J Soils Sediments (2013) 13:1213–1222
DOI 10.1007/s11368-013-0701-7

SOILS, SEC 4 • ECOTOXICOLOGY • RESEARCH ARTICLE

Effectiveness of amendments to restore metal-arsenic-polluted soil functions using *Lactuca sativa* L. bioassays

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Received: 10 January 2013 / Accepted: 9 April 2013 / Published online: 24 April 2013

Abstract
Purpose Because the success of the stabilisation of contaminants from amendments depends on the pollutants involved and the amendments used, the goals of this study were to assess whether selected amendments are able to restore highly polluted soils and to advance the knowledge of both the most suitable amendments to restore polluted soils and the most appropriate bioassays to estimate soil toxicity.
Materials and methods An acidic and polluted soil from mining waste was amended with marble sludge, compost and iron in nine different combinations. The soils were placed in plastic pots and bioassays, including the different stages in the development of lettuce (*Lactuca sativa* L.), were carried out. Pore water was analysed at the different stages of the development of lettuce. At the end of the experiment, pollutant concentrations in lettuce leaf were analysed and the sequential extraction of trace elements was performed.
Results and discussion The effectiveness of the amendments in reducing the toxicity of contaminated soils varied depending on the bioassay used. Marble sludge was the most effective in increasing pH and in reducing pollutant concentrations in pore water, clearly encouraging germination, root elongation and emergence. Throughout the emergence phase, marble sludge decreased in its effectiveness, probably because the pollutants precipitated as hydroxides and carbonates were taken up by the lettuce. In contrast, the compost began to improve the elongation of the seedling and the growth of lettuce. Although the amendments were effective in reducing the negative impact of pollutants in soils, none of them was able to successfully restore the functions of highly polluted soil.
Conclusions The development of the plant until the end of the establishment phase is the best index to estimate soil phytotoxicity, although the effect on the health of potential consumers can only be evaluated from the toxic element concentrations in the plant. The uptake of pollutants stabilised by the amendments would explain why the reduction of easily available pollutant concentrations does not necessarily imply the restoration of the normal functioning of the ecosystem.

Keywords Amendment · Bioassays · Bioavailability · Phytotoxicity · Pollutants

Responsible editor: Kerstin Hund-Rinke

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1 Introduction

One of the major problems for environmental quality is the heavy metal contamination of soil (Purves 1999). Mining activity results in the accumulation of highly polluted waste scattered throughout the landscape. This pollution not only degrades the quality of soil, aquatic and atmospheric environments but can also accumulate in crop (Verma and Dubey 2001), causing serious problems to human health (Adriano 1992; Lee and Chon 2003). Urban and agricultural demands have led to the occupation of land situated near disused metalliferous mines, increasing the environmental risks associated with the pollution of soil, surface water, and groundwater and the transfer of contaminants to plants (Alloway et al. 1998; Leita et al. 1998). The application of organic and inorganic amendments is a potential low-cost method of decreasing the bioavailability of trace elements in contaminated soil (Brown et al. 2004, 2005; Peng et al. 2009; Ping et al. 2008; Satoa et al. 2010; Melgar-Ramírez et al. 2012). However, the success of stabilisation of the contaminants in soil depends on the pollutants involved and the amendments used (Kumpiene et al. 2008). Analysis of the concentration of pollutants in aqueous extracts (lixiviates and pore water) of contaminated soil is one of the most commonly used methods to assess the effectiveness of an amendment (Hartley et al. 2004; Schwab et al. 2007; Simón et al. 2010), whereas bioassays with seeds are a common technique to evaluate the phytotoxicity of these solutions (Escoto et al. 2007; Bagur- González et al. 2011). However, some authors (Brown et al. 2005; Alvarenga et al. 2008) have observed that amendments of polluted soil reduce the extractable pollutants, but do not necessarily restore the normal function of the system (plant growth and microbial activity), suggesting that soil extracts may not be a sufficient measure to evaluate in situ soil amendments. Therefore, in addition to the chemical characteristics of the polluted soil, additional bioassays to better assess the level of restoration of contaminated ecosystem functions are needed (Leitgib et al. 2007; Alvarenga et al. 2012).

Lettuce grows better than other crops in contaminated soil and can accumulate relatively high amounts of metals and arsenic in its leaves (Cobb et al. 2000); thus, it has been one of the most used species in bioassays. Accordingly, tests with *Lactuca sativa* L. using a soil–water solution (germination and root elongation indices) and solid phase (emergence and growth indices) are considered useful tools to assess soil phytotoxicity (Escoto et al. 2007; Pandard et al. 2006).

The goals of this study were to assess whether selected amendments are able to restore highly polluted soils and to advance the knowledge of both the most suitable amendments to restore polluted soils and the most appropriate bioassays to estimate soil toxicity. To this end, we compared different combinations of amendments added to a highly polluted soil through a series of bioassays that measured different stages in the development of lettuce plants.

