Phytoextraction of lead by *Helianthus annuus*: effect of mobilising agent application time

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ABSTRACT

Pot experiments were conducted to determine the best time for application of (ethylenediaminetetraacetic acid) EDTA and sheep manure extract (SME) in phytoremediation of a contaminated soil by *Helianthus annuus.* The plant was grown in a mine calcareous soil treated with increasing concentrations of EDTA or SME in 30 and 10 days before sowing (T1 and T2) and 10 and 30 days after sowing (T3 and T4). The best time for EDTA application was T4. The EDTA application before seed germination significantly reduced sunflower seedling emergence and dry weight. Soil available Pb and lead concentrations in plant organs increased with EDTA concentration but the actual amount of phytoextracted Pb decreased at high EDTA concentrations significantly, due to severe growth depression. SME application after sowing can increase plant dry weight and Pb concentration in the soil solution, enhancing the accumulated metal concentrations in shoots and roots. However the results showed that the most efficient treatments for Pb phytoextraction by sunflower are applications of 0.5 and 2 g EDTA/kg soil at T3 and T4, respectively.

Keywords: lead; phytoextraction; *Helianthus annuus*; EDTA; sheep manure extract; application time

High biomass producing plant species, such as *Helianthus annuus*, have potential for removing large amounts of trace metals by harvesting the aboveground biomass if sufficient metal concentrations in their biomass can be achieved. However, the low bioavailability of heavy metals in soils and the limited translocation of heavy metals to the shoots by most high biomass producing plant species limit the efficiency of the phytoextraction process. The success of phytoremediation depends upon the ability of a plant to uptake and translocate heavy metals, which is a function of the specific phenotype and genotype (Chen et al. 2003). Several studies have documented that chelating agents such as ethylene diamine triacetic acid (EDTA), *N*-(2-hydroxyethyl)-ethylenediaminetriacetic acid (HEDTA), and citric acid (CA) can be used to increase metal mobility, thereby enhancing phytoextraction (Huang et al. 1997, Elless and Blaylock 2000, Chen et al. 2003, Turgut et al. 2004, Liphadzi and Kirkham 2006). For instance, 1.0 g/kg EDTA was reported to be the most effective chelator, increasing shoot Pb concentration in pea and corn cultivars (Huang et al. 1997). A similar study on Pb accumulation with HEDTA increased the Pb concentration from 40 μg/g to 10 600 μg/g (Huang and Cunningham 1996). When Cd was used, 2 mmol/kg CA was more effective than EDTA or HEDTA, and increased the Cd shoot uptake from 125 to 256 μg/g (Elkhatib et al. 2001). Lesage et al. (2005) reported that heavy metal concentrations in harvested shoots increased with EDTA concentration but the actual amount of phytoextracted heavy metals decreased at high EDTA concentrations, due to severe growth depression.

Recent studies have led to significant advances in phytoremediation; however several questions at both the basic research and practical application levels remain unanswered. Basic research is needed on the use of synthetic and organic chelating agents for inducement of hyperaccumulation as well as ascertaining the fate of the chelating agents once in the soil (McIntyre 2003). Therefore, the aim of this study was to evaluate the impact of synthetic and natural mobilising agents on the uptake of Pb. The chelator concentration and source were varied to determine if distribution within the plant tissue could be enhanced. In addition, the influence of application time was investigated with application of the mobilising agents in 4 different times.

MATERIAL AND METHODS

Soil source characterization and preparation

The soil used for these experiments is representative of a mine soil in Isfahan province, Iran. The Irankoh region is one of several sites in Iran that mine for zinc and lead. It is located 20 km southwest of Isfahan city (50°31–45' N, 32°28–37' W; 1670 m above sea level). Some physical and chemical soil characteristics together with total and available Pb and Cd contents were summarised in Table 1. The soil was classified as Fine Loamy, Mixed Thermic, Typic, Haplo Calcids. The texture of the soil was loam. It was nonsaline (EC 0.28 dS/m), calcareous (equivalent calcium carbonate 25% and pH 7.76), with relatively low cation exchange capacity (CEC 18.4 cmolc/kg), organic matter (OM 0.86%) and total nitrogen (TN 0.083%). Soil available P and K were relatively high (27.4 and 170.2 μg/g, respectively). Total and available Cd were 3.2–4.2 μg/g and 0.03–0.1 μg/g, respectively. Soil total and available Pb were also considerably high (183–320 μg/g and 9–34 μg/g, respectively).

