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Application of bioassays with *Enchytraeus crypticus* and *Folsomia candida* to evaluate the toxicity of a metal-contaminated soil, before and after remediation

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Abstract

Purpose A contaminated soil was amended to reduce bioavailability of metals (As, Cd, Cu, Pb, and Zn) and to modify its potential environmental impacts. Reproduction toxicity tests using two different soil invertebrates, *Enchytraeus crypticus* and *Folsomia candida*, were used to evaluate efficiency of soil amendments to reduce metal availability.

Materials and methods This study has been carried out on a very contaminated soil from El Arteal mining district (SE Spain). The amendments used were marble sludge from the cutting and polishing of marble, compost from greenhouse wastes, and synthetic iron oxides. Soils were analyzed for cation exchange capacity, organic carbon and calcium carbonate content, particle size distribution, pH, electrical conductivity, and total metal content. Porewater and 0.01 M $CaCl_2$-extractable concentrations were measured in un-amended and amended soils. Soil organisms were exposed to all treatments and to untreated soil. The parameters evaluated in both bioassays were survival and reproduction.

Results and discussion All treatments decreased the porewater and $CaCl_2$-extractable concentrations of Zn, Pb, Cd, and Cu. The amendments increased survival and reproduction of *E. crypticus*, reducing toxicity. Survival of *F. candida* was also increased by the treatments; its reproduction did, however, not improve. These differences may be due to other factors that may affect collembolan reproduction. The different sensitivity of each test organism to some soil properties such as pH and electrical conductivity, which can affect reproduction, should be considered before interpreting results from bioassays focussed on toxicity due to pollutants.

Conclusions Reproduction toxicity bioassays with soil invertebrates are a good complement of chemical analysis to properly assess the ecological risk of remediation processes. Organisms with different exposure routes and different sensitivities to soil properties should be used simultaneously to assess the environmental risk of metal-contaminated sites and to evaluate the effectiveness of remediation processes.

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1 Introduction

Remediation of soils contaminated with trace elements is mainly based either on the extraction or on the stabilization of the contaminants. Physicochemical extraction techniques generally imply degradation of soil structure and high costs, while stabilization techniques can improve soil physico-chemical and biological properties, do not generate by-products, are less expensive, and therefore are more suitable for remediation of extensive areas of low-value land (Mench et al. 2003). Stabilization is based on the use of

amendments to accelerate the processes of sorption, precipitation, and complexation that naturally take place in soils to reduce mobility and bioavailability of trace elements (Hartley et al. 2004). Many amendments have been used to decrease metal solubility and bioavailability, including phosphate (Ownby et al. 2005); numerous industrial by-products of inorganic nature, like red mud, fly ash, or wastes left after the cutting of marble (Pérez-Sirvent et al. 2007); different organic materials (Del Moral et al. 2010); and iron oxides (Lumsdon et al. 1984). Stabilization by using amendments may offer an effective alternative to conventional physico-chemical extractions.

Evaluation of contaminated sites is usually performed by chemical analysis of metals in soil. This is not enough either to evaluate the environmental risk of metal-contaminated soil or the efficiency of soil remediation techniques. In this sense, metal bioavailability, rather than total content, is increasingly used as a key indicator of the potential risk to the environment (Adriano et al. 2004). Metal availability may be assessed with different extraction methods, using water or diluted salt solutions (e.g., 0.01 $CaCl_2$), or in the soil solution (porewater). Assessment of the bioavailability requires biological tests (bioassays) that determine metal uptake or effects on selected test organisms. The integration of chemical and biological (toxicological) information is necessary to properly assess the ecological risk of remediation processes.

The aim of this study was to apply a combination of chemical and biological assays to assess the effectiveness of different amendments in remediating an acid metal-contaminated soil.

2 Materials and methods

2.1 Contaminated soil and amendments

The top layer (20 cm) of a contaminated soil from the El Arteal mining district (Almeria, SE Spain) was collected, air-dried at 25°C, 2-mm-sieved, and thoroughly mixed to ensure homogeneity.

Three different amendments were applied to the contaminated soil: marble sludge from the cutting and polishing of marble, compost from agricultural greenhouse wastes, and synthetic Fe oxides (Bayferrox 920). The Fe oxide was goethite, which was composed of 85–87% Fe_2O_3.

In a preliminary test, all amendments were applied at three different levels, as well as the possible combinations, resulting in 27 different treatments. Nine of these 27 combinations proved to be effective in reducing the toxicity of the soil to the germination and root elongation of *Lactuca sativa* (results not shown). Therefore and for practical reasons, we only selected nine combinations for this study. The amended soils were labeled with a three-digit number (4-0-0, 0-2-0, 0-6-0, 4-2-0, 8-2-0, 8-6-0, 4-2-3, 8-2-3, and 8-6-3), the first digit representing the marble sludge content; the second, the compost content; and the third, the content of Fe oxides, all expressed in percent (*w/w*). Contaminated soil without amendment was labeled as 0-0-0 and included as a control in all tests. Amendments used were 2-mm-sieved, and the amended soils were homogenized by hand for 15 min.

