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A contribution towards the risk assessment of soils from the São Domingos Mine (Portugal): Chemical, microbial and ecotoxicological indicators

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ABSTRACT

This study is a contribution towards a risk assessment of the São Domingos Mine area (Portugal), integrating information from: soil physicochemical characteristics, pseudo-total and bioavailable trace elements (As, Cd, Cr, Cu, Ni, Pb and Zn), ecotoxicological evaluation, and microbial indicators. The bioassays using soil eluates (seed germination, luminescent inhibition of *Vibrio fischeri* and *Daphnia magna* immobilization) confirmed the soil toxicity categorization obtained with the bioassays using soil (plant growth tests, *Eisenia fetida* mortality and avoidance behaviour). However, the soil identified as the most toxic using bioassays, was different from the expected when considering the results from pseudo-total and effective bioavailable trace elements. Taking in consideration the observations, it is highly recommended to complement the results from environmental chemistry with results from bioassays, in order to provide a more complete and relevant information on the bioavailability of contaminants and to characterize the risk of contaminated soils.

1. Introduction

The São Domingos mine, located in SE Portugal, is one of a number of volcanogenic massive sulphide deposits within the Iberian Pyrite Belt, which extends from Spain along the south region of Portugal. The mine was extensively exploited from 1857 to 1966, when the production was discontinued. During all that time, pyrite and sulphides of several trace elements (TE) became exposed to the air and were responsible for the pollution observed in soils, superficial water and sediments, mainly through water erosion and eolian dispersion (Batista, 2000; Quental et al., 2002; Oliveira et al., 2002; Matos and Martins, 2006; Álvarez-Valero et al., 2008). In a study which surveyed 85 abandoned mines in Portugal, the São Domingos mine was assigned to the highest level of environmental danger (Oliveira et al., 2002).

Almost every risk assessment procedure for contaminated sites includes a preliminary evaluation of risks based on the total concentration of contaminants. These concentrations can be compared with soil quality guidelines values, from dose–response relationships, to assess the likelihood of harm (Weeks and Comber, 2005; Harmsen, 2007; Pereira et al., 2008). However, this conservative approach, described in a regulatory context, assumes that the total concentration of a contaminant present in a soil is available for uptake by organisms, which can sometimes lead to an overestimation of risk. In fact, organisms respond only to the fraction of the contaminant that is biologically available and several studies have demonstrated that biological effects are not always related to the total concentration of a contaminant (Alexander, 2000; Ehlers and Luthy, 2003; Semple et al., 2004; Harmsen, 2007). Taking that into account, bioavailability is increasingly being used as a key indicator of potential risks that contaminants pose to both environmental and human health (Adriano et al., 2004; ISO/DIS 17402, 2006). However, the concept of bioavailability is not easily defined and it should be described in relation to the assessment of soil functions to be monitored (Alexander, 2000; Ehlers and Luthy, 2003; Semple et al., 2004; Adriano et al., 2004; ISO/DIS 17402, 2006; Harmsen, 2007).

The concentration-based bioavailability, assessed by chemical methods, is of primary importance from an experimental point of view, but chemical data alone are not sufficient to evaluate the toxic effects of the contaminants and characterize contaminated environments, because they are not able to provide information on the

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effects of the chemical compounds and do not take into consideration the interactions between contaminants, matrix and biota (Leitgib et al., 2007; Antunes et al., 2008; Pereira et al., 2008). As a result, a variety of bioassays should be used to evaluate the potential risk posed by contaminants to organisms: when combined with analytical environmental chemistry, bioassays provide a more complete and relevant information on the contaminants' bioavailability in soils (Leitgib et al., 2007; Alvarenga et al., 2008; Antunes et al., 2008; Pereira et al., 2008).

To our best knowledge, the risk assessment of soils from the São Domingos Mine area was based only on chemical and mineralogical data (Batista, 2000; Oliveira et al., 2002; Quental et al., 2002; Matos and Martins, 2006; Pinto et al., 2007; Álvarez-Valero et al., 2008; Pérez-López et al., 2008; Abreu et al., 2009; Tavares et al., 2009). One exception was a study by Pereira et al. (2006), which reported the effects on microbial community activity and associated functions.

The aim of this study was to contribute to the risk assessment of soils from sulphide mines, considering the environmental bioavailability of TE, obtained by chemical methods, complemented by toxicological bioavailability, obtained by observing effects on organisms. Soils characteristics were assessed by means of: (i) general physicochemical characterization; (ii) pseudo-total TE quantification; (iii) effective and potentially bioavailable TE quantification; (iv) ecotoxicological evaluation; and (v) soil enzymatic activities.

2. Materials and methods

2.1. Study area and characterization of the sampling sites

The São Domingos mining area is characterized by a Mediterranean mesothermic humid climate, with hot dry summers and short winters (Quental et al., 2002).

The intensive mining activity produced considerable amount of residues, which caused the environmental deterioration of the zone (Batista, 2000; Oliveira et al., 2002; Matos and Martins, 2006; Matos et al., 2006; Álvarez-Valero et al., 2008). Nowadays, a large negative environmental impact is observed in the mining area along the São Domingos stream valley and an intensive acid mine drainage in the old Achada do Gamo sulphur factories, marked by significant Pb, As, Sb, Cu, Zn and Fe anomalies in stream sediments, soils and waters (Batista, 2000; Oliveira et al., 2002; Matos et al., 2006).

In this study, topsoil (0–20 cm) was collected in three different locations, designated sites A, B and C, in April 2009. Sampling site A was located near the open cast pit, in the place where the mined raw material was crushed in a mill. It had a reddish-brown colour, typical of gossanous schists and gossan (iron oxyhydroxides-bearing rocks) (Quental et al., 2002).

Sampling site B was located at Achada do Gamo, a place where the crushed material was smelted to obtain high grade copper ore and sulphur products. The ruins of the mining infrastructures still remain, namely the important smelting plant, and enormous dark dumps of slag, generally enriched in many of the same metals that were present in the ore being smelted (Batista, 2000; Quental et al., 2002).

Sampling site C is located in the São Domingos stream valley, middle way between sites A and B. The residual materials dumped there are mainly composed of low grade schist with disseminated sulphide mineralization still active in acid drainage production (Quental et al., 2002).

2.2. General physicochemical characterization

Soil sub-samples were air-dried, passed through a 2 mm sieve and subjected to general physicochemical characterization. Particle-size distribution was determined by the pipet method (Gee and Bauder, 1986). Soil pH was determined in a soil to deionised water suspension of 1:2.5 (w/v), and electrical conductivity (EC) in a soil to deionised water suspension of 1:5 (w/v). Total oxidizable organic carbon (C_{org}) was determined according to Walkley and Black (1934). Total nitrogen was analysed by the Kjeldahl method. Extractable P and K were determined using the Egner–Riehm method (Riehm, 1958). All measurements, except particle-size distribution, were carried out in triplicate.

2.3. Quantification of trace elements

Pseudo-total TE (As, Cd, Cr, Cu, Ni, Pb and Zn) concentrations were determined by either flame or electrothermal atomic absorption spectrometry after digestion of the samples with aqua regia according to ISO 11466 (1995). Three independent replicates were performed for each sample and blanks were measured in parallel.

Trace elements bioavailable fractions were determined using two different single step extractions: a mobile fraction (extracted by 0.01 M $CaCl_2$, pH 5.7, without buffer) (Houba et al., 1996; Pueyo et al., 2004), sometimes referred as the effective bioavailable metal fraction, and a mobilisable fraction (extracted by a solution of 0.5 M NH_4CH_3COO, 0.5 M CH_3COOH and 0.02 M EDTA, pH 4.7) (Hammer and Keller, 2002), considered as a potentially bioavailable metal fraction (Gupta et al., 1996; Harmsen, 2007). Extractions were performed with 2 h horizontal reciprocate shaking, on a 1:10 (w/v) soil to solution ratio, at room temperature. The extract was separated from the solid residue by centrifugation at 3000 *g* for 10 min.

2.4. Ecotoxicity evaluation

Direct toxicity bioassays were performed using the whole soil, and dilutions with an artificial soil prepared according to OECD 207 (1984), which was also used as the control (10% sphagnum peat, 20% kaolin clay, 70% industrial sand, pH adjusted to 6.0 ± 0.5 with calcium carbonate, concentrations refer to dry weight basis). Direct acute toxicity bioassays were: plant growth test with *Avena sativa* L. and *Brassica rapa* L. (ISO/DIS 15799, 1999), *Eisenia fetida* mortality (OECD 207, 1984) and *E. fetida* avoidance behaviour (ISO/DIS 17512-1, 2008).

The indirect exposure bioassays were performed using soil water-leachable extracts, obtained according to DIN 38 414-S4 (1984), using a batch test with a single leaching cycle (distilled water in a 1:10 (w/v) solid-to-liquid ratio, 24 h under constant agitation, room temperature). The leachate was separated by centrifugation at 3000 *g* for 10 min and filtered through a membrane filter of pore size 0.45 μm. The soil water-leachable extract was used in the indirect exposure bioassays: seed germination tests with *A. sativa* L., *Lepidium sativum* L. and *B. rapa* L. (Fuentes et al., 2004), luminescent inhibition of *Vibrio fischeri* (ISO 11348-2, 1998) and *Daphnia magna* immobilization (ISO 6341, 1996).

Whenever possible, the EC_{50} values (test soil concentration, % w/w, or leachate concentration, % v/v, at which a toxic effect on 50% of the exposed organisms can be observed) were calculated.

2.4.1. Plant growth bioassays

Plant growth tests were carried out according to ISO/DIS 15799 (1999), using a monocotyledonous and a dicotyledonous plant, *Avena sativa* L. and *Brassica rapa* L., respectively. Plastic pots with 500 ± 5 g DW of test soil, or its dilutions with artificial soil (25, 50, 75%, w/w), were used.

These mixtures were moistened to reach 60% of the water-holding capacity (WHC). Sixteen seeds of each plant were sown at a maximum depth of 1 cm from the surface (four replicates per concentration). The bioassay was carried out in environmental chambers maintained at 20 ± 2 °C, with a photoperiod of 16 h light and 8 h darkness. The 60% WHC was daily re-established by the addition of distilled water. Fourteen days after emergence of 50% of the seedlings in the control, plants were harvested and weighed. Growth was expressed in terms of plant dry biomass, after drying at 60–70 °C for 48 h.

2.4.2. Eisenia fetida mortality

The test was performed according to the OECD guideline for testing of chemicals "Earthworm, Acute Toxicity Tests" (OECD 207, 1984). Adult clitellate worms used in the test (individual weight 300–600 mg) came from a synchronised culture, reared under controlled environment (temperature 20 ± 2 °C and a photoperiod of 16 h:8 h light:dark).

Rectangular plastic containers were used (approximately 1 L capacity, 190 × 111 mm). For each test, 500 ± 5 g DW of the test soil, or dilutions of it with artificial soil (25, 50, 75% w/w), was placed into each container (four replicates per concentration). Mixtures were moistened in order to reach 60% WHC, which was monitored during the test period. Ten earthworms, which have been conditioned for 24 h in artificial soil and then washed quickly before use, were placed on the test medium surface. The containers were kept at 20 ± 2 °C, in continuous light (to ensure that worms remained in the test medium throughout the duration of the test). The test duration was 14 d, after which the containers were emptied and mortality was registered.

When two consecutive concentrations resulted in 0 and 100 per cent mortality, these two values were considered sufficient to indicate the range within which the EC_{50} fell (OECD 207, 1984).

2.4.3. Avoidance test with E. fetida

The test was performed using a two section chamber following the description provided by the International Standard Organization Guideline (ISO 17512-1, 2008), using *E. fetida*. The test principle is to assess whether organisms will distribute randomly between uncontaminated and contaminated soil or if, on the contrary, they will avoid contaminated soil. Rectangular plastic containers were used (190 × 111 mm), divided into two compartments by a removable plastic split. The artificial soil was placed in one of the compartments (±200 g DW), and the test soil, or dilutions of it with artificial soil, at concentrations of 25, 50, 75% (w/w), were placed in the opposite compartment (±200 g DW). The soil humidity was adjusted to 60% WHC. For each concentration, four replicates were used with 10 earthworms

per replicate. At the beginning of the test the plastic split was removed and the individuals were placed in the middle of the container. Earthworms were kept at 20 ± 2 °C, with a photoperiod of 16 h:8 h light:dark. After 48 h test period, the split was reintroduced in the marked position and the earthworms were counted in each compartment containing the control and the test soil. Animals that were cut by the split were considered as being in the soil to which the animal's head was directed. The evaluation of soil toxicity was performed using the "habitat function" of soil (ISO 17512-1, 2008). In this case, the habitat function of soils is considered to be limited if less than 20% of the test organisms (on average) are found in the test soil, which indicates an impact on behaviour.

2.4.4. Seed germination bioassays

Germination tests were performed following Fuentes et al. (2004). *A. sativa* L., *L. sativum* L. and *B. rapa* L. seeds were used. A volume of 5 ml of each soil water-leachate, or a dilution of it with distilled water (25, 50 and 75% v/v), was added to a Petri dish (∅ = 8.5 cm) with a Whatman no. 1 ashless filter paper. Distilled water was used as control. Fifteen seeds were placed in each dish (four replicates per concentration). Plates were incubated at 20 ± 2 °C in the dark. Seed germination and root length in each plate were measured after 72 h. Percentages of relative seed germination (RSG) and relative root growth (RRG), after exposure to the extracts, were calculated, using the values obtained with distilled water as the non-toxic control. The germination index (GI), expressed in percentage GI = [RSG × RRG]/100 (Zucconi et al., 1985), was also determined.

2.4.5. Daphnia magna immobilization bioassay

The *Daphnia magna* acute immobilization tests were performed according to the standardised method ISO 6341 (1996), using soil water-leachable extracts and the appropriated dilutions to allow the calculation of EC_{50} values: 3.1, 6.3, 12.5, 25, 50 and 75% (v/v). Holding and dilution water was prepared according to ISO 6341 (1996) and was used as the non-toxic control (four replicates per concentration). Five young daphnids, aged less than 24 h old at the start of the test, were exposed to 50 ml of the test solution for a period of 48 h. Tests were conducted in environmental chambers at 20 ± 2 °C. A 16 h light and 8 h dark cycle was used. Immobilization was recorded after 24 and 48 h exposure and compared with the control.

2.4.6. Luminescent bacteria bioassay

Inhibitory effects of soil water-leachable extracts on the light emission of *Vibrio fischeri* (NRRL B-11177) were determined according to ISO 11348-2 (1998). This is an acute toxicity test where the inhibition of the natural light emission of the bacteria in contact with soil water-leachable extracts is measured against the non-toxic control (2% w/v NaCl solution). Dilutions of the soil water-leachable extracts were performed with the non-toxic control (3.1, 6.2, 12.5, 25.0 and 50.0%, v/v). The decrease of luminescence was measured after 30 min contact of a given volume of the sample to be tested with a suspension of the luminescent bacteria. Tests were carried out with a LUMIStox 300 equipment. All measurements were carried out in duplicate.

2.5. Soil enzymatic activities

"Fresh" soil samples were used to quantify dehydrogenase activity, no longer than 48 h after sampling, according to Tabatabai (1994), with modifications. Soil subsamples were kept refrigerated (4 °C) at their "field moisture content" to perform other enzymatic activities. The acid phosphatase activity was measured according to Eivazi and Tabatabai (1977), as described by Alef et al. (1995), and the β-glucosidase activity was measured according to Eivazi and Tabatabai (1988), as described by Alef and Nannipieri (1995). The methodologies used are described elsewhere (Alvarenga et al., 2009).

2.6. Statistical analysis

All data were checked for homogeneity of variance and normality (Kolmogorov–Smirnov test) and, when possible, subjected to one-way ANOVA. Whenever significant differences were found ($P < 0.05$) a post hoc Tukey HSD test was used to further elucidate differences among means ($P < 0.05$). Pearson's correlation coefficients (r) were calculated between soil physicochemical properties, TE bioavailable content, and soil enzymatic activities ($P < 0.05$). All statistical analysis was carried out with the software Statistica 6.0 (StatSoft, Inc., 2001).

In the *D. magna* acute immobilization and in the *E. fetida* mortality tests the EC_{50} values were calculated using the Probit Method with the software Minitab (2000). In the *V. fischeri* bioluminescence inhibition test EC_{50} values were determined using the software of the equipment Lumistox, from Lange®. The other EC_{50} values were determined applying a non-linear regression model with a Four Parameter Logistic Curve, using the software SigmaPlot 10.0 (Systat, Inc., 2006).

Avoidance was calculated according to the International Standard Organization Guideline (ISO 17512-1, 2008). As the control soil and the test soil differ in more properties than the contaminants, statistical calculations are not appropriate (ISO 17512-1, 2008). In this case, the application of a fixed threshold value is recommended: test soils with less than 20% of the total number of worms, are classified as having a "limited habitat function".

3. Results

3.1. General physicochemical characterization

In all three locations the soils were generally acid, poor in organic matter and in plant nutrients (Table 1). Soil A was less acidic, but had the lowest OM and nitrogen contents, and the lowest salinity. Soil B presented the highest OM and nitrogen contents, while Soil C was more saline (more than 10 times the value for soil A). It is important to emphasize the considerable difference found in the soil textures: soil A was sandy and soil B a sandy loam, while soil C was a silt loam, which, when wet, had a very high cohesiveness and a small water permeability.

3.2. Trace elements pseudo-total and bioavailable contents

Pseudo-total Cd, Cr, Ni and Zn concentrations were low (Table 2), below the Canadian Soil Quality Guidelines considering industrial use and, with the exception of Cd, considering agricultural use (CCME, 2006). The opposite was true for As, Cu and Pb, which exceeded many times those guideline values. Taking into account the pseudo-total TE concentrations, soil A has considerably higher pseudo-total As and Pb concentrations, when compared with soils B and C, while soil B presented the highest pseudo-total concentrations for Cu and for Zn. Although severely contaminated, soil C was less contaminated than soils A and B, when considering pseudo-total TE concentrations.

In the majority of the cases, the bioavailable fractions were small, both for the mobilisable and the mobile fractions, with less than 10% of the total TE content in the mobile fraction. The difference between total and bioavailable contaminant concentrations was particularly large in soil A, and when considering Pb, which was the TE with overall lower bioavailability values. Despite the fact that the bioavailable fractions were small, which is in accordance with the results found by other authors in similar environments (Alvarenga et al., 2008; Santos et al., 2009), some discrepancies should be highlighted. Arsenic presented high mobilisable contents in all sampled soils, especially in soils B and C (46% and 27% of the pseudo-total content, respectively), suggesting a high risk posed by that element. Moreover, despite the fact that its effective bioavailable fraction represented less than 10% of the pseudo-total content, the value was still very high and above the guideline value for As (12 mg kg^{-1}), which refers to a total concentration (CCME, 2006). Considering Zn, despite the fact that it presented pseudo-total concentrations below the soil quality guideline value in all sampling sites, considerable high bioavailable values were found in soil C: 28% and 25% of the pseudo-total in the mobilisable and mobile

Table 1
Soil main physicochemical characteristics (mean values ± standard deviation, $n = 3$).

Parameter		Soil sampling site		
		A	B	C
pH (1:2.5)		4.34 ± 0.01a	3.31 ± 0.01b	3.53 ± 0.01c
EC (dS m^{-1})		0.105 ± 0.001a	0.58 ± 0.03b	1.16 ± 0.02c
OM (%)		0.13 ± 0.04a	2.0 ± 0.4b	0.51 ± 0.4c
$N_{Kjeldahl}$ (%)		0.02 ± 0.07a	0.190 ± 0.001b	0.14 ± 0.05b
Extractable P (mg P_2O_5 Kg^{-1})		19 ± 7a	17 ± 3a	15.4 ± 0.3a
Extractable K (mg K_2O Kg^{-1})		15 ± 3a	4 ± 2b	4.0 ± 0.6b
Texture	Sand (%)	88.8	59.7	26.5
	Silt (%)	4.6	31.5	68.1
	Clay (%)	6.6	8.9	5.4
		Sand	Sandy loam	Silt loam

Values in a row marked with the same letter are not significantly different (Tukey HSD test, $P > 0.05$).

Table 2
Trace elements concentrations in the different soil fractions (mean values ± standard deviation, $n = 3$). Values in parenthesis indicate the percentage of the pseudo-total fraction that is found in the bioavailable fraction.

Parameter		Soil sampling site			Soil Quality Guidelines
		A	B	C	
As (mg kg^{-1})	Pseudo-total	7955 ± 91	674 ± 22	961 ± 21	12[a,b]
	Mobilisable	1225 ± 385 (15%)	311 ± 44 (46%)	259 ± 39 (27%)	
	Mobile	157 ± 9 (2%)	56 ± 7 (8%)	87 ± 34 (9%)	
Cd (mg kg^{-1})	Pseudo-total	3.38 ± 0.08	1.868 ± 0.003	2.6 ± 0.3	22[a]; 1.4[b]
	Mobilisable	0.20 ± 0.01 (6%)	0.23 ± 0.01 (12%)	0.258 ± 0.004 (10%)	
	Mobile	0.14 ± 0.01 (4%)	0.16 ± 0.01 (9%)	0.20 ± 0.01 (8%)	
Cr (mg kg^{-1})	Pseudo-total	17.0 ± 0.4	8.8 ± 0.3	24.7 ± 1.0	87[a]; 64[b]
	Mobilisable	<LD	<LD	<LD	
	Mobile	<LD	<LD	<LD	
Cu (mg kg^{-1})	Pseudo-total	202 ± 12	434 ± 8	224 ± 6	91[a]; 63[b]
	Mobilisable	2.9 ± 0.6 (1%)	9.8 ± 0.2 (2%)	27 ± 5 (12%)	
	Mobile	1.1 ± 0.1 (0.5%)	5.2 ± 0.5 (1%)	16 ± 2 (7%)	
Ni (mg kg^{-1})	Pseudo-total	14.2 ± 0.3	10.0 ± 0.1	12 ± 1	50[a,b]
	Mobilisable	0.72 ± 0.02 (5%)	0.83 ± 0.02 (8%)	1.51 ± 0.02 (13%)	
	Mobile	0.60 ± 0.02 (4%)	0.2 ± 3 (2%)	1.3 ± 0.1 (11%)	
Pb (mg kg^{-1})	Pseudo-total	26,975 ± 576	3920 ± 248	1624 ± 160	600[a]; 70[b]
	Mobilisable	823 ± 68 (3%)	7 ± 1 (0.2%)	5.2 ± 0.3 (0.3%)	
	Mobile	72 ± 5 (0.3%)	2.7 ± 0.2 (0.1%)	2.5 ± 0.1 (0.2%)	
Zn (mg kg^{-1})	Pseudo-total	84 ± 6	168 ± 9	137 ± 24	360[a]; 200[b]
	Mobilisable	1.3 ± 0.1 (2%)	8.3 ± 0.6 (5%)	38 ± 2 (28%)	
	Mobile	1.1 ± 0.1 (1%)	5.8 ± 0.3 (3%)	35 ± 5 (26%)	

[a] Canadian Soil Quality Guidelines for the Protection of Environmental and Human Health, considering industrial use (CCME, 2006).
[b] Considering agricultural use (CCME, 2006).

forms, respectively. Copper behaviour in soil C followed that pattern, also with higher bioavailable fractions: 12% and 7% of the pseudo-total in the mobilisable and mobile forms, respectively. Because of that, although soil C could be considered "the least contaminated", when considering pseudo-total TE quantification, the same was not true when the bioavailable TE quantification was taken into consideration: soil C has the highest bioavailable Cu and Zn contents.

3.3. Soil ecotoxicity assessment

The *E. fetida* mortality was the least sensitive of the direct bioassays used, namely because earthworms appeared to adopt an avoidance strategy: they arrange themselves in a cluster, near the wall of the box, avoiding contact with the soil. Nevertheless, it was possible to calculate $EC_{50} = 76\%$ (w/w) for soil C, $EC_{50} = 94\%$ (w/w) for soil B, and some mortality in the 100% (w/w) replicates for soil A (Table 3). In contrast, the behavioural response bioassay, with the same organism, was found to be an extremely sensitive test, allowing the classification of these soils as toxic or with impaired "habitat function", as less than 20% of the organisms were found in the test soil for concentrations above 6.3% (w/w) for soil A and B, and above 4.7% for soil C.

B. rapa was more sensitive than *A. sativa* to this type of soil contamination, but they both allowed the classification of soil C as the most toxic.

The seed germination bioassay identified soil C as the most toxic of the three, allowing the calculation of EC_{50} values only for soil C (Fig. 1).

In the bioluminescence inhibition bioassay, the observed toxic effect was insufficient to allow calculation of the EC values in the leachate from soil B. The same was not true for soils A and C, allowing the calculation of EC values, with similar toxic responses for both.

D. magna was a more sensitive organism to this type of soil contamination, with an EC_{50} of 2% (v/v) for the leachate from soil C, more toxic than soil B (13% v/v) and soil A (26% v/v), identifying the leachate from soil C as the most toxic.

3.4. Soil enzymatic activities

Soil B had significantly higher enzymatic activities ($P > 0.05$) than the soils sampled in the other two locations, A and C (Table 4). No significant differences were found between soil A and soil C, when considering β-glucosidase and acid phosphatase activities, but

Table 3
Results from the ecotoxicological tests of the soils (mean values, $n = 4$).

Contact	Endpoint of the measurement	Organism	Interpretation of the results	Soil sampling site		
				A	B	C
Direct (whole soil)	Growth	Avena sativa	EC_{50} (%)	99	NT	64
		Brassica rapa	EC_{50} (%)	NT	91	42
	Mortality	*Eisenia fetida*	EC_{50} (%)	NT	94	76
	Avoidance	*Eisenia fetida*	Av (%) > 20, "habitat function is limited" for soil concentrations above (% w/w)	6.3	6.3	4.7
Indirect (soil-water extract)	Germination index (GI)	*Avena sativa*	EC_{50} (%)	NT	NT	37
	GI (%) = [RSG × RRG]/100	*Brassica rapa*	EC_{50} (%)	NT	NT	74
		Lepidium sativum	EC_{50} (%)	NT	NT	74
	Immobilization/mortality	*Daphnia magna*	EC_{50} (%) (48 h)	26	13	2
	Bioluminescence inhibition	*Vibrio fischeri*	EC_{50} (%) (30 min)	34	NT	39

EC_{50}: contaminated soil dose (%) that caused 50% inhibition (or lethality); NT: Non-toxic; Av (%): avoidance; RSG (%): relative seed germination; RRG (%): relative root growth; GI (%): germination index, GI = [RSG × RRG]/100.

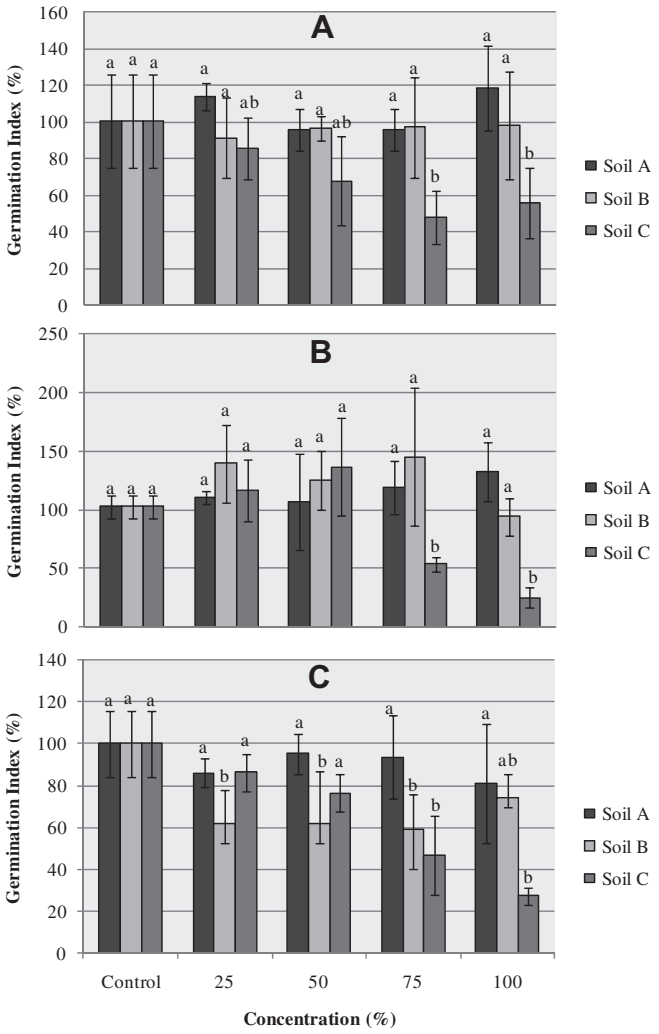

Fig. 1. Germination index (%) (mean ± SD, $n = 4$, Tukey test, $P < 0.05$). (A) *Avena sativa* L., (B) *Lepidium sativum* L. and (C) *Brassica rapa* L. Columns marked with the same letter are not significantly different (Tukey test, $P > 0.05$).

a significantly higher value for dehydrogenase activity was found in the soil from location C, when compared to the soil from location A.

Significant positive correlations were found between soil OM content and soil enzymatic activities, and between the different soil enzymatic activities that were calculated (Table 5). Dehydrogenase activity was significantly negatively correlated with soil pH, with bioavailable As and Pb, and positively correlated with soil OM and Kjeldahl nitrogen content. Although the correlations found between β-glucosidase and acid phosphatase activities, and TE bioavailable fractions, were negative, r values were not significant ($P > 0.05$). So, in this particular case, the differences found in soil enzymatic activities could be better explained by the differences found in soil OM content (Table 1), and not by the contaminants concentrations, as can be observed by the strong correlation found between soil OM and the different soil enzyme activities (Table 5).

4. Discussion

Taking a "bird's eye view" over the results, it becomes clear that the results from the bioassays provide a more meaningful and holistic risk assessment of contaminated sites. In fact, taking for granted that environmental risk assessment based on the total concentration of a contaminant can overestimate risks, and that organisms respond only to the fraction of the contaminant that is biologically bioavailable (Alexander, 2000; Ehlers and Luthy, 2003; Semple et al., 2004; Adriano et al., 2004; ISO/DIS 17402, 2006; Harmsen, 2007), a very important doubt is cast on availability results obtained by chemical methods: do they really translate into "biological availability"? The only way to answer that question is by comparing the results of those tests with the effects on organisms, preferably with simple, cost- and time-effective bioassays, covering a variety of effects across different taxa (ISO/DIS 17402, 2006; Harmsen, 2007; Antunes et al., 2008).

In this study, the bioassays identified soil from site C as the most toxic, while the results from pseudo-total and effective bioavailable trace elements concentration suggested that soil from sites A or B should be the most toxic. In fact, soil C presented smaller As and Pb pseudo-total concentrations than the values found in soil A, and smaller Cu, Pb and Zn pseudo-total concentrations than the values found in soil B. More surprising was the fact that this toxicity could not be predicted either by taking into account the As and Pb effective bioavailable concentrations, which were higher in soil A than in soil C. These responses can be tentatively explained by the fact that soil C had higher bioavailable Cu and Zn than soils A and B, or to some overlooked ionic toxicity. In fact, the high salinity of soil C (1.16 mS cm^{-1}) could be indicative of high overall bioavailability, even for other ions that were not tested. The results arising from the investigation support the idea defended by other authors: when evaluating the potential risk posed by metals in soil, results from analytical environmental chemistry should be combined with results from bioassays in order to provide a more complete and relevant information on the bioavailability of contaminants (van Gestel et al., 2001; Loureiro et al., 2005a, 2005b; Alvarenga et al., 2008; Antunes et al., 2008).

Acute tests and chronic bioassