iron and Manganese Oxides"; F4, Fraction 4 "Bound to Organic Matter"; and F5, Fraction 5 "Residual".

Table 4
Pseudototal Cu concentrations in soil compartments (Bulk and *Bidens pilosa* and *Tagetes minuta* rhizospheres) and their percentages in each fraction of the sequential extraction around a Cu smelter in Córdoba, Argentina. ANOVA results comparing concentrations among sites and compartments.

Site	Compartment	Cu Pst *** (mg kg^{-1})	Cu F1 *** (%)	Cu F2 *** (%)	Cu F3 *** (%)	Cu F4 *** (%)	Cu F5 *** (%)
1	Bulk	137 ± 12 b	10 ± 4 b	10 ± 3 a	42 ± 4 a	24 ± 1 b	14 ± 3 c
	Rhiz (*B. pilosa*)	89 ± 12 c	22 ± 9 b	13 ± 4 a	19 ± 4 b	37 ± 7 b	9.9 ± 0.8 c
	Rhiz (*T. minuta*)	74 ± 3 c	5 ± 4 c	nd	28 ± 14 b	63 ± 7 a	6.9 ± 0.8 d
2	Bulk	132 ± 26 b	nd	3 ± 2 b	55 ± 14 a	31 ± 13 b	12 ± 3 c
	Rhiz (*B. pilosa*)	103 ± 3 b	18 ± 4 b	11 ± 4 a	24 ± 4 b	11 ± 3 c	35.2 ± 0.8 a
	Rhiz (*T. minuta*)	109 ± 12 b	15 ± 9 b	4 ± 2 b	28 ± 14 b	36 ± 7 b	17 ± 3 c
3	Bulk	83 ± 12 c	3 ± 1 c	5 ± 2 b	61 ± 4 a	25 ± 7 b	6.2 ± 0.8 d
	Rhiz (*B. pilosa*)	64.4 ± 0.5 c	49 ± 4 a	3 ± 2 b	8 ± 4 b	20 ± 7 b	20.2 ± 0.1 b
	Rhiz (*T. minuta*)	156 ± 12 a	12 ± 4 b	13 ± 4 a	27 ± 1 b	22 ± 1 b	25 ± 3 b
4	Bulk	128 ± 3 b	12 ± 4 b	26 ± 11 a	39 ± 14 a	19 ± 7 b	4.5 ± 0.1 d
	Rhiz (*B. pilosa*)	122 ± 3 b	8 ± 4 b	13 ± 4 a	18 ± 14 b	55 ± 1 a	5.8 ± 0.8 d
	Rhiz (*T. minuta*)	104 ± 26 b	28 ± 9 b	9 ± 3 a	18 ± 4 b	31 ± 7 b	14 ± 3 c
5	Bulk	115 ± 12 b	27 ± 16 b	27 ± 12 a	13 ± 4 b	27.8 ± 0.1 b	5.2 ± 0.8 d
	Rhiz (*B. pilosa*)	100 ± 26 b	12 ± 1 b	22 ± 8 a	28 ± 14 b	20 ± 3 b	19 ± 3 b
	Rhiz (*T. minuta*)	72 ± 12 c	15 ± 1 b	8 ± 3 a	39 ± 1 a	39 ± 3 b	nd
6	Bulk	104 ± 26 b	8 ± 4 b	17 ± 5 a	45 ± 4 a	25 ± 7 b	6.0 ± 0.8 d
	Rhiz (*B. pilosa*)	34 ± 12 d	16 ± 9 b	9 ± 3 a	33 ± 4 b	20 ± 13 b	21 ± 3 b
	Rhiz (*T. minuta*)	37 ± 0.5 d	10 ± 1 b	39 ± 12 a	24 ± 14 b	17 ± 7 b	10.0 ± 0.1 c
N		54	54	54	54	54	54

ANOVA references: Interaction was found between Cu pseudototal concentrations and the five fractions. Values in each column followed by the same letter do not differ significantly at $p < 0.05$, those followed by different letters differ significantly: significance levels: ns, not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Abbreviations: Pst: pseudototal. F1, Fraction 1 "Exchangeable"; F2, Fraction 2 "Bound to Carbonates"; F3, Fraction 3 "Bound to Iron and Manganese Oxides"; F4, Fraction 4 "Bound to Organic Matter"; and F5, Fraction 5 "Residual", nd: not detectable.

its three compartments, with 4 to 6 lines. However, there was a significantly higher bacterial diversity, with 10 lines, for site 5 in the *T. minuta* rhizosphere. Two lines were present in all the samples, indicating that two bacteria can resist extreme Pb concentrations in soils and extend in these conditions.

3.2. Pb, Cu and Zn concentrations in plants

Fig. 3 presents the results of the Pb (A), Zn (B) and Cu (C) concentrations for *B. pilosa* and *T. minuta*, while Fig. 4 shows the total Pb extraction per plant and the absolute (A) and percentage (B) distribution among the three studied organs for *B. pilosa* and *T. minuta*. A bifactorial ANOVA in cells was performed for total Pb extraction by crossing the factor categories (sites and species), with the aim of enhancing the statistical power and reducing type II errors.

Both species presented higher Pb concentrations in roots, followed by stems and finally leaves (Fig. 3-A). Using ANOVA to compare the Pb concentrations in each organ among sites indicated that *B. pilosa* accumulated higher Pb concentrations in the root at sites 2, 3 and 5; in stems at sites 3 and 5; and in leaves at sites 2 and 3. Higher concentrations of Zn were founded in root at sites 3 and 5; in stems at sites 3, 5 and 6; and in leaves at sites 3 and 6. *T. minuta* accumulated higher Pb concentrations in the three organs at sites 2, 3 and 5, while it accumulated similar Zn and Cu concentrations in root among sites, but presented higher values for Zn at sites 2, 3 and 6. All these variables revealed the same general trend, with higher concentrations being found at sites 2, 3 and 5 for both species.

Regarding the total Pb extraction per plant and its distribution among the plant organs (Fig. 4), *T. minuta* extracted more Pb than *B. pilosa* at sites 2, 3, while the opposite was found at site 5. At these

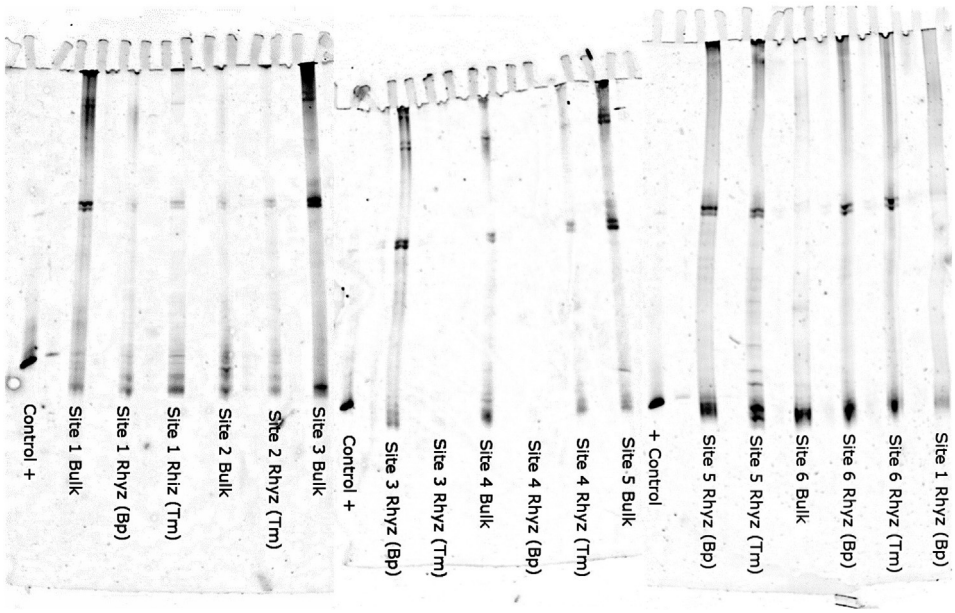

Fig. 2. DGGE pictures for bacterial 16S rRNA gene.

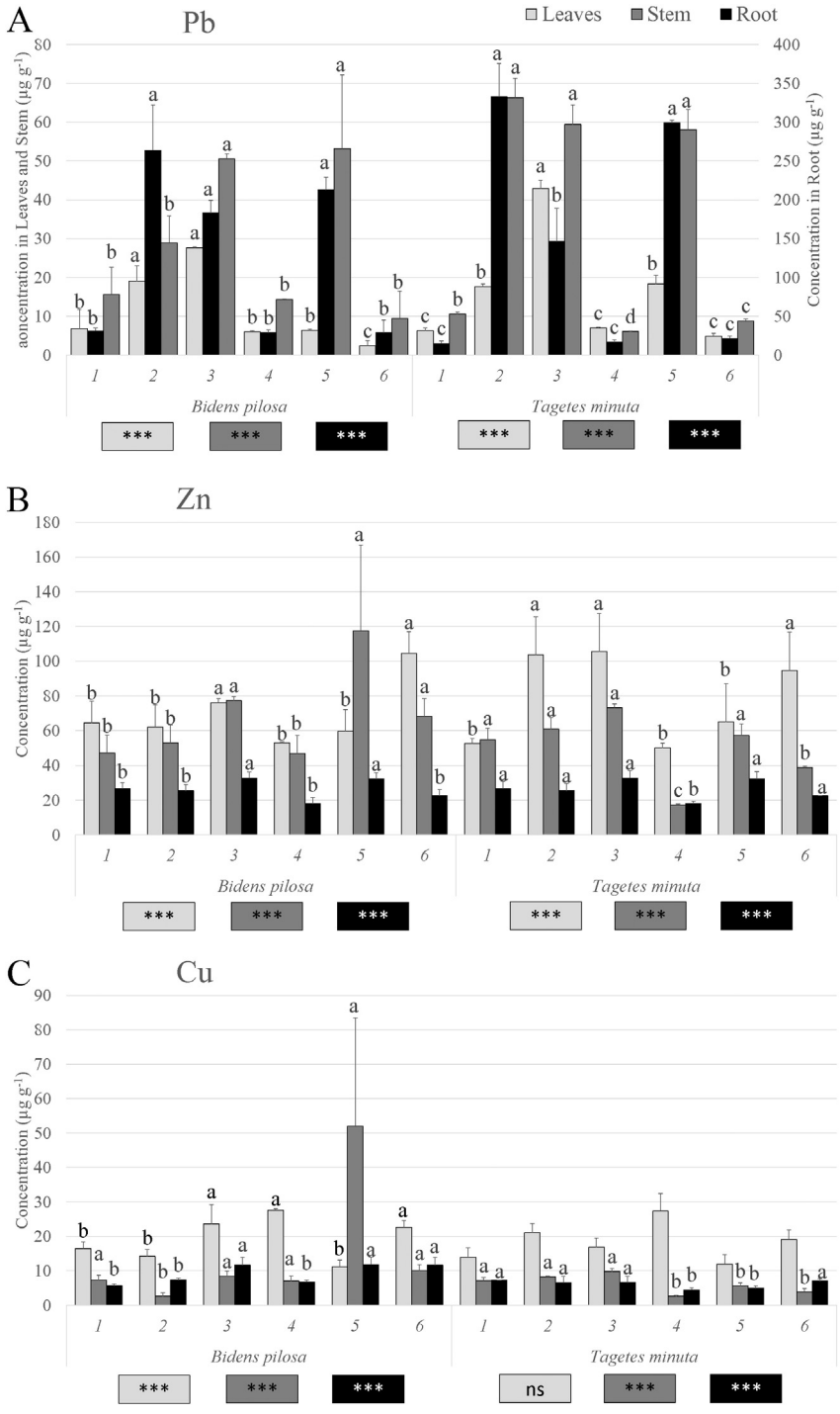

Fig. 3. Pb (A), Zn (B) and Cu (C) mean concentrations ± standard deviation (indicated by the error lines over bars) in leaves, stem and root in *Bidens pilosa* and *Tagetes minuta* growing in Pb polluted soils at six sampling sites. ANOVA and mean comparison were performed for each organ within each species among sites. Bars with same letter do not differ significantly at $p < 0.05$. Significance levels: ns, not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. N = 18 for each species and each organ.

three abovementioned sites, the difference between species was concentrated in the stem, with Pb extraction by root and leaves being similar. There were no significant differences between species noted at the other sites. If we consider the total Pb extraction per plant (sum of the three organs), *B. pilosa* did not reveal significant differences among sites, but *T. minuta* extracted significantly more Pb at sites 2 and 3 than at the others.

The Pb percentage distribution among organs (Fig 4-B) showed that at almost every site (with the only exception being *B. pilosa* at site 2) the pollutant mainly resided (more than 50%) in the aerial organs of the plants. The lowest Pb percentages for root were found at site 1, with the absolute amount of Pb extracted being relatively low for both species.

Results for the dry biomass, bioconcentration factor (BCF) and translocation factor (TF) calculated for *B. pilosa* and *T. minuta* are shown in Table 5. With the purpose of categorizing these results, it should be noted that a value of TF > 1 indicates an effective translocation of metals from root to shoots and a value of BCF > 10 indicates hyper-accumulator species, with BCF > 1 showing accumulator species and BCF < 1 revealing excluder species (Bu-Olayan and Thomas, 2009). BCF was calculated

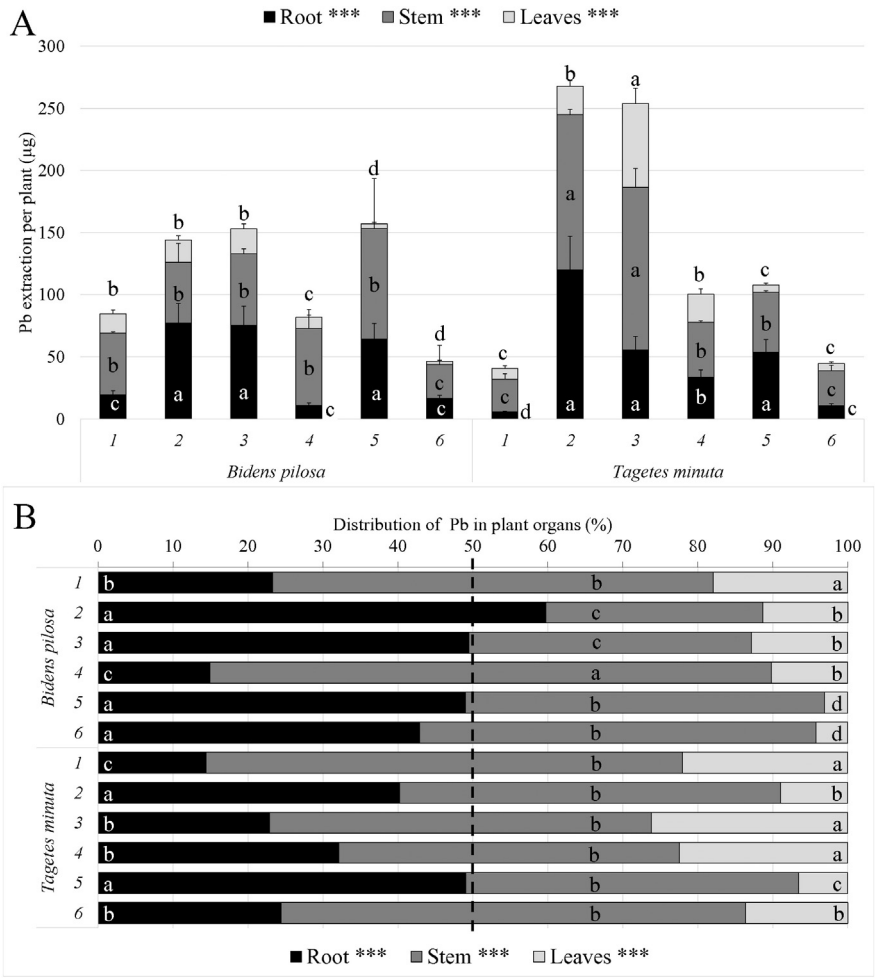

Fig. 4. A) Pb mean total extraction per plant ± standard deviation (indicated by the error lines over bars) in each organ of *Bidens pilosa* and *Tagetes minuta* growing in Pb polluted soils at six sampling sites. B) Percentage distribution of the total Pb extracted among organs. Bifactorial ANOVA and mean comparison were performed by crossing the factor categories (6 sites and 2 species). Bars with same letter do not differ significantly at $p < 0.05$. Significance levels: ns, not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. N = 36.

twice, once using the Pb content in Fraction 1 (indicating a bioconcentration capability related to the bioavailable Pb) and also using the pseudo-total content (traditional approach). With respect to the bioavailable Pb in soil, *B. pilosa* did not act as an accumulator species (BCF > 1), where as *T. minuta* did this at two sites. Traditional BCF presented minimal values for both species, but *T. minuta* revealed higher values.

Concerning TF, it appeared that none of the studied species translocated Pb from root to shoots. However, this result is in contrast with Fig. 4 B, where *B. pilosa* presented a mean of 60% of the extracted

Table 5
Dry biomass in each organ and the whole plant, bioconcentration factor (BCF) of the pseudo-total and fraction 1 Pb concentration, root to shoot translocation factor (TF) and Total Transfer Factor (TTF), mean values ± SD for *Bidens pilosa* and *Tagetes minuta* growing in Pb polluted soils in Córdoba, Argentina.

Species	Site	Dry biomass (g per plant)				BCF *** (F1)	BCF *** (Pseudototal)	TF *** (root-shoot)	TTF *** (root-shoot)
		Leaves ***	Stem ***	Root ***	Total ***				
Bidens pilosa	1	2.43 ± 0.56 a	4.51 ± 0.86 a	0.66 ± 0.10 b	7.59 ± 1.41 a	0.84 ± 0.23 b	0.007 ± 0.003 d	0.02 ± 0.004 d	3.49 ± 0.80 a
	2	0.88 ± 0.12 b	1.59 ± 0.19 b	0.32 ± 0.05 b	2.79 ± 0.34 b	0.12 ± 0.02 d	0.009 ± 0.002 d	0.01 ± 0.003 d	0.79 ± 0.31 b
	3	0.71 ± 0.09 b	1.14 ± 0.13 c	0.42 ± 0.07 b	2.27 ± 0.26 b	0.29 ± 0.06 c	0.040 ± 0.002 b	0.05 ± 0.001 b	1.03 ± 0.33 b
	4	1.48 ± 0.25 a	4.29 ± 0.80 a	0.42 ± 0.07 b	6.20 ± 1.04 a	0.79 ± 0.21 b	0.015 ± 0.003 c	0.03 ± 0.003 c	6.23 ± 2.14 a
	5	0.57 ± 0.07 b	1.44 ± 0.17 b	0.29 ± 0.05 b	2.30 ± 0.26 b	0.18 ± 0.04 c	0.008 ± 0.003 d	0.01 ± 0.001 d	1.19 ± 0.35 b
	6	0.83 ± 0.11 b	2.64 ± 0.39 a	0.58 ± 0.09 b	4.05 ± 0.56 a	0.67 ± 0.17 b	0.013 ± 0.002 c	0.02 ± 0.002 d	3.52 ± 0.81 a
Tagetes minuta	1	1.49 ± 0.26 a	2.53 ± 0.37 a	0.50 ± 0.08 b	4.53 ± 0.66 a	1.65 ± 0.51 a	0.018 ± 0.002 c	0.03 ± 0.004 c	7.22 ± 3.06 a
	2	1.31 ± 0.21 a	1.92 ± 0.25 b	0.32 ± 0.05 b	3.55 ± 0.47 a	0.21 ± 0.04 c	0.011 ± 0.004 c	0.01 ± 0.003 d	2.05 ± 0.48 a
	3	1.61 ± 0.29 a	2.28 ± 0.32 a	0.42 ± 0.07 b	4.32 ± 0.62 a	0.86 ± 0.23 b	0.062 ± 0.005 a	0.12 ± 0.004 a	3.78 ± 0.89 a
	4	3.22 ± 0.89 a	7.31 ± 1.81 a	1.93 ± 0.30 a	12.5 ± 3.0 a	1.61 ± 0.49 a	0.019 ± 0.008 c	0.09 ± 0.02 a	2.28 ± 0.52 a
	5	0.40 ± 0.05 c	0.87 ± 0.09 c	0.18 ± 0.03 c	1.45 ± 0.15 c	0.16 ± 0.03 c	0.014 ± 0.008 c	0.01 ± 0.002 d	1.04 ± 0.33 b
	6	1.20 ± 0.18 a	3.15 ± 0.50 a	0.53 ± 0.08 b	4.88 ± 0.73 a	0.60 ± 0.15 b	0.013 ± 0.005 c	0.02 ± 0.004 d	3.17 ± 0.72 a
	N	36	36	36	36	36	36	36	36

ANOVA references: Values in each column followed by the same letter do not differ significantly at $p < 0.05$, those followed by different letters differ significantly: significance levels: ns, not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Pb localized in shoots, a percentage which rose to 70% in *T. minuta*. For this reason, we propose a new factor, with the aim of complementing the traditional TF rather than replacing it, namely the Total Transfer Factor (TTF), which considers the absolute content of Pb instead of concentrations and is calculated as follows:

$$TTF = (C_{leaves}.BM_{leaves} + C_{stem}.BM_{stem})/(C_{root}.BM_{root})$$

where C is the concentration of an element in leaves, stem or root and BM is the biomass production. This factor could be useful for phytoextraction purposes because it combines the pollutant concentration in plant organs and biomass production, thereby indicating how much Pb was transferred above ground from the total Pb uptake, which effectively gives the extracted Pb. The traditional formula may indicate one species being a better translocator than another when it is in fact extracting a smaller absolute quantity of a contaminant, which constitutes a serious mistake in the phytoextraction context.

The results of TTF for *B. pilosa* and *T. minuta* are shown in Table 5, where the values found indicate that both species are effective Pb translocators.

4. Discussion

The relation among soil variables id shown in Fig. 5(A and B), where Fig. 5-Arelates the fractioning of Pb in soil with the physicochemical variables, as usually considered in bibliography (pH, EC, OM%, texture) and also with soil bacterial diversity (SBD). It was found that soil Pb bioavailability (fractions 1 and 2) was positively correlated with soil electrical conductivity and soil bacterial diversity, but negatively correlated with soil pH, Pb content in fraction 5 and clay percentage, whereas the organic matter, silt and sand contents did appear to be not related to Pb fractioning. These results are in agreement with the bibliography reviewed by Kabata (2011). This PCA result associates sites 5, 3 and the *Tagetes minuta* rhizospheric soil at site 2 with the bioavailable Pb. The addition of other element contents in soil to the PCA, such as Zn and Cu (Fig. 5-B), revealed that the Cu concentration in soil fractions 2 and 4 was positively correlated to Pb bioavailability. Related to this, there are several important publications that have studied simultaneously the content of these metals in soils and their accumulation in plants, but none of these authors have reported a relationship among the different metals or different soil fractions (Deng et al., 2004; Hale et al., 2012; Mignardi et al., 2012; Nolan et al., 2003; Tipping et al., 2003). In this context, the positive relation found between Pb

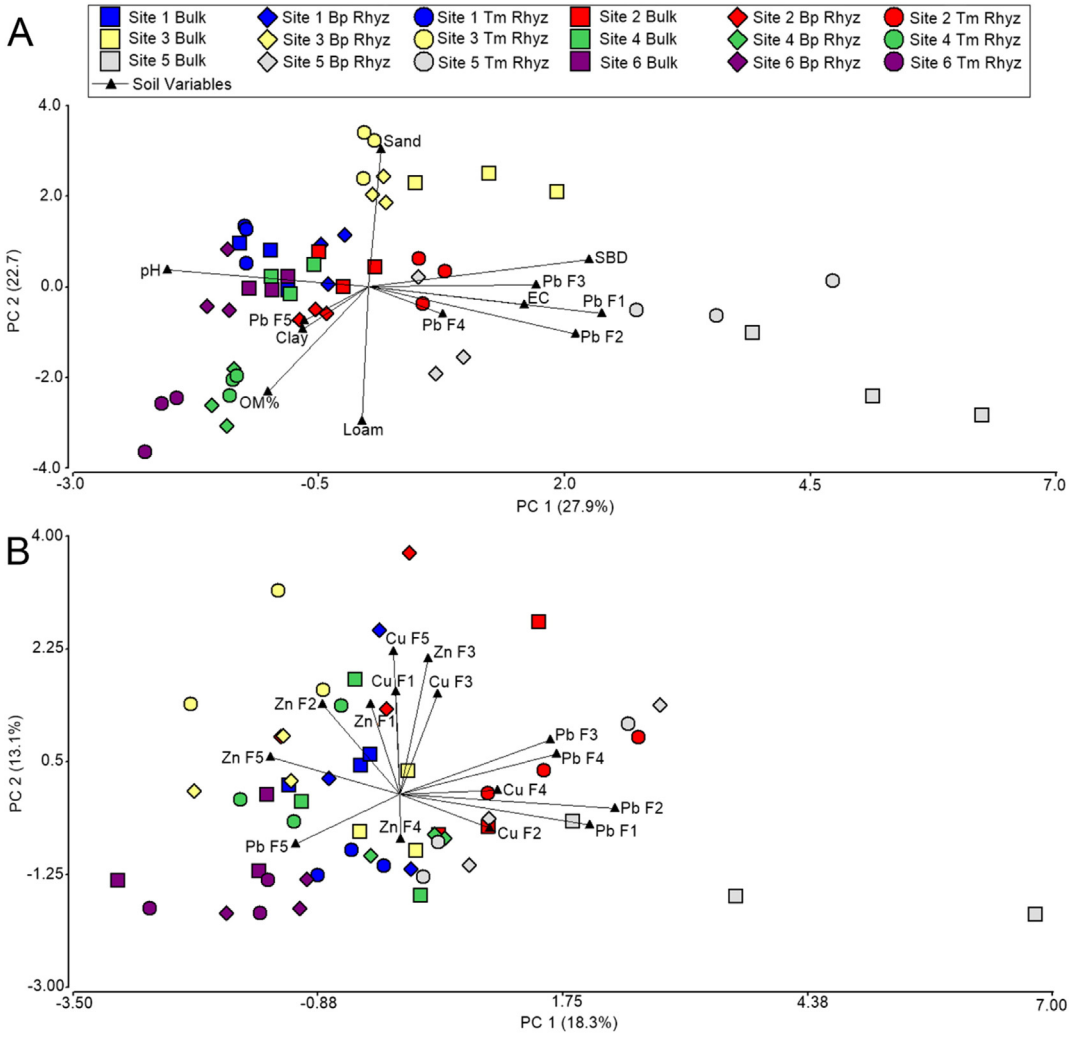

Fig. 5. A) Principal component analyses for Pb concentration in five soil fractions, pH, electrical conductivity (EC), organic matter percentage (OM%), soil bacterial diversity (SBD), and texture (sand, silt and clay percentage), measured in topsoil samples (bulk, *Bidens pilosa* rhizosphere, and *Tagetes minuta* rhizosphere) collected at a Pb polluted site. B) Principal component analyses for Pb, Zn and Cu concentrations in five soil fractions measured in topsoil samples (bulk, *Bidens pilosa* rhizosphere, and *Tagetes minuta* rhizosphere) collected at a Pb polluted site.

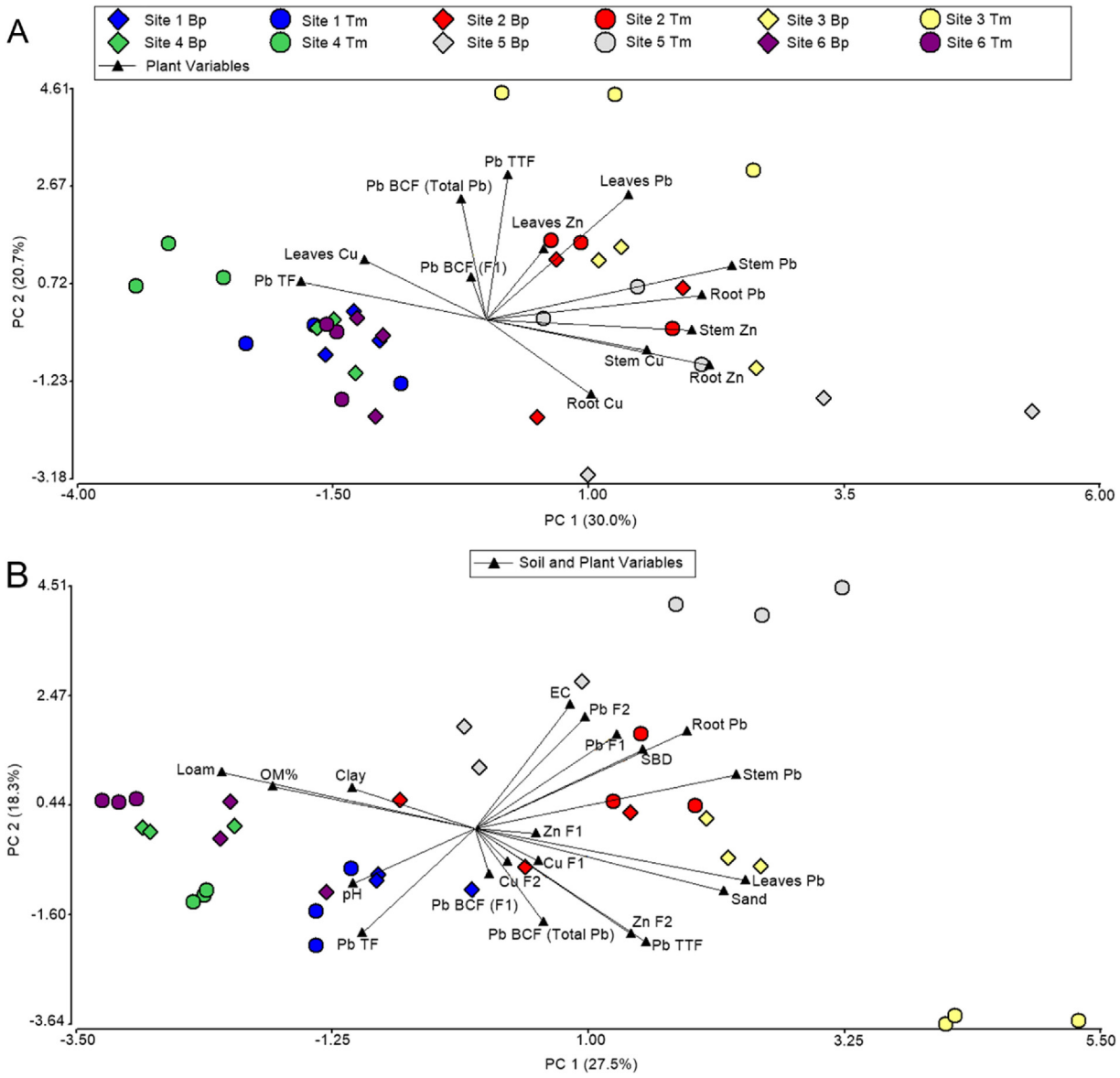

Fig. 6. A) Principal component analyses for Pb, Zn and Cu concentrations in plant organs, Pb bioconcentration factor (calculated using Pb total concentration in soil [Pb BCF (Total Pb)] and using Pb concentration in fraction 1 [Pb BCF (F1)]), Pb translocation factor (PB TF) and Pb Total Transfer Factor (Pb TTF), measured in *Bidens pilosa* and *Tagetes minuta* samples collected at a Pb polluted site. B) Principal component analyses for Pb concentration in plant organs, Pb bioconcentration factor (calculated using Pb total concentration in soil [Pb BCF (Total Pb)] and using Pb concentration in fraction 1 [Pb BCF (F1)]), Pb translocation factor (PB TF) and Pb Total Transfer Factor (Pb TTF), measured in *Bidens pilosa* and *Tagetes minuta* samples, and pH, electrical conductivity (EC), organic matter percentage (OM%), soil bacterial diversity (SBD), texture (sand, silt and clay percentage) and Pb, Zn and Cu concentrations in fractions 1 and 2 measured in their rhizospheric topsoil samples.

bioavailability and Cu (F2 and F4) in soils in the present study is a new contribution to the subject. This PCA result associates sites 2 and 5 with the bioavailable Pb in soil.

Fig. 6-Apresents a PCA that examines the correlation between the variables related to Pb phytoextraction efficiency with the content of Zn and Cu in plant organs. This revealed that Pb concentration in leaves was positively correlated to Zn concentration in leaves, while the Pb concentrations in the stem and root was positively correlated to the Zn and Cu concentrations in the same organs, but negatively correlated to Cu content in leaves. Other authors have also studied and reviewed the interaction among plant uptake of different toxic metals (Haiyan and Stuanes, 2003; Israr et al., 2011; Kabata Pendias and Pendias, 1984; Wong et al., 1986; Yoon et al., 2006) and have found similar results for the relationship between Zn and Pb, with both metals appearing to enter the plant together. On the other hand, there is disagreement about Cu and Pb interaction, with some authors finding that Cu enhances Pb uptake (Israr et al., 2011) while others reported the opposite trend (Wong et al., 1986). However, it should be noted that most of the above studies involved hydroponic experiments with all the toxic metals present at high concentrations. In the current investigation, we contemplated an in situ context and considered Pb, Cu and Zn fractioning in real soils, with Pb being the only contaminant and other elements being micronutrients. Our results revealed that Cu and Pb enter the plant together, but their translocation to leaves involves a competitive effect.

As the bioconcentration factor presented the same trend when it was calculated using Pb content in fraction 1 or the total concentration, we do not propose a new formula to calculate this. Concerning Pb TF, this was not related to the Pb concentration in leaves, but Pb TTF was positively correlated with the Pb concentration in leaves and stem (Fig. 6-B), indicating that this new factor could be useful when species are compared for phytoextraction aims. Fig. 6-Bpresents a PCA that examines the correlation between the variables related to Pb phytoextraction efficiency and Pb, Cu and Zn bioavailability in soil,

Table 6
Multiple lineal regression for Pb concentration in root, stem and leaves and total Pb extraction per plant.

Dependent variable	Model	R^2	N
Pb_{Root}	$0.38^{***} Pb_{F1} + 0.07^{**} Pb_{F2}$	0.65^{***}	36
Pb_{Stem}	$85.51^{**} + 0.11^{***} Pb_{Root} + 0.35^{***} Zn_{Stem} + 0.25^{*} Cu_{F1} - 12.82^{*}$ pH	0.89^{**}	36
Pb_{Leaves}	$0.44^{***} Pb_{Stem} - 0.01^{*} Pb_{F2} + 0.83^{**} Zn_{F2} - 0.2^{**} Cu_{Stem}$	0.85^{**}	36
Total Pb extraction per plant	$113.8^{*} + 0.22^{**} Pb_{F1} - 1.13^{*} Zn_{F3} - 0.3^{*}$ CE $+ 1.67^{*} Zn_{Stem} + 4.32^{*} Zn_{Root} - 2.76^{*} Cu_{Stem} - 2.63^{**} Cu_{Root}$	0.69^{***}	36

* Significant at 0.05 probability level.
** Significant at 0.01 probability level.
*** Significant at 0.001 probability level.

physicochemical variables in soils and SBD. Using this approach, it was found that the root and stem Pb concentrations were related to the bioavailability of this metal and SBD, indicating that these variables are important for Pb uptake. The influence of SBD might be related to the phytohormones that appear in the rhizosphere, because microorganisms produce and release these in the context of a positive interaction with plants. In addition, plants produce root exudates with organic compounds that act as nutrients for microorganisms, which liberate hormones that promote root growth and obtain more root exudates (Rajkumar et al., 2010). The root exudates may be an important factor for solubilizing Pb in soil, which is shown in Fig. 5-A, and for enhancing Pb uptake (Quartacci et al., 2014). Furthermore, Pb concentration in leaves was positively correlated to Zn and Cu bioavailability and sand content in soils but negatively correlated to organic matter percentage and clay and silt content in soil, thus indicating that these variables are responsible for Pb translocation.

Bearing in mind the above findings, multiple lineal regressions with all the data set were performed to determine the most significant variables for predicting the Pb concentration in each organ and the total Pb extraction per plant (Table 6). These results showed that Pb concentration in roots depended on Pb bioavailability in soils, as was expected. Moreover, Pb concentration in stems was increased by the Pb concentration in the root, as was also predicted. However, a new finding was that this was also increased by the Zn concentration in stem and the Cu content in the soil fraction 1, while it decreased with pH. In addition, the Pb concentration in leaves increased with Pb concentration in the stem and with Zn content in soil fraction 2, while it decreased with Pb content in soil fraction 2 and with Cu concentration in the stem. The total Pb extraction increased with Pb content in soil fraction 1 and with Zn concentration in stem and roots, but it was reduced by Zn content in soil fraction 3, Cu concentration in stem and roots, and EC. These results suggest that the presence of bioavailable Cu in soils favors Pb uptake in plants, while the presence of Cu in plant tissues obstructs the translocation process. However, in the case of Zn, its incorporation to the plant favors Pb translocation, which is an important finding because it helps in determining the metabolic route used by plants to uptake Pb.

Although there is extensive knowledge about the mechanism used to uptake elements such as Cd, Zn, Cu, Ni from polluted soils (Clemens et al., 2002), Pb uptake is still not well understood (Pourrut et al., 2011). Our findings indicate that Zn is necessary to incorporate and translocate Pb into the plants, with both metals entering the plant together, while Cu represents a competition factor. In fact, competition between Pb and other cations has been previously observed for Ca (Pourrut et al., 2008; Wang et al., 2007), but not for Cu. Moreover, this result indicates that the relationship among Zn, Cu and Pb is determinant not only for the first uptake in root but also for the translocation process. Taking into account that both Cu and Zn are micronutrients, and that therefore every plant species has mechanisms established for their uptake, then these mechanisms could be the key for the phytoextraction of Pb.

5. Conclusions

The present study has demonstrated that Pb uptake from polluted soils and its translocation to aerial tissues by two Asteraceae species are related not only to the traditional physico-chemical soil parameters (pH, OM%, EC, texture), but also, and more importantly, to the Zn and Cu content in plant and soil systems. These results provide evidence that supports one of the three possible input pathways of Pb into the roots proposed by Pourrut et al. (2011): that Pb uptake could be facilitated by families of Cation Diffusion Facilitator (CDF), ZRT/IRT-like Protein (ZIP) or the Natural resistance-associated macrophage proteins (Nramps) associated with the transport of Cu, Zn, Cd and Mn. It was also found that soil bacterial biodiversity is important for Pb uptake by plants. A new factor was proposed to evaluate phytoextraction efficiency, namely the "Total Transfer Factor" (TTF) which indicates how much Pb is transferred above of the total Pb uptak, which gives the effectively extracted Pb. The TTF was better correlated to the Pb concentration in leaves than the traditional Translocation Factor.

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Appendix A Supplementary data

Supplementary data associated with this article can be found in the online version, at doi: http://dx.doi.org/10.1016/j.geoderma.2016.06.011.These data include the Google map of the most important areas described in this article.

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