2.2 Analytical methods

The particle size distribution was determined by the pipette method (Loveland and Whalley 1991). Calcium carbonate equivalent content (%$CaCO_3$) was estimated manometrically (Williams 1948). Total carbon content was analyzed by dry combustion in a LECO SC-144DR analyzer. Organic carbon (OC) content was determined by the difference between the amounts of total carbon and inorganic carbon from $CaCO_3$. Cation exchange capacity (CEC) was determined with 1 M Na acetate at pH 8.2, and pH was measured in a 1:2.5 soil/water suspension. The water holding capacity was determined according to ISO (1999).

To determine total metal concentrations, the soil, very finely ground (<0.05 mm), was digested in strong acids (HNO_3+HF). A certified reference soil (Standard Reference Material SRM2711) was included to control quality of the analysis. The accuracy of the method was confirmed with analysis (six replicates) of the Standard Reference Material SRM2711. For all metals (As, Cd, Cu, Pb, and Zn) analyzed, average recoveries ranged between 94% and 101% of the certified reference values.

In order to simulate the natural conditions of irrigated soils, all soil samples were placed in 16-cm-long glass columns with 5 cm inner diameter, which narrowed towards the bottom to 0.4 cm. The bottom of the column was filled with fiberglass to keep the sample in the column. The column was hand-packed with 200 g of the sample, which was covered with a 2-cm layer of fiberglass to facilitate the uniform flow. Next, 120 cm^3 of distilled water were added to the column, at 10 cm^3/h, in the dark to avoid algal growth. Porewater was collected using Rhizon soil-moisture samples (Rhizon Research product, Wageningen, The Netherlands), immediately filtered through cellulose filters (0.45 μm pore) by vacuum suction into Pyrex™ flasks previously washed with acid and stored at <4°C until analysis by inductively coupled plasma-mass spectrometry (ICP-MS; Hewlett Packard 4500 STS). Available metal concentration was obtained by shaking 5 g soil in 50 ml 0.01 M $CaCl_2$ solution for 2 h at 200 rpm and subsequent filtration through a 0.45 μm cellulose membrane filter (Gelman Sciences, MI, USA). Trace elements were measured by ICP-MS using a Hewlett Packard 4500 STS spectrometer.

2.3 Bioassays with *Enchytraeus crypticus* and *Folsomia candida*

Before starting the tests, all soils were dried completely at 50°C and moistened at 60% of their water holding capacity. To check for the performance of the test organisms, in all tests, two additional controls were included, using the OECD (Organization for Economic Cooperation and Development) artificial soils. The OECD artificial soil was prepared as described by OECD (1984) by mixing 70% sand, 20% Kaolin clay, and 10% finely ground Sphagnum peat, adjusted to pH 6 with a small amount of $CaCO_3$. Additional artificial OECD soil with pH 2 (without $CaCO_3$) was included as a control for acidic soil conditions.

2.3.1 E. crypticus

Toxicity tests with *E. crypticus* were carried out according to OECD Guideline 220 (OECD 2004). The animals were cultured in dishes containing Bacto agar, kept at $20\pm1°C$ with a 12/12 h photo period, and fed boiled oatmeal. Sexually mature animals (with clearly visible clitellum) and of approximately the same size were used in the experiment. Individuals were transferred from the culture dishes to a Petri dish with water and then introduced in the test container that was filled with 30 g of moist soil. For each test, five replicate test containers were used. Test containers were closed with perforated aluminum foil, and 3 mg crushed oatmeal was added as food. The test containers were placed in a climate room at 18°C, with 75% of relative humidity and a 12/12 h light/dark cycle. Once a week, containers were checked for water loss and compensated for by weighing, and additional food was added if needed.

After 21 days, the number of surviving adults and juveniles produced were determined in each test container. To facilitate counting, enchytraeids were fixated by adding a solution of 10 ml ethanol and stirring the container. After 1 min, the suspension was flushed out into a plastic jar with 100 ml of distilled water. The enchytraeids were stained by adding 300 µl of 1% Bengal rose solution. The samples were again shaken and incubated for 24 h at approximately 4°C. Then, the adults and juveniles were isolated by sieving over 160 µm and counted in photo trays using a magnifying glass.

2.3.2 F. candida

Tests with the springtail *F. candida* were carried out according to ISO (1999). The test organism was cultured in plastic pots with a layer of moist plaster of Paris mixed with activated charcoal (9:1, *w/w*) in a climate room at 18°C, 75% relative humidity, and a 12/12 h light/dark regime. To obtain synchronized cultures, adult animals were incubated in new containers to lay eggs and taken out after

2 to 3 days. The eggs hatched after approximately 14 days, and the emerging juveniles (10–12 days old) were used in the experiments. Ten individuals were placed in each container filled with 30 g of moist soil. For each test, five replicate test containers were used.

Test containers were incubated in a climate room at 18°C, with 75% of relative humidity and a 12/12 h light/dark cycle. The animals were fed with 3 mg of yeast at the start of the assay and after 14 days. Twice a week, all test containers were opened to aerate and weighed to correct for water loss if needed.

After 28 days of incubation, the content of each container was flushed with 100 ml water in a beaker to make all animals float to the surface. Surviving adults per test container were counted by eye. The number of juveniles produced was assessed by making a photograph and applying a digital imaging software (Cell^D).

2.4 Data analysis and statistical methods

Because the test soils contained different trace elements, a factorial analysis (principal component analysis (PCA)) was made in order to minimize the number of variables with high saturation per factor and to detect structure in the relationship between variables, missing values were removed pairwise, and the factor matrix was subjected Varimax rotation, facilitating data interpretation.

Data from the bioassays (numbers of surviving adults and juveniles produced), and porewater and $CaCl_2$-extractable metal concentrations in the different treatments were

Table 1 Physicochemical properties and total trace element concentrations in the contaminated soil (CSoil) and the organic (CM) and inorganic (MS and Fe-ox) amendment used in this study

	CSoil	Amendment		
		MS	CM	Fe-ox
CEC (cmolckg^{-1})	1.12	3.6	47.9	–
CaCO$_3$ (g kg^{-1})	nd	990	–	–
pH	3.06	8.5	8.8	–
EC (dS m^{-1})	34.8	2.0	7.2	–
OC (g kg^{-1})	7.8	–	417	–
Sand (g kg^{-1})	699	36	–	–
Silt (g kg^{-1})	288	642	–	–
Clay (g kg^{-1})	13	322	–	–
Cd (mg kg^{-1})	6.2	0.2	0.1	nd
Cu (mg kg^{-1})	46.5	5.1	0.61	0.91
Pb (mg kg^{-1})	3541	1.2	17.2	3.8
Zn (mg kg−1)	3137	7.1	70.4	504
As (mg kg^{-1})	178	3.8	1.3	28.5

CEC cation exchange capacity, *EC* electrical conductivity, *OC* organic carbon, *MS* marble sludge, *CM* compost, *Fe-ox* iron oxide, *nd* not detected

Table 2 pH, cation exchange capacity, electrical conductivity, organic carbon content, and total trace element concentrations (mean±SD, $n=3$) of the different amended soils used in this study

TREATMENTS	pH-H$_2$O	CEC (cmolc kg^{-1})	EC (dSm^{-1})	OC (%)	Total concentration mg kg^{-1}				
					Cd	Cu	Pb	Zn	As
0-0-0 (untreated)	3.1	1.12	3.79	0.77	6.2±0.5	50.2±3.4	3542±180	3137±184	178±12
4-0-0	7.6	1.22	3.95	0.74	5.2±0.6	45.6±5.8	3560±219	2808±314	166±18
0-2-0	5.0	2.06	3.79	1.09	6.0±0.6	53.1±5.3	3568±166	3044±227	178±7
0-6-0	6.9	3.93	4.92	1.71	5.7±0.2	56.1±1.1	3680±155	3121±137	178±8
4-2-0	7.3	2.16	4.33	1.05	5.9±0.4	48.5±3.3	3404±172	2782±237	161±27
8-2-0	7.1	2.12	4.58	1.03	6.2±0.5	49.6±2.4	3405±185	2898±175	167±9
8-6-0	7.2	2.26	5.21	1.02	5.8±0.6	56.6±4.1	3463±185	3029±167	177±2
4-2-3	7.6	2.22	6.96	1.01	5.3±0.9	48.7±3.2	3524±83	2796±164	159±2
8-2-3	7.3	4.13	6.10	1.65	5.6±0.7	46.5±7.5	3237±311	2756±362	158±10
8-6-3	7.5	4.09	5.72	1.63	5.4±0.5	56.4±3.0	3030±60	2692±124	154±13

The three-digit number for each treatment represents the amounts of marble sludge, compost, and Fe oxides (MS-CM-Fe ox) added all expressed as percent (w/w)

Table 3 Porewater and 0.01 M CaCl$_2$ extractable metal concentrations measured in the untreated and treated contaminated soils

Treatments	Porewater concentration					CaCl$_2$-extractable concentration				
	Cd	Cu	Pb	Zn	As	Cd	Cu	Pb	Zn	As
		(mg dm^{-3})			(µg dm^{-3})			(mg kg^{-1})		(µg dm^{-3})
0-0-0	0.92±0.13[a]	0.22±0.01[a]	2.08±0.02[a]	71.6±6.40[a]	10.4±2.9[c]	0.843±0.24[a]	0.549±0.43[a]	1395±451[a]	229±88[a]	13.4±69[ab]
4-0-0	0.05±0.01[c]	0.06±0.01[b]	0.20±0.02[c]	1.99±0.40[c]	6.74±1.9[b]	0.279±0.15[c]	0.388±0.39[ab]	30.0±7.4[c]	7.78±4.8[c]	24.8±18[c]
0-2-0	0.31±0.02[b]	0.07±0.01[b]	0.83±0.15[b]	39.5±7.98[b]	2.52±0.52[a]	1.55±0.18[b]	0.133±0.14[bc]	527±26[b]	61.2±33[b]	10.0±2.9[b]
0-6-0	0.06±0.01[b]	0.07±0.01[b]	0.21±0.06[b]	6.70±2.41[b]	2.32±0.94[a]	1.19±0.31[b]	0.413±0.12[bc]	142±21[b]	31.5±13[b]	12.6±5.5[b]
4-2-0	0.03±0.01[c]	0.06±0.01[b]	0.18±0.01[c]	1.96±0.80[c]	3.30±0.33[a]	0.182±0.20[c]	0.032±0.01[c]	16.6±6.4[c]	3.49±3.8[c]	16.4±8.9[ab]
8-2-0	0.01±0.02[c]	0.07±0.02[b]	0.16±0.08[c]	1.22±0.25[c]	3.28±0.55[a]	0.215±0.10[c]	0.011±0.01[c]	18.9±2.9[c]	4.43±3.4[c]	17.7±9.7[ab]
8-6-0	0.01±0.02[c]	0.10±0.03[b]	0.15±0.03[c]	1.03±0.48[c]	1.32±0.38[a]	0.237±0.14[c]	0.138±0.02[c]	18.9±5.1[c]	4.96±2.9[c]	17.4±9.3[ab]
4-2-3	0.06±0.01[c]	0.08±0.01[b]	0.20±0.01[c]	1.09±0.14[c]	15.6±1.9[d]	0.194±0.08[c]	0.065±0.01[c]	13.7±6.6[c]	4.14±2.7[c]	17.5±9.9[ab]
8-2-3	0.03±0.01[c]	0.06±0.01[b]	0.14±0.02[c]	0.71±0.06[c]	13.5±0.98[d]	0.205±0.10[c]	0.067±0.02[c]	14.5±4.0[c]	5.44±4[c]	17.4±8.9[ab]
8-6-3	0.03±0.01[c]	0.09±0.02[b]	0.14±0.01[c]	0.50±0.04[c]	13.4±0.72[d]	0.170±0.07[c]	0.084±0.01[c]	8.77±3.3[c]	4.05±2.2[c]	14.5±6.6[ab]

Different letters denote significant differences (ANOVA; Duncan's post hoc test, $p<0.05$) between different treatments. See Table 2 for treatment codes

analyzed by one-way analysis of variance (ANOVA), and the significance of the differences between the mean values was calculated by Duncan's post hoc test at $P<0.05$. Correlation analysis using metal concentrations, soil properties, and survival and reproduction data was also performed to find factors best explaining the responses of the test organisms in the bioassays. The software package SPSS 15.0 was used in all statistical analysis.

3 Results

3.1 Soil and amendments properties

The contaminated soil was acidic sandy loam with high electrical conductivity (EC), low CEC, low OC content, and high content of Zn, Pb, Cd, As, and Cu (Table 1). The marble sludge had a very high $CaCO_3$ content, basic pH,

low CEC and EC, and a low concentration of trace elements. The compost from greenhouse agriculture had very high OC content, high EC, and relatively low metal concentrations. The synthetic Fe oxides used in this study contained traces of other metals, especially of Zn.

After amendment of compost (Table 2), soil pH-H_2O increased from 3.1 to values up to 6.9, and EC of soil porewater also tended to increase. Marble sludge alone or in combination with other amendments increased the pH to values as high as 7.6. Levels of other soil properties, such us OC and CEC, were also increased. Total concentrations of trace elements in the soil were not affected by the different amendments.

3.2 Available metal concentrations

The addition of marble sludge, alone or in combination with other amendments, markedly reduced the porewater

Fig. 1 Survival of *E. crypticus* (**a**) and *F. candida* (**b**) after 28 days exposure to a contaminated soil before and after remediation by adding different amendments. *Asterisk* denotes significant difference (ANOVA; Duncan's post hoc test, $p<0.05$) compared with untreated soil, while different *letters* denote significant differences ($p<0.05$) between different treatments and control soils. See Table 2 for the amendment codes and their properties. *Error bars* show standard deviation ($n=5$)
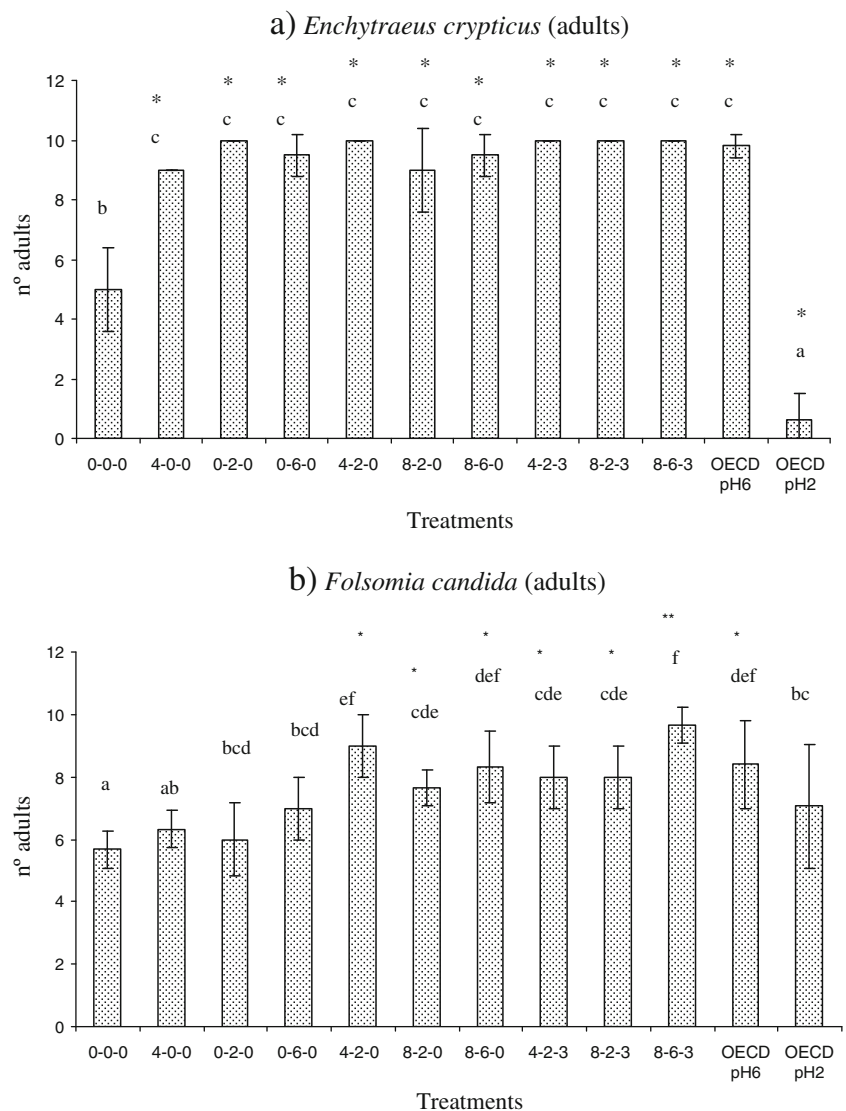
and CaCl$_2$-extractable concentrations of trace elements (Table 3). After the application of marble sludge, alone or in combination with other amendments, concentrations of Zn, Pb, and Cd in porewater and CaCl$_2$ extracts were significantly (ANOVA; Duncan's post hoc test, $p<0.05$) lower compared with untreated soil or the organic treatment alone. All treatments significantly ($p<0.05$) reduced Cu concentration in porewater, but no significant differences between the different treatments were found. All amendments reduced metal concentrations in porewater compared with the untreated soil, with combinations of marble sludge and compost being more effective than compost alone. CaCl$_2$-extractable Cu concentration was significantly lower ($p<0.05$) after application of marble sludge and compost together. CaCl$_2$-extractable As concentration was only significantly lower ($p<0.05$) after application of compost alone.

3.3 Survival of soil organisms

No *E. crypticus* mortality was seen in the OECD control soil at pH 6, while, at pH 2, survival was only 6%. Survival was 50% in the untreated soil and >90% in the amended soils (Fig. 1a). Differences between amended and unamended soils were significant (ANOVA; Duncan's post hoc test, $p<0.05$), although no significant differences between treatments were found. Survival of *F. candida* in the control soil (OECD pH 6) was 96% (see Fig. 1b), so exceeding the limit of 80% set by the ISO guideline 11267 (ISO 1999). Survival was slightly lower (84%) in the acid control soil (OECD pH 2). In the contaminated soil, collembolan survival was lowest (58%). All amendments improved survival compared with the untreated soil (see Fig. 1b). Survival was significantly ($p<0.05$) higher after the combined treatments (marble sludge and compost, with or without iron oxides) compared with untreated soil or to soil treated with a single amendment (only marble sludge or only compost).

For both test species, the number of adults, soil properties, trace elements in porewater, and CaCl$_2$ extractable concentrations were selected in order to perform PCA. Three factors together explained 85.0% of the variability in the data (Table 4). The first factor explained 54.3% of the variance and showed that survival of *E. crypticus* and *F. candida* (with a low load) was directly related to pH-H$_2$O and inversely to concentrations of Zn, Pb, Cd, and Cu in porewater and CaCl$_2$ extracts concentrations. The second factor grouped two different soil properties, CEC and OC content, with survival of *F. candida* (with a low load). The third factor included As concentrations in porewater, electrical conductivity, and CaCl$_2$-extractable Cd concentrations. The fourth factor grouped CaCl$_2$ extractable As and Cu concentrations.

3.4 Reproduction of soil organisms

Reproduction of the enchytraeids in the OECD control soil at pH 6 was high, with an average of 319 juveniles per test container (Fig. 2a) by far exceeding the minimum number of 25 juveniles per vessel prescribed by the OECD Guideline 220 (OECD 2004). In the OECD control soil at pH 2, no juveniles were produced, showing that reproduction was sensitive to low pH. In the contaminated soil, the enchytraeids produced almost no juveniles. Reproduction significantly (ANOVA; Duncan's post hoc test, $p<0.05$) improved after amending the soils (see Fig. 2a), although treatments with iron oxide (4-2-3, 8-2-3, and 8-6-3) were significantly ($p<0.05$) more toxic than without iron oxides (4-2-0, 8-2-0, and 8-6-0). Reproduction of *F. candida* was high in the OECD control soil at pH 6, with the number of juveniles (219) exceeding the minimum of 100 per test container set by ISO (1999). In the OECD control soil at pH 2, the number of juveniles was slightly lower but still much higher than in the contaminated and amended soils (see Fig. 2b).

For both test organisms, the number of juveniles, soil properties, porewater, and CaCl$_2$-extractable metal concentrations were selected in order to perform PCA analysis. Four factors explained 83.3% of the variability in the data

Table 4 Principal component analysis of the survival of the test organisms (*E. crypticus*_A and *F. candida*_A) in the bioassays on untreated and treated contaminated soils, related to soil properties, porewater (pw), and 0.01 M CaCl$_2$ extractable (ext) concentrations of Zn, Pb, As, Cd, and Cu with Varimax rotation

	Component			
	1	2	3	4
pH	−0.923			
CEC		0.930		
OC		0.890		
EC			0.803	
pw_Cd	0.955			
pw_Cu	0.876			
pw_Pb	0.950			
pw_Zn	0.945			
pw_As			0.845	
ext_Cd	0.427		−0.672	
ext_Cu	0.470			0.791
ext_Pb	0.915			
ext_Zn	0.920			
ext_As				0.848
*E. crypticus*_A	−0.783			
*F. candida*_A	−0.476	0.448		
Var. exp	54.3%	12.2%	9.6%	8.9%

Only rotated factor loadings greater than 0.4 are shown

Fig. 2 Reproduction of *E. crypticus* (**a**) and *F. candida* (**b**) after 28 days exposure to a contaminated soil before and after remediation by adding different amendments. *Asterisk* denotes significant difference (ANOVA; Duncan's post hoc test, $p<0.05$) compared with untreated soil, while different *letters* denote significant differences ($p<0.05$) between different treatments and control soils. See Table 3 for the amendment and their properties. *Error bars* show standard deviations ($n=5$)

a) *Enchytraeus crypticus* (juveniles)

b) *Folsomia candida* (juveniles)
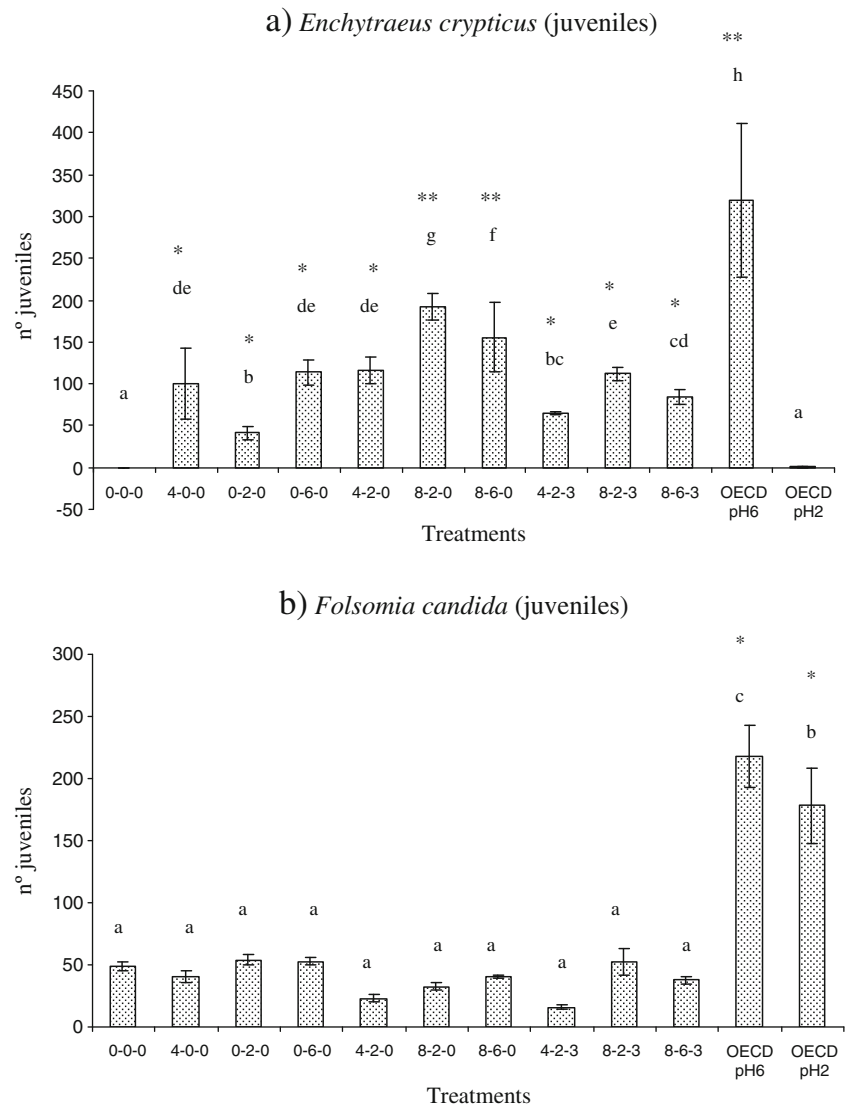
(Table 5). The first factor, explaining 51.9% of the variance, shows that reproduction of *E. crypticus* was directly related to pH-H$_2$O and indirectly to Zn, Pb, and Cu porewater concentrations and CaCl$_2$-extractable concentrations of Zn and Pb. The second factor grouped *F. candida* reproduction, electrical conductivity, porewater As concentrations, and CaCl$_2$-extractable Cd concentrations. The third factor grouped two different soil properties, CEC and OC content. The fourth factor grouped CaCl$_2$-extractable As and Cu concentrations.

4 Discussion

4.1 Soil characteristics

The soil used in this study was acid and extremely contaminated with the total concentrations of As, Cd, Zn,

and Pb between 3.3 and 29 times exceeding the baselines proposed for South-East Spain (Sierra et al. 2007). Amendments used in this study increased soil pH and CEC, which are the two most important parameters affecting metal availability and toxicity to soil invertebrates (Lock and Janssen 2001). Although the amendments had no effect on total metal concentrations in soils, porewater and CaCl$_2$-extractable concentrations of all metals and As decreased significantly after the soils were amended (see Table 3). The addition of compost alone reduced porewater and CaCl$_2$-extractable metal concentrations, indicating the importance of adsorption to the added organic matter (Sekaly et al. 1999). The combination of compost with marble sludge, except for Cu, was more effective in decreasing metal concentrations in the extracts, which can be attributed to a significant increase in soil pH and subsequent precipitation (Simón et al. 2005). Compared with these treatments, the addition of Fe oxides did not significantly affect porewater

Table 5 Principal component analysis of the reproduction of the test organisms (*E. crypticus*_J and *F. candida*_J) in untreated and treated contaminated soils related to soil properties, porewater (pw), and 0.01 M CaCl$_2$ extractable (ext) concentrations of Zn, Pb, As, Cd, and Cu with Varimax rotation

	Component			
	1	2	3	4
pH	−0.939			
CEC			0.903	
OC			0.862	
EC		−0.768		
pw_Cd	0.955			
pw_Cu	0.836			
pw_Pb	0.952			
pw_Zn	0.950			
pw_As		−0.827		
ext_Cd		0.648		
ext_Cu				0.784
ext_Pb	0.926			
ext_Zn	0.904	0.648		
ext_As				0.851
*E. crypticus*_J	−0.757			
*F. candida*_J		0.545		
Var. exp	51.8%	13.4%	9.7%	8.8%

Only rotated factor loadings greater than 0.5 are shown

and CaCl$_2$-extractable metal concentrations in the soil but increased electrical conductivity of the porewater.

After amendments, porewater concentration of Cd was higher than the maximum concentration recommended for irrigation water (0.01 mg Cd dm^{-3}; Pais and Benton 1997), but Zn, Pb, and Cu concentrations no longer exceeded the maximum values (2 mg Zn dm^{-3}, 5 mg Pb dm^{-3}, and 0.2 mg Cu dm^{-3}). Porewater concentration of As was lower than the maximum concentration recommended in irrigation water (0.1 mg As dm^{-3}), before and after remediation. Application of marble sludge, alone or in combination with other amendments, reduced CaCl$_2$-extractable concentrations of Zn, Pb, and Cd to values lower than the action values for extractable trace elements in soil (5 mg Zn dm^{-3}, 3 mg Pb dm^{-3}, and 0.08 mg Cd dm^{-3}; Prueb 1997). CaCl$_2$-extractable concentration of As was very high in all cases, between 10 and 24 times higher than the action value (0.1 mg As dm^{-3}). CaCl$_2$-extractable concentration of Cu, before and after remediation, was always lower than the action value (0.4 mg Cu dm^{-3}).

4.2 Survival of soil organisms

The contaminated soil was very toxic for *E. crypticus*. Survival was 50% reduced in the untreated soil and 94% in

the acid OECD control soil at pH 2. Consequently, survival was very sensitive to pH even when no metals were present, although survival at the acid pH was higher than found by other authors (Amorim et al. 2005a, b). After amendment toxicity to *E. crypticus* was reduced, survival was higher than 90% in all treatments and related to pH and inversely to porewater CaCl$_2$-extractable metal concentrations (see Table 3). So, the increase in pH and decrease in available metal concentrations were the two most important parameters in reducing toxicity for *E. crypticus* survival.

The contaminated soil was very toxic for *F. candida*. Survival was reduced by 56% in untreated soil and 16% in the acid OECD control soil at pH 2, suggesting that *F. candida* was less sensitive to pH than *E. crypticus*. This means *F. candida* survival in untreated soil was affected by other factors, such as metal concentration and other soil properties. After treatment, toxicity to *F. candida* was reduced with survival never being lower than 76%. Survival of *F. candida* was related, although with low load, to pH and inversely to porewater and CaCl$_2$-extractable metal concentrations (see Table 3). After amendments, available metal concentrations were lower than in untreated soil, although the improvement in survival was no more than 20%. Sandifer and Hopkin (1996) found a reduction of more than 22% in survival when total concentration was higher than 1,200 mg Cd kg^{-1}, 40 mg Cu kg^{-1}, 2,000 mg Pb kg^{-1}, and 350 mg Zn kg^{-1}. Consequently, total concentration of Zn, Pb, and Cu in our test soils, which were higher than those tested by other authors, could also affect survival of *F. candida*. In addition, the survival of *F. candida* was also related with other soil properties, such as CEC and OC content (see Table 3).

4.3 Reproduction of soil organisms

No juvenile *E. crypticus* were found in untreated soil and the acid OECD control soil at pH 2, suggesting that pH is also a stress factor for reproduction. After treatment, the number of juveniles was never higher than 49% of the neutral OECD control (pH 6), even when CaCl$_2$-extractable metal concentrations were lower than EC$_{50}$ values (144 mg Zn kg^{-1}, 61.6 mg Cd kg^{-1}, and 5.25 mg Cu kg^{-1}; Weltje et al. 1995). Total concentrations of As and Zn in the treated soils exceeded EC$_{50}$ values (212 mg Zn kg^{-1}, Weltje et al. 1995; and 229 mg As kg^{-1}, Lock and Janssen 2001). In addition, the number of juveniles was lower in soils with iron amendment (see Fig. 2a), probably due to increased electrical conductivity (see Table 2) of the porewater (Frouz et al. 2005). Thus, pH, EC, and total metal concentrations in the amended soils could explain the effect on *E. crypticus* reproduction.

Reproduction of *F. candida* in untreated soil was only 22%, while it was 82% of that in the neutral OECD control

soil (pH 6) in the acid control soil (OECD pH 2). So, collembolan reproduction was less sensitive to pH than that of *E. crypticus*. Porewater metal concentrations in untreated soil were lower than EC_{50} values (73.2 mg Zn kg^{-1}, 7.31 mg Pb kg^{-1}, 16.6 mg Cd kg^{-1}, and 8.51 mg Cu kg^{-1}; Bongers 2007), while CaCl$_2$-extractable concentrations of Zn and Pb in untreated soil were higher than EC_{50} values (26.5 mg Zn kg^{-1}; Bongers 2007). Thus, only CaCl$_2$-extractable concentrations of Zn could partly explain the effect on reproduction of *F. candida* in untreated soil. However, reproduction for *F. candida* was not improved by the amendments, although CaCl$_2$-extractable concentrations were lower than EC_{50} values for all metals.

On the other hand, total concentrations of Pb and Zn, before and after amendment, exceeded the EC_{50} values at pH 6 (2970 mg Pb kg^{-1}, 900 mg Zn kg^{-1}; Sandifer and Hopkin 1996), suggesting that these metals could partly explain the decreased *F. candida* reproduction. In addition, other factors could affect *F. candida* reproduction. According to Crommentuijn et al. (1997), pH may act as a stress factor (optimum pH=6), with reproduction decreasing at soil pH>7.0. EC could also affect reproduction for *F. candida*. After amendment, EC values were higher than 3.7 dS m^{-1} (see Table 2) and, according to Domene et al. (2007), such values might inhibit *F. candida* reproduction.

5 Conclusions

Application of a small amount of marble sludge and compost to an acid metal-contaminated soil improved soil properties, such as pH and CEC, and reduced porewater and 0.01 M CaCl$_2$-extractable metal concentrations. After the addition of the amendments, the toxicity to the potworm *E. crypticus* and the springtail *F. candida* was reduced when considering survival. In terms of reproduction, toxicity was reduced compared with the untreated soil for of *E. crypticus*, but not for *F. candida*.

This study illustrates the difficulty in evaluating the toxicity of natural contaminated soil using bioassays, which is quite complex because of the impact of several environmental factors and their combinations. The different routes of metal exposure and the different sensitivities of each test organism should be taken into account when bioassays are used to assess the effectiveness of stabilization techniques. Applying a battery of different bioassay methods, using test organisms representative of different taxonomic groups that have different physiologies, and different routes of exposure is recommended Frouz et al. (2005).

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