Table 1. Some soil characteristics used in this study

Parameter	Unit	Amount
Texture	–	loam
Clay	(%)	21.40
Silt	(%)	42.13
Sand	(%)	36.47
EC	(dS/m)	0.28
pH	–	7.76
ECC	(%)	25
CEC	(cmolc/kg)	18.4
OM	(%)	0.86
TN	(%)	0.083
Available P	(μg/g)	27.4
Available K	(μg/g)	170.2
Total Pb	(μg/g)	214
Total Cd	(μg/g)	3.6
Available Pb	(μg/g)	18
Available Cd	(μg/g)	0.08

EC – electrical conductivity; ECC – equivalent calcium carbonate; CEC – cation exchange capacity; OM – organic matter; TN – total nitrogen

Chelator and amendment sources and levels

EDTA was purchased in reagent grade form Sigma Chemical. Sheep manure extract (SME) was prepared by extracting 1:5 manure/distilled water suspension with shaking (120 round per min for 20 min) and centrifugation. Sheep manure extract had alkaline pH (7.9). The total solid of SME was measured for calculating the amounts of amendment in each treatment. Cadmium and lead concentrations in SME were 0.05 and 0.06 mg/l, respectively.

Three and half kg of the soil was put in pots of 20 cm diameter; the pots were arranged in a completely randomized experimental design with three replicates. A standard nutrient solution of distilled water containing 150 mg N [$(NH_4)_2SO_4$], 70 mg P (KH_2PO_4) and 100 mg K (K_2SO_4) was then added per kg of soil dw to rehydrate the soil.

Increasing concentrations of EDTA (0.5 and 2 g/kg soil) or SME (0.5 and 2 g/kg soil) were applied on soil at 4 different times [30 or 10 days before sowing (T1, T2) and 10 or 30 days after sowing (T3, T4)].

The additions of Cd were 0.004 and 0.016 μg/pot and those of Pb were 0.005 and 0.02 μg/pot in the SME 0.5 and 2 g/kg treatments, respectively.

Plant culture, harvesting, and analysis

Sunflower seeds (*Helianthus annuus* strain Alestar) were sown in soil. The plant grew in greenhouse conditions. After two weeks, 3 seedlings were maintained in each pot with three replicates. Three seedlings were used per 3.5 kg soil in each pot to yield a total of nine seedlings per treatment condition. After 14 weeks, at the flowering stage, the plants were carefully harvested. Soil total and available Pb were extracted by HNO_3-H_2O_2-HCl (USEPA, 2000) and DTPA (diethylene triamine pentaacetic acid) (Lindsay and Norvell 1978). Plant samples were washed with distilled water and HCl (0.01N). The roots and shoots (leaves and stems) were then sectioned and dried at 70°C during 48 h. After drying, the tissue sections were weighed. They were ground and stored in glass containers until analysis. Each sample of root or shoot organs was separately analyzed for Pb concentration with the wet ash method. Each sample was weighed and digested with trace pure HNO_3 and H_2O_2 using a hot plate (Soon and Abboud 1993). After digestion, the volume of each sample was adjusted to 50 ml using deionized water. Total Pb of samples was analyzed by atomic absorption on a Varian 220 instrument (Varian Australia Pty Ltd., Mulgrave, Australia) using an air-acetylene flame (Ramos et al. 1994). Translocation factors (shoot/root ratios) and enrichment factors (shoot/soil ratios) of Pb were also studied. The treatments were compared to ascertain concentration, uptake, translocation, and selectivity. Statistical analyses were performed on the SAS 6.12 software. All tests were done at 95% confidence level.

RESULTS AND DISCUSSION

Plant dry weight

Potential decreases in total plant dry weight were observed when the soil treatment was EDTA (Table 2). EDTA decreased significantly root and shoot dry weight, whereas SME stimulated root and shoot dry weight compared to control. The time of application of chelator and amendment on plant dry weight is very important. The negative effect of EDTA was more obvious when it was applied 30 and 10 days before sowing (T1 and T2). The plant biomass decreased with increasing the EDTA levels. However in T4, this effect was lower than in other times.

After only 7 days of seedling growth, shoot biomass was decreased by all EDTA treatments compared with the zero-EDTA control and SME treatments. The leaves showed chlorosis symptoms and then they necrosed. Hence the Pb concentration in sunflower was not determined for EDTA presowing treatments. Lesage et al. (2005) reported that *Helianthus annuus* suffered heavy metal stress due to the significantly increased bioavailable metal fraction in the soil. Vassil et al. (1998) however showed that free EDTA is more toxic than the complexes with metals. The high EDTA dose of 2.0 g/kg soil reduced seedling emergence and height of the plants grown in the composted biosolids (Liphadzi and Kirkham 2006).

Plant dry weight was significantly higher in all SME treatments compared to control. Plant biomass increased with increasing of SME levels. The effect of SME application time on root and shoot dry weight was not significant and as important as application time of EDTA treatments. Still, the root was more sensitive to time of SME application than shoot. Root dry weight was higher in presowing application times (T1 and T2).

Plant Pb concentration

Table 3 shows Pb concentrations in roots and shoots of sunflower. According to Pendias and Pendias (1992) Pb accumulations in root and shoot organs were above the normal level (0.2–20 μg/g). In most cases it reached the critical levels (30–300 μg/g). Lead bioaccumulation in roots and shoots of plants was significantly different. Lead concentration in root organs of the plant was higher than that in shoot organs in all treatments.

Generally, Pb concentration increased in both organs of the plant with increasing EDTA level, but it decreased with increasing SME level, especially at T1, T2 and T3. This decrease was not significant at T4. Applying EDTA increased Pb concentration in root organs significantly ($P < 0.05$) from 37.73 μg/g in control up to 72.96 μg/g in the case of application of 2 g EDTA/kg soil at T3. It is interesting that this treatment had relatively low effect on Pb concentration in shoot organs of sunflower. It may be concluded that EDTA

Table 2. The effect of mobilising agents on roots and shoots dry weights of *Helianthus annuus* in a contaminated soil

Treatment	Root biomass* (g/pot)				Shoot biomass* (g/pot)			
	T1	T2	T3	T4	T1	T2	T3	T4
Control	2.45^{cd}	2.45^{cd}	2.45^{cd}	2.45^{cd}	15.53^{e}	15.53^{e}	15.53^{e}	15.53^{e}
0.5 g/kg EDTA	0.253^{f}	0.28^{f}	1.26^{e}	2.357^{cd}	0.593^{j}	0.82^{j}	12.43^{g}	14^{f}
2 g/kg EDTA	0.087^{f}	0.137^{f}	0.847^{e}	2^{d}	0.243^{j}	0.433^{j}	3.98^{i}	10.47^{h}
0.5 g/kg SME	3^{b}	2.8^{bc}	2.7^{bc}	2.6^{bc}	16.4^{d}	18.4^{b}	18.77^{ab}	17.13^{cd}
2 g/kg SME	3.5^{a}	3^{b}	2.8^{bc}	2.6^{bc}	17.85^{bc}	19.33^{a}	19.5^{a}	18.33^{b}

SME – sheep manure extract

*values with different letters are significantly different at the 0.05 probability level for each parameter

Table 3. The effect of mobilising agents on roots and shoots Pb concentrations in *Helianthus annuus* in a contaminated soil

Treatment	Root Pb concentration* (μg/g)				Shoot Pb concentration* (μg/g)			
	T1	T2	T3	T4	T1	T2	T3	T4
Control	37.73^{g}	37.73^{g}	37.73^{g}	37.73^{g}	10.13^{j}	10.13^{j}	10.13^{j}	10.13^{j}
0.5 g/kg EDTA	–	–	67.50^{b}	64.66^{bc}	–	–	38.40^{c}	31.8^{d}
2 g/kg EDTA	–	–	72.96^{a}	62.36^{c}	–	–	55.53^{a}	46.10^{b}
0.5 g/kg SME	43.00^{f}	45.00^{f}	52.10^{de}	56.13^{d}	14.26^{h}	15.85^{fg}	17.00^{ef}	17.83^{e}
2 g/kg SME	42.08^{fg}	41.96^{fg}	49.90^{e}	52.53^{de}	12.23^{i}	13.50^{hi}	14.76^{gh}	17.43^{e}