2 Materials and methods

2.1 Contaminated soil and amendments

The top layer (20 cm) of contaminated soil from the El Arteal mining district (Almeria, SE Spain) was collected, air-dried at 25 °C, sieved through a 2-mm pore size mesh and thoroughly mixed to ensure homogeneity. Three different amendments—marble sludge from the cutting and polishing of marble (MS), compost from agricultural greenhouse waste (CM) and synthetic Fe oxides (Bayferrox 920, BF)—were applied to the contaminated soil. In a previous column assay study (González et al. 2012), these amendments were applied at two different levels and in all possible combinations, resulting in 26 different treatments. Only the most effective treatments in reducing the toxicity to *L. sativa* L. (MS, CM, CM-MS and CM-MS-BF) were selected for the present study. The amended soils were labelled with three-digit numbers representing the supplemental amounts (in per cent, *w*/*w*) of MS (first digit), CM (second digit) and BF (third digit). Soil without amendment was labelled '000'. The amendments used were sieved through a 2-mm pore size mesh and the amended soils homogenised by hand for 15 min. A total of ten treatments were studied: 000, 020, 060, 400, 420, 423, 820, 823, 860 and 863.

2.2 Greenhouse experiment

Uncontaminated natural soil (the control soil), unamended contaminated soil and amended contaminated soils (three replicates) were moistened to field capacity with distilled water (≈200 cm^3 kg^{-1} dry soil) and allowed to air-dry in cycles of approximately 5 days (eight repetitions) to reach equilibrium (Martínez and Motto 2000). Plastic (PVC) pots with a drainage system were filled with 150 g of each soil and 90 cm^3 of distilled water was added to each pot. When the excess water was drained, pore water (PW1) was collected using Rhizon soil-moisture samples (Rhizon Research, Wageningen, the Netherlands) and the redox potential (E_h), electrical conductivity (EC) and pH were immediately measured. A 5-cm^3 aliquot of PW1 was immediately filtered through a cellulose filter (0.45-µm pore) under vacuum suction into a Pyrex flask previously washed with acid, acidified with HNO_3 and stored at <4 °C until the analysis. In all the PW1 samples, seed germination and root elongation were determined according to the US EPA procedure (US EPA 1996). Fifteen seeds of *L. sativa* L. (variety Villena RZ) were placed in 90-mm diameter Petri dishes containing filter paper in the bottom for support and 5 cm^3 of each PW1 was added. As a control, 5 cm^3 of distilled water was added to a similar Petri dish (water control, three replicates). The dishes were incubated for 120 h at 24±0.1 °C; the number of germinated seeds was counted and the lengths of the roots were measured. The seed germination index (GI; the proportion of germinated seeds compared to the water control) and the root elongation index (REI; percentage of root elongation in relation to the water control) were estimated.

After PW1 was collected, ten lettuce seeds were sown in each pot and the pots were maintained in a greenhouse under a natural day/night regime. Throughout the experiment, 25 cm^3 of distilled water was added three times per week at 50 cm^3 h^{-1}. Additionally, to prevent stress in the seedlings due to deficiencies of elements essential for development (Smical et al. 2008), 25 cm^3 of the nutrient solution prepared using analytical grade reagents [4 mmol L^{-1} $Ca(NO_3)_2$, 2 mmol L^{-1} KNO_3, 2.5 mmol L^{-1} K_2HPO_4 and 2 mmol L^{-1} $MgSO_4$] was supplied once per week. After 15 days, the emergence index (EI; the proportion of emerged seedlings in relation to the soil control) was estimated, pore water was again collected (PW2) following the same procedure as for PW1, and all the seedlings except one were carefully removed. The seedlings removed were washed with distilled water, and the shoots and roots were measured, dried and weighed. The seedling elongation index (SEI; proportional mean length of the roots and leaves of the removed seedlings in relation to the soil control) was estimated. The experiment with one seedling per pot and the same inputs of water and nutrient solution as applied during the emergence phase was continued until the establishment of the plant (14 weeks from sowing). The established plants were carefully removed and washed with distilled water and the roots and shoots were measured, dried and weighed. The dry weight index (DWI) of the established plants (the proportion of the total dry weight of the plant in relation to the soil control) was calculated. Lastly, sequential extraction of trace elements was performed in the soils in which the lettuce plants were established.

2.3 Soil and pore water analysis

The calcium carbonate equivalent content (%$CaCO_3$) in the soils was estimated manometrically (Williams 1948). The total carbon content was analysed by dry combustion using a LECO SC-144DR analyser. The organic carbon (OC) content was determined by the difference between the amount of total carbon and inorganic carbon from $CaCO_3$. Particle size distribution was determined using the pipette method (Loveland and Whalley 1991). pH was measured in a 1:2.5 soil/water suspension. To determine the total element concentrations, the soil was very finely ground (<0.05 mm) and digested with strong acids (69 % HNO_3, 35 % HCl, 40 % HF and 5 % H_3BO_3). The content of trace elements in each digested sample and in the pore water was measured with inductively coupled plasma–mass spectrometry (ICP-MS) using a Hewlett Packard 4500 STS spectrometer. The accuracy of the method was confirmed by the analysis (six replicates) of the standard reference material SRM2711 (soil with moderately large trace element concentrations). For arsenic (As), copper (Cu), cadmium (Cd), lead (Pb) and zinc (Zn), the average recoveries ranged between 94 and 101 % of the certified reference values.

2.4 Plant analysis

Due to the environmental risk from metals entering the food chain and because the aboveground parts of the plants are most relevant to human health, only the leaves of the established lettuce plants were analysed. The leaves were dried and digested with strong acid (HNO_3) and H_2O_2 using a microwave and closed digestion vessels (Kingston and Jassie 1986; Sah and Miller 1992). The trace elements in each digested sample were measured using ICP-MS. The accuracy of the method was confirmed with an analysis (six replicates) of the standard reference material 1572 (citrus leaves). For all the elements analysed, the average recoveries ranged between 95 and 109 % of the certified reference values.

2.5 Sequential extraction procedure

The sequential extraction of the trace elements in the amendment soils in which the lettuce was established was performed according to a modified Tessier et al. (1979) procedure, with a total of six steps: step 1—water-soluble fraction extracted with distilled water; step 2—exchangeable fraction extracted with $CaCl_2$; step 3—acid-soluble fraction extracted with acetic acid and presumably precipitated as hydroxides and bounded to carbonates; step 4—reducible fraction extracted with hydroxylamine hydrochloride and presumably bound to Fe oxyhydroxides; step 5—oxidisable fraction extracted with H_2O_2 and ammonium acetate and presumably bound to organic matter; and step 6—residual fraction. The trace element concentrations in all the extracts were measured using ICP-MS.

2.6 Statistical methods

The data distribution in the different treatments and amendments was established by calculating the mean values and standard deviations; the differences between the individual means were compared using Tukey's test ($p<0.05$). To reduce the number of variables and to detect structure in the relationships between them, a principal component analysis (PCA) including the amount of each amendment added, pH, Eh, EC and REI in the case of PW1 and the same parameters and SEI in the case of PW2, was performed using the varimax orthogonal rotation option. Pearson's correlation coefficients were calculated between the pollutant concentration in lettuce leaves and the pollutant concentration extracted with each reagent. The software package SPSS (PASW Statistics 18) was used for all the statistical analyses.

3 Results and discussion

3.1 Soils and amendments

The properties and the total contents of trace elements in uncontaminated and contaminated soils and the amendments used are given in Table 1. The polluted soil was saline and acidic, with a Cu concentration similar to that of the baseline proposed by Sierra et al. (2007) for Almería province (48.4 mg kg^{-1}). The concentrations of As, Cd, Zn and Pb were

Table 1 Mean values and SD of the main properties and total trace element concentrations (*n*=3) in UCS and CS and organic (CM) and inorganic (MS and BF) amendments

	UCS		CS		Amendments					
					CM		MS		BF	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
$CaCO_3$ (g kg^{-1})	nd	–	nd	–	nd		982	2	nd	
pH	7.2	0.1	3.1	0.1	8.7	0.2	8.5	0.1	7.6	0.1
EC (dS m^{-1})	1.3	0.1	34.9	1.0	7.5	0.2	2.1	0.1	1.7	0.1
OC (g kg^{-1})	11.7	0.2	7.8	0.4	412	1	7.9	0.8	nd	–
Sand (g kg^{-1})	437	8	683	6	–	–	38	4	–	–
Silt (g kg^{-1})	384	6	279	5	–	–	646	8	–	–
Clay (g kg^{-1})	166	3	38	2	–	–	323	7	–	–
Cu (mg kg^{-1})	8.4	0.4	47	2	0.64	0.08	5.1	0.2	0.91	0.03
Zn (mg kg^{-1})	28.7	1.2	3127	57	71.3	3.1	7.1	0.2	499	16
As (mg kg^{-1})	11.6	0.3	179	5	1.3	0.3	3.8	0.1	28.2	1.6
Cd (mg kg^{-1})	0.33	0.04	6.1	0.3	0.65	0.03	0.21	0.02	nd	–
Pb (mg kg^{-1})	19.6	1.0	3564	39	17.3	0.7	1.3	0.1	3.8	0.1

UCS uncontaminated soil, *CS* contaminated soil, *nd* not detected

between 3- and 29-fold higher than the baselines proposed by previous authors (As, 54.6 mg kg^{-1}; Cd, 0.3 mg kg^{-1}; Zn, 145.1 mg kg^{-1}; and Pb, 120.7 mg kg^{-1}). The MS material showed a very high $CaCO_3$ content, basic pH, low EC value and low trace element concentrations. The CM material from the greenhouse crop waste showed a very high OC content, high EC value and relatively low metal concentrations. The BF amendment composed of goethite with 70 % Fe showed relatively high contents in Zn and As (although much lower than in the contaminated soil), basic pH and a low EC value. The uncontaminated soil was basic, with low salt content, loamy (textural class), an OC content similar to the mean values of Almería province (11.0 g kg^{-1}), lower concentrations of Zn, As, Pb and Cu, and a similar Cd concentration compared to the baselines proposed for this province.

3.2 Germination bioassay

The physicochemical parameters of PW1 drastically changed in the amended soils compared to the unamended soil (Table 2). The pH values increased, particularly in the soils amended with MS, among which the differences were not significant. In the soils amended with CM, the pH values also increased, but to a lesser extent compared to the MS treatments, and the increase was significantly higher when more CM was added. The EC values increased significantly in the soils amended with Fe and the maximum amount of CM (6 %). The E_h values significantly decreased in all the amended soils, with the lowest values in the soils amended with Fe.

The trace element concentration in PW1 significantly decreased in all the amended soils in relation to the unamended soil (see Table 2), indicating that the solubility of potentially toxic elements was pH-dependent (Kumpiene et al. 2008). However, the behaviour was different depending on the element considered and the amendment added. Zn, Cd and Pb concentrations decreased sharply in the soils amended with MS and with only 6 % of CM (between 90 and 99 %), presumably because the heavy metals were precipitated as hydroxides or carbonates (Gray et al. 2006). González et al. (2012) suggested that $CaCO_3$ plays an active role in reducing the solubility of Zn, Cd and Cu. The decrease was lower (between 60 and 77 %) in the soils amended with only 2 % of CM. The total concentration of As also decreased sharply in the amended soils: more in the soils amended with MS and CM, singly or in combination (between 74 and 91 %) and less when Fe was added (between 39 and 47 %). The Cu concentrations decreased between 61 and 74 % in all the amended soils, with the highest concentration in the soils amended with 6 % CM (060, 860 and 863).

All the seeds germinated in PW1 (see Fig. 1), most likely because embryonic plants obtain their nutritional requirements from material stored in the seed and were thus less affected by the physicochemical parameters of the solution (Kapustka 1997; Araujo and Monteiro 2005). Therefore, GI could not be used to assess the effectiveness of the amendments. In contrast, the REI values were strongly affected by the physicochemical parameters of the solution, showing greater differences between the amended soils, and this parameter can be used to assess the effectiveness of amendments. The highest REI value was registered in the soils amended with MS (high pH) and low concentration (2 %) of CM (relatively low EC), particularly in amended soil 820. These results were similar to those obtained by González et al. (2012) using column bioassays.

The PCA including the amount of each amendment added, pH, EC, E_h, trace element concentration and REI in PW1

Table 2 Mean of the pH, EC, E_h and pollutant concentration in pore water prior to sowing (PW1) and after the emergence phase (PW2)

Pore water	Amendment	pH	EC (dS m^{-1})	E_h (mV)	Zn (mg L^{-1})	Pb	As (μg L^{-1})	Cd	Cu
PW1	000	3.47a	3.95a	418a	78.7a	2.09a	23.0a	1065a	220a
	020	5.00b	3.79a	310b	29.1b	0.83b	2.33c	244b	60.3de
	060	6.20c	5.66cd	275c	3.10c	0.20c	2.00c	29.0c	85.7b
	400	6.50cd	3.95a	331b	1.47d	0.19c	6.00c	48.7c	68.7cd
	420	6.70de	4.43ab	316b	1.38d	0.18c	3.00c	55.0c	50.7e
	423	6.60de	6.29d	150d	0.86d	0.19c	12.3b	27.7c	60.0de
	820	6.80de	3.98a	277c	0.74d	0.14d	2.33c	12.3c	65.7cd
	823	6.67de	6.43d	142d	0.73d	0.14d	14.0b	27.3c	70.3cd
	860	6.97e	4.88bc	249c	1.19d	0.15d	2.00c	14.0c	76.3bc
	863	6.73de	5.72d	170d	0.51d	0.14d	12.3b	30.3c	85.3b
PW2	000	3.45a	3.21a	410a	81.5a	2.45a	23.0a	1356a	284a
	020	5.24b	3.18a	302bcd	34.5b	1.31b	2.50d	348b	68.0cd
	060	6.12c	4.62cd	287cd	4.73c	0.32c	2.07d	46.5cde	101b
	400	7.36e	3.05a	333b	3.53cd	0.25c	5.57c	98.3cd	80.3bcd
	420	7.10d	3.64b	317bc	3.40cd	0.26c	2.77d	103c	54.7d
	423	7.03d	4.93d	157f	1.08e	0.24c	11.7b	36.4cde	71.7bcd
	820	7.35d	3.69b	273de	2.13de	0.16c	2.60d	26.0de	76.7bcd
	823	7.24de	4.33c	139f	1.13e	0.17c	13.6b	35.3cde	81.0bcd
	860	7.43d	4.67cd	242e	1.71de	0.19c	1.93d	15.9	83.0bcd
	863	7.34d	4.84d	165f	0.78e	0.17c	12.3b	40.7cde	94.0bc

In each column, means followed by the same letter do not differ significantly (Tukey's test: $p<0.05$)

EC electrical conductivity, E_h redox potential

(Table 3) confirm that the addition of MS and CM (factor PW11) raised the pH and lowered the soluble trace element concentrations, thereby stimulating root elongation (see Fig. 1). The addition of BF and MS (factor PW12) decreased E_h and increased EC values, but did not affect root elongation.

3.3 Emergence bioassay

During the emergence phase, which lasted 15 days, washing diminished the EC values in PW2 (approximately 20 % lower) compared to PW1 (see Table 2), whereas the pH increased, particularly in the MS-amended soils, which was most likely the result of more intense mineral weathering, including $CaCO_3$. The E_h values tended to remain constant. The metal concentrations were markedly higher in PW2 in relation to PW1, increasing in the amended soils by 20–190 % for Zn, 15–110 % for Cd and 15–60 % for Pb. The largest increases in Zn (between 140 and 190 %) and Cd (between 90 and 110 %) were associated with the soils amended with only MS and with MS and 2 % of CM (400, 420 and 820), suggesting that the increased concentration of pollutants in PW2 could have originated from the partial solubilisation of the metals precipitated

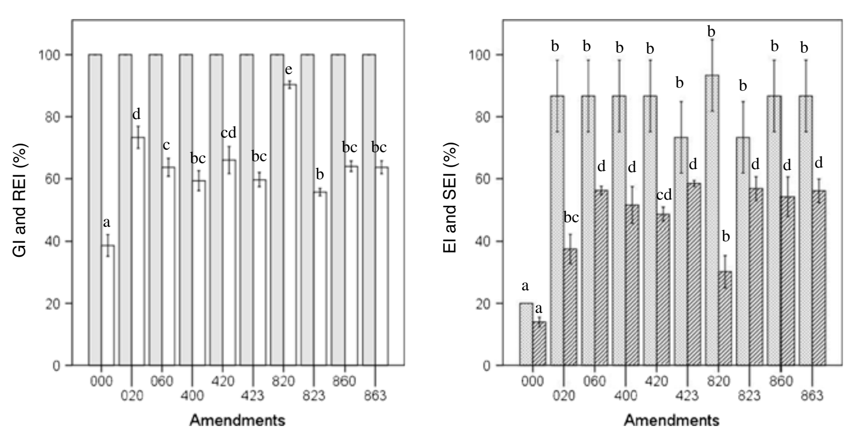

Fig. 1 Mean values and standard deviation (*error bars*) of the germination index (*gray bars*) and root elongation index (*white bars*) during the germination bioassay and the emergence index (*dotted bars*) and seedling elongation index (*striped bars*) during the emergence bioassay. For each index, *mean values followed by the same letter* do not differ significantly (Tukey's test: $p<0.05$)

Table 3 Principal component analysis (load matrix) in PW1 and PW2, including pH, E_h, EC, trace element concentration and REI in the case of PW1 and the same parameters and SEI in the case of PW2

	PW1 factors		PW2 factors		
	PW11	PW12	PW21	PW22	PW23
MS (%)	−0.458	0.540	−0.568	0.578	
CM (%)	−0.414				0.920
BF (%)		0.945		0.940	
pH	−0.845	0.452	−0.919		
E_h (mV)		−0.892	0.433	−0.767	
EC (dS m^{-1})		0.916		0.530	0.788
Cu (mg L^{-1})	0.905		0.925		
Zn (mg L^{-1})	0.886	−0.420	0.920		
As (mg L^{-1})	0.878		0.751	0.594	
Cd (mg L^{-1})	0.921		0.941		
Pb (mg L^{-1})	0.893	−0.415	0.909		
REI	−0.808				
SEI			−0.634		0.507
Accum. var. (%)	49	81	49	72	90

Only ≥0.400 loads included

as hydroxides or from the weathering of the carbonates that had previously bound these pollutants. The increase in Cu concentrations only ranged between 8 and 20 %, whereas the As concentration was similar to that of PW1.

The differences in the EI values were not significant (see Fig. 1); therefore, as the GI, the EI value cannot be used to assess the impact of amendments on soil toxicity. SEI also increased in the amended soils and, similar to REI, could be used to assess differences between amendments because it was more strongly affected by the physicochemical parameters of the solution (PW2) and showed significant differences among the amendments.

The PCA including the amount of each amendment added, pH, EC, Eh, trace element concentration in PW2 and SEI (see Table 3) revealed that the amendments altered the physicochemical parameters of PW2 in a way similar to the alteration of PW1, even though some differences were noted. Thus, the addition of MS raised the pH and lowered the soluble heavy metal concentrations in PW2, stimulating seedling elongation (factor PW21). The addition of MS and BF decreased E_h and increased EC values and As concentration, but did not affect SEI (factor PW22); meanwhile, the addition of CM increased SEI (factor PW23). Thus, during seedling emergence, the addition of MS and CM tended to stimulate seedling elongation.

Although REI and SEI were affected by similar parameters of PW1 and PW2, respectively, the indices were inversely related (see Fig. 2). Because the pH increased and the EC decreased in PW2 in relation to PW1, the only parameter that

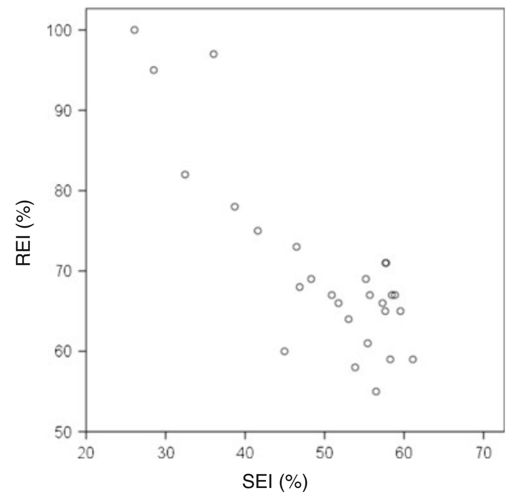

Fig. 2 Root elongation index (*REI*) versus seedling elongation index (*SEI*) for amended soils

could justify the inverse relationship between the indices would be the concentration of soluble trace elements in PW2 in relation to PW1. In this case, the increasing concentration of pollutants in PW2 was enhanced when the concentration in PW1 was low. Thus, the largest increases in heavy metals in PW2 were associated with the soils amended with MS and MS and 2 % of CM (see Table 2), for which the highest REI values were registered. Therefore, although the addition of MS and CM stimulated SEI in relation to untreated soil, when only the amended soils were compared, the addition of these amendments tended to decrease seedling elongation. These results support the idea that the pollutants precipitated as carbonates or hydroxides could be mobilised and absorbed by the radicles, adversely affecting seedling elongation. The acidification of the rhizosphere by H^+ extrusion from roots (Ric de Vos et al. 1986; Bernal and McGrath 1994) could be the mechanism that caused the weathering of carbonates and the solubilisation of the trace elements previously immobilised by this amendment.

3.4 Establishment bioassay

After 14 weeks, only the seedlings grown in the soils amended with the highest proportion of CM (060, 860 and 863) became established; the others died, indicating that CM was the most effective amendment for reducing phytotoxicity. The effectiveness of the organic matter in improving the development of the lettuce plants was already evident during the emergence phase (see Table 3, factor PW23). The degree of lettuce development was different depending on the combination of amendments, which, in turn, affected the uptake and accumulation of pollutants in the leaves (Table 4).

The copper concentration in the leaves was within the range of healthy lettuce plants (see Table 4) and was not phytotoxic; therefore, this metal will not be considered

Table 4 Mean and SD of the DWI and the concentrations of trace elements in lettuce leaves in control and amended soils

		Amended soil			Control	Range in healthy lettuce
		060	860	863		
DWI (%)	Mean	87.7a	47.3b	88.2a	100c	
	SD	±5.2	±1.3	±3.3	–	
Zn (mg kg^{-1})	Mean	103b	246a	85.3c	29.0d	33–196[a]
	SD	±5	±6	±4.2	±3.2	
Pb (mg kg^{-1})	Mean	71.8b	215a	52.8c	4.93d	0.28–0.71[b]
	SD	±1.4	±12	±2.2	±0.31	
As (mg kg^{-1})	Mean	7.78b	11.5a	2.72c	0.35d	0.02–1.5[b]
	SD	±0.80	±0.6	±0.32	±0.03	
Cd (mg kg^{-1})	Mean	2.06b	3.57a	1.25c	0.05d	0.6–1.6[b]
	SD	±0.18	±0.39	±0.13	±0.03	
Cu (mg kg^{-1})	Mean	6.38b	11.1a	5.19c	4.76d	6–16[a]
	SD	±0.13	±0.6	±0.23	±0.38	

The range in healthy lettuce is also included. In each row, different letters denote significant differences (Tukey's test: $p<0.05$)

DWI dry weight index

[a]Range for mature healthy lettuce in Mills and Jones (1996)

[b]Range for mature healthy lettuce in Mench and Baize (2004)

further. In contrast, the concentration of other pollutants exceeded the range of healthy lettuce plants in one or more of the amended soils. The combination of MS and CM (amended soil 860) was the least effective in decreasing phytotoxicity, with the lowest DWI values (<50 %) and higher foliar concentrations of pollutants (ranging from 1.3 times higher for Zn to 300 times higher for Pb compared to healthy lettuce). The addition of CM only (060) or a combination of the three amendments (863), particularly the latter, was more effective in reducing phytotoxicity, significantly increasing DWI (≈88 %) and decreasing the pollutant concentrations in the leaves compared to the levels for 860. In any case, even with the most effective amendment (863), the DWI value decreased by approximately 12 % and the foliar Pb, As, Zn and Cd concentrations were much higher than in the control, with foliar concentrations of As (2-fold) and Pb (70-fold) higher than those of healthy lettuce. Therefore, none of the amendments used (alone or in combination) were able to successfully restore the normal functions of highly contaminated soil.

The sequential extraction of the soils in which the lettuce plants were established (Fig. 3) showed that the major fraction of Zn was oxidisable (extracted with H_2O_2-ammoniun acetate, HP), followed by the acid-soluble fraction (extracted with acetic acid, AA) and the reducible fraction (extracted with hydroxylamine hydrochloride, HA), with minor amounts of soluble (extracted with water, W) and exchangeable (extracted with $CaCl_2$, CC) fractions. In the case of Cd and Pb, the major fraction was reducible, followed by the oxidisable and acid-soluble fractions. For all three heavy metals, the oxidisable and reducible fractions were significantly higher in amended soil 863 and lower in 860, whereas the acid-soluble fraction was significantly higher in amended soil 860 and lower in 863. These results indicate that the development of the established lettuce plants was increased when the metals were bound to organic matter (oxidisable fraction) and Fe oxyhydroxides (reducible fraction) and was reduced when the metals were bound to carbonates or precipitated as hydroxides (acid-soluble fraction), suggesting that the lettuce roots could take up metals precipitated as carbonates or hydroxides but not bound to organic matter and Fe oxyhydroxides. Some authors (Conesa et al. 2007; González-Alcaraz et al. 2011) found high contents of metals in plants grown in acid mine tailings amended with $CaCO_3$, even though very low concentrations of metals were detected in the soil solution.

Proton excretion by the roots and precipitation of iron oxyhydroxides on the $CaCO_3$ particles to form coatings (Simón et al. 2005) could explain the effect of the amendments on the uptake and accumulation of metals in the lettuce leaves. Thus, because H^+ extrusion is reduced when the pH decreases (Bernal and McGrath 1994), the lower pH of amended soil 060 (pH 6.1) in relation to 860 (pH 7.4) explains the reduced mobilisation and uptake of the acid-soluble metals and the fastest plant growth in 060 with respect to 860. The lowest metal concentration in the leaves of plants grown in 863 with respect to 860, both soils with the same $CaCO_3$ concentration and similar pH values (7.3 and 7.4, respectively), could be due to the precipitation in 863 of Fe oxyhydroxide on the $CaCO_3$ particles to form coatings that protected them from weathering (Simón et al. 2005), thus reducing the acid-soluble fraction and metal uptake by the lettuce.

In the case of As, the major fraction was the reducible fraction, followed by the exchangeable and minor amounts of the oxidisable, acid-soluble and water-soluble fractions. The high concentration of As exchangeable in the amended soils with a high pH may be due to the dominance of more negatively charged arsenate species under basic conditions

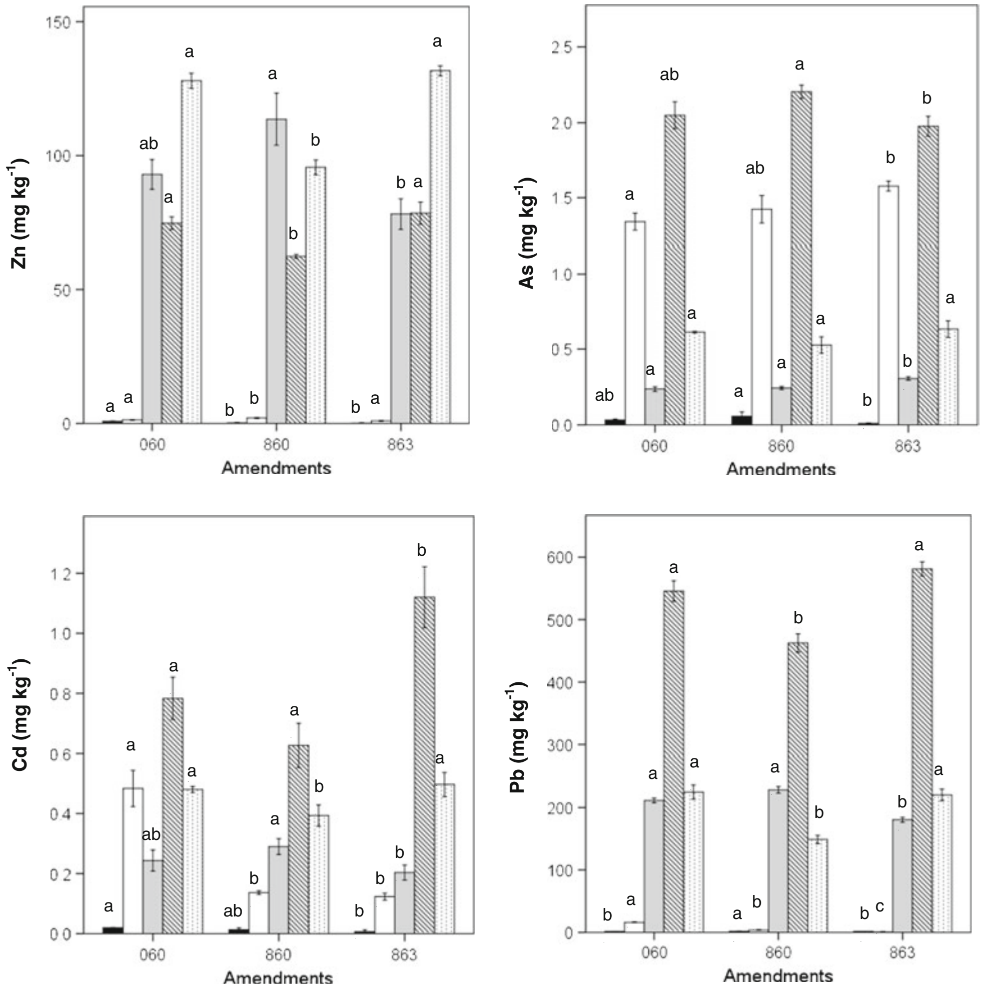

Fig. 3 Pollutant fraction extracted with water (*black bars*), $CaCl_2$ (*white bars*), acetic acid (*gray bars*), hydroxylamine hydrochloride (*striped bars*) and H_2O_2–ammonium acetate (*dotted bars*) in soils in which lettuce plants were established. For each fraction, *mean values followed by the same letter* do not differ significantly (Tukey's test: $p<0.05$)

($HAsO_4^{2-}$), species that tend to be repelled by negatively charged surfaces and are less strongly retained in the diffuse double layer. In addition, the highest As exchangeable fraction in 863 indicates that this fraction did not adversely affect the development of the lettuce plants. The mean value of the reducible fraction was significantly higher in amended soil 860 (2.21 mg As per kilogram) and lower in 863 (1.98 mg As per kilogram), suggesting that As bound to Fe oxyhydroxides could be taken up by the lettuce. The lower fraction of reducible arsenic in amended soil 863, in which one might expect that the amount of arsenic bound to Fe oxyhydroxides was higher, could be due to the lower E_h values (see Tables 2 and 3) that conditioned higher iron reduction and mobilisation of bound arsenic, both elements being extracted from the soil through lixiviates (González et al. 2012).

Pearson's correlation coefficients between the trace element concentration in the lettuce leaves and the sequentially extracted fractions (Table 5) confirm that the uptake and accumulation of Cd, Zn and Pb in the leaves were significantly higher when the acid-soluble fraction was high; in contrast, with increasing oxidisable and reducible fractions, the leaf concentration significantly decreased. In the case of As, the leaf concentration significantly increased when the water-soluble and reducible fractions increased and decreased when the acid-soluble fraction increased, suggesting that the soluble and reducible fractions could be absorbed by the lettuce plants, but not the acid-soluble fraction.

Table 5 Pearson's correlation coefficients between pollutant concentrations in lettuce leaves and pollutant concentrations extracted using W, CC, AA, HA and HP

Extractants	Leaf concentrations			
	Cd	Pb	Zn	As
W	0.332	0.794*	−0.328	0.752*
CC	−0.133	−0.222	0.938**	−0.586
AA	0.841**	0.679*	0.833**	−0.811**
HA	−0.817**	−0.946**	−0.954**	0.825**
HP	−0.867**	−0.954**	−0.991**	−0.654

W water, *CC* calcium chloride, *AA* acetic acid, *HA* hydroxylamine hydrochloride, *HP* H_2O_2–ammoniun acetate

*$p<0.05$; **$p<0.001$

4 Conclusions

The effectiveness of the amendments in reducing the toxicity of contaminated soils was different depending on the bioassay used. Taking into account the different stages of plant development, a comparison of different bioassays is needed before evaluating the effectiveness of amendments to reduce soil toxicity. Relatively high values of germination and emergence indices (≈100 %) do not necessarily indicate that the soil is not phytotoxic. In our germination and emergence bioassays, marble sludge application was the most effective amendment to increase the pH and reduce trace element concentrations in pore water, clearly encouraging the germination, root elongation and emergence. During the establishment bioassay, the pollutants that precipitated as hydroxides and carbonates could be mobilised by the lettuce roots, and the iron amendment tended to decrease the mobilisation of the bound pollutants. The organic matter-bound pollutants were found to be in a non-bioavailable form. Thus, the combination of the three amendments was the most effective method in reducing soil toxicity.

The development of the plant until the end of the establishment phase is the best index to estimate soil phytotoxicity. In any case, the effect on the health of potential consumers can only be evaluated from the concentration of toxic elements in plants. The uptake of pollutants stabilised by the amendments would explain why the reduction in the concentration of easily available pollutants (extracted with water and/or calcium chloride) did not necessarily imply the restoration of the normal functioning of the ecosystem.

None of the amendments used in this study was able to successfully restore the functions of highly polluted soil, which does not mean that they were not effective in reducing the negative impact of pollutants in soils. These results suggest that the goal of restoring highly polluted soil should not be to reuse the polluted area for food production, whilst a new line of research to determine the maximum level of pollution at which amendments could completely restore the normal functions of the polluted system is open.

Acknowledgments This study was funded by the projects CTM2009-07921 (Science and Innovation Ministry of Spain and FEDER) and P07-RNM-03303 (Andalusian Government and FEDER). Rijk Zwaan Company is thanked for providing lettuce seeds. The first author thanks the Innovation and Science Ministry of Spain for a FPI fellowship.

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