SME – sheep manure extract

*values with different letters are significantly different at the 0.05 probability level for each parameter

can solubilize soil Pb and Pb-EDTA can be easily absorbed by roots of plant. This finding is supported by Lai and Chen (2004), Turgut et al. (2004), Lesage et al. (2005), and Liphadzi and Kirkham (2006). It was reported that applying 5 mmol EDTA/kg can significantly increase the Cd, Zn, or Pb concentrations both in the soil solution or extracted using deionized water in single or combined metals-contaminated soils, thus increasing the accumulated metals concentrations in rainbow pink grass shoots.

It was reported that adding chelants to soil increases not only the total dissolved metal concentration but also changes the uptake mechanism and depending on metal, plant species, and chelant concentration, significantly increases not only of plant Pb but also of plant Cu and Zn are likely (Nowack et al. 2006).

Komárek et al. (2007) reported that the addition of 9 mmol EDTA/kg led to a 214-fold increase in soluble Pb content (as much as 60% of Pb mobilised) and the addition of 9 mmol EDDS/kg led to a 33-fold increase in soluble Pb content compared to the control. However the competition of other metals [e.g. Fe from dissolving Fe (hydr)oxides] in the soil for the EDTA ligand is one of the most important efficiency-controlling parameter (Nowack et al. 2006, Komárek et al. 2007). It was reported that the significantly lower Pb-extraction efficiency of EDDS is to a great extent influenced by the competition of Cu to form the soluble metal-EDDS complex. The extraction efficiency of chelating agents is not only dependent on the stability constants (logK), but largely on the metals and ligand (chelant) concentrations. Therefore, the extraction efficiency of Pb in time is influenced by the partial dissolution of other metals like Fe oxides and hydroxides and consequent remobilisation and complexation of the adsorbed metal (Nowack et al. 2006).

Nowack et al. (2006) reported that in the absence of chelants, Zn and Cu accumulation in plants is governed by uptake of free metal ions in the symplastic pathway, which is efficient at low solution concentrations, and only a little Pb is taken up into the shoots. If the same metal concentrations in solution were chelated, then the uptake would occur through the apoplastic pathways and Cu and Zn uptake would be reduced while Pb uptake would be strongly increased. Therefore it becomes clear why there is an increase in Pb uptake in the presence of the chelants in most cases. In contrast, the few hydroponic data on Cu and Zn uptake in the presence of chelants mostly show a decrease in metal uptake. With a high dissolved metal concentration, translocation of all three metals to the shoots would increase in the presence of chelants because the nonselective uptake in the presence of chelants would exceed selective uptake along the symplastic pathway for both essential and nonessential metals. However, it depends on the plant species and may vary considerably.

The stimulating effect of SME on Pb concentration in both organs of the plant was significant ($P < 0.05$) compared to control. The increasing effect of SME on Pb concentration in root and shoot organs of the plant was more obvious in T4. The rapid mineralization of SME and the high buffering capacity of the calcareous soil made SME less efficient in increasing the phytoextracted amounts of heavy metals at T1, T2 and T3 compared to T4. Strikingly Pb concentration in both organs of the plant decreased with increasing SME level especially at T1, T2 and T3. This novel finding may be due to the negative effect of SME on Pb availability in soil. Organic matter like SME can act in two ways: decrease the mobility of metals by sorbing them or increase their mobility by complexing them with dissolved organic matter. In this study the first action of SME is more obvious at T1 and T2 and the second action of SME is more obvious at T3 and T4. However these finding need more study.

Plant Pb enrichment factor and available Pb content in soil

Table 4 shows Pb enrichment factors (shoot/soil Pb concentration ratios) in all treatments. Although soil total Pb was considerably high (183–320 μg/g), the available Pb (DTPA extractable Pb) in soil was markedly low (9–34 μg/g, respectively). The enrichment factors calculated on the basis of total Pb in soil were considerably low in all treatments (from 0.047 in control to 0.26 in 2 g EDTA/kg soil at T3). The increasing EDTA levels increased Pb enrichment factor markedly, however increasing SME levels decreased Pb enrichment factor in sunflower. The effect of chelating application time on enrichment factor was important. The highest enrichment factor was obtained for EDTA at 10 days after sowing (T3), whereas the enrichment factor of Pb in SME treatments was higher at 30 days after sowing (T4).

Soil available Pb after plant harvest was shown in Table 4. The addition of EDTA increased soil available Pb from 22.6 μg/g in control up to 72.3 μg/g in 2 g EDTA/kg soil at T4. This finding is supported by many reports. Lai and Chen (2004) reported that the concentrations of Cd, Zn, and Pb in a soil solution of rainbow pink significantly increased following the addition of EDTA ($P < 0.05$). It was also reported that the addition of EDTA and EDDS increased soluble metals in soil significantly (Komárek et al. 2007).

Soil available Pb increased with increasing EDTA level significantly, but increasing SME level had no significant effect on soil available Pb. However, in both EDTA and SME treatments, available Pb was higher in after-sowing application times (T3 and T4). Thus, it would be better to apply mobilising agents after plant germination (in early vegetative growth of plant). After the sensitive stage of plant growth the available form of heavy metals increases significantly with a few phytotoxicity symptoms.

Translocation factor and Pb uptake by plant

The effect of EDTA and SME on shoot/root Pb concentration ratio (translocation factor) was also significant, compared to control (Table 5). Translocation factor increased with increasing EDTA levels, but it decreased with increasing SME levels at T1, T2 and T3. Applying a higher level SME at 30 days after sowing (T4) increased Pb translocation factor. The effect of EDTA on the translocation factor was significantly higher than that of SME. The translocation factor increased from 0.268 in control up to 0.762 with application of 0.5 g EDTA/kg soil in 10 days after sowing (T3). Once again, these results show that Pb-EDTA complex is relatively mobile in plant root and shoot systems (Vassil et al. 1998), whereas the mobility of Pb complexes with individual organic acids in SME in plant tissues is negligible. Luo et al. (2005) reported that the application of EDTA significantly increased the shoot-to-root ratios of

Table 4. Lead enrichment factors (shoot/soil ratios) in *Helianthus annuus* and soil available Pb (μg/g) after plant harvest

Treatment	Pb enrichment factor based on total concentration in soil*				Soil available Pb after plant harvest*			
	T1	T2	T3	T4	T1	T2	T3	T4
Control	0.047^{j}	0.047^{j}	0.047^{j}	0.047^{j}	22.6^{g}	22.6^{g}	22.6^{g}	22.6^{g}
0.5 g/kg EDTA	–	–	0.18^{c}	0.149^{d}	40.5^{d}	42.6^{d}	53.5^{c}	58^{bc}
2 g/kg EDTA	–	–	0.26^{a}	0.215^{b}	62^{b}	60.85^{b}	67.7^{a}	72.3^{a}
0.5 g/kg SME	0.067^{h}	0.074^{fg}	0.079^{ef}	0.083^{e}	25^{fg}	23.4^{g}	28.7^{ef}	31^{e}
2 g/kg SME	0.057^{i}	0.063^{hi}	0.069^{gh}	0.082^{e}	26.3^{efg}	22^{g}	26^{efg}	28.8^{ef}

SME – sheep manure extract

*values with different letters are significantly different at the 0.05 probability level for each parameter

Table 5. Lead translocation factor (shoot/root ratio) and its uptake by *Helianthus annuus*

Treatment	Translocation factor*				Pb uptake (μg/pot)*			
	T1	T2	T3	T4	T1	T2	T3	T4
Control	0.268^{h}	0.268^{h}	0.268^{h}	0.268^{h}	156.9^{g}	156.9^{g}	156.9^{g}	156.9^{g}
0.5 g/kg EDTA	–	–	0.569^{b}	0.492^{c}	–	–	477.4^{a}	445.1^{b}
2 g/kg EDTA	–	–	0.762^{a}	0.74^{a}	–	–	221^{f}	482.2^{a}
0.5 g/kg SME	0.333^{de}	0.352^{d}	0.327^{de}	0.318^{efg}	233.9^{f}	291.8^{d}	318.9^{c}	305^{cd}
2 g/kg SME	0.29^{gh}	0.322^{ef}	0.296^{gh}	0.332^{de}	218.3^{f}	260.7^{e}	287.7^{d}	319.4^{c}

SME – sheep manure extract
*values with different letters are significantly different at the 0.05 probability level for each parameter

the concentrations of Cu, Pb, Zn and Cd in corn (*Zea mays* L. cv. Nongda 108) and bean (*Phaseolus vulgaris* L. white bean).

The effect of mobilising agents on Pb uptake by sunflower is shown in Table 5. Application of EDTA and SME significantly increased Pb uptake compared to control ($P < 0.05$). However, this effect depends on the mobilising agent and time of application. Although applying 2 g EDTA/kg soil at T3 improved Pb concentration in root and shoot organs of the plant as well as its enrichment and translocation factors significantly, Pb uptake in EDTA treatment was higher when it was applied at T4 due to lower heavy metal toxicity and higher plant biomass production. Lead uptake increased from 156.9 μg/pot in control to 482.2 μg/pot in 2 g EDTA/kg soil at T4.

The results showed that the best time for EDTA application for Pb phytoextraction is 30 days after sowing (T4). Application of EDTA before sowing (T1 and T2) reduces plant growth and shoot dry weight because of heavy metal toxicity, especially in the sensitive growth stage. The effect of increased transpiration rate of the plants on metal uptake under stress conditions (increased EDTA concentration) may be an important process. Lai and Chen (2004) found that EDTA treatment significantly increased the total uptake of Pb in the shoots of rainbow pink and vetiver grass over the values obtained with the control treatment ($P < 0.001$), but it did not significantly increase the total uptake of Cd and Zn. Lesage et al. (2005) found that heavy metal concentrations in harvested shoot of *Helianthus annuus* increased with EDTA concentration but the actual amount of phytoextracted heavy metals decreased at high EDTA concentrations, due to severe growth depression.

The results showed that shoot dry weight was considerably high in application of EDTA 30 days after sowing (T4) and it might be suitable time for effective Pb absorption and high Pb uptake (Table 5).

Although application time of SME had no significant effect on shoot dry weight, it had a significant effect on Pb uptake by *Helianthus annuus* (Table 5). In 0.5 g SME/kg treatment Pb uptake increased from 233.9 μg/pot in T1 to 318.9 μg/pot in T3. There was a similar result for 2 g SME/kg treatment. In 2 g SME/kg treatment Pb uptake increased from 218.3 μg/pot in T1 to 319.4 μg/pot in T4. The rapid mineralization of SME and the high buffering capacity of the soil made SME at T1 and T2 less efficient in increasing Pb uptake by *Helianthus annuus*.

It was reported that dissolved organic matter from cattle-manure slurry and sludge and sludge compost increases the solubility of cadmium, copper and zinc soil (Del Castilho et al. 1993, Zhou and Wong 2001). The results showed that soil available Pb was higher in SME treatments at T3 and T4.Therefore the positive effects of SME on Pb uptake by *Helianthus annuus* is the result of plant growth improvement and soil Pb solubilization.

The negative effect of high EDTA dose on germination, seedling emergence, height and dry weight of plants grown in heavy metal contaminated soil has been reported by many scientists (Chen et al. 2003, Turgut et al. 2004, Lesage et al. 2005, Li et al. 2005, Liphadzi and Kirkham 2006). The application time of synthetic chelating agent (i.e. EDTA) for increasing heavy metal bioavailability in soil and phytoextraction by a sensitive plant like *Helianthus annuus* is very important. Comparing the effect of EDTA and SME on Pb phytoextraction, the study showed that SME is not more effective than EDTA. The efficiency of EDTA on Pb phytoextraction by *Helianthus annuus* depends on the time of application. In the stage of fast vegetative growth (10 or 30 days after sowing) EDTA application may be more efficient for Pb phytoextraction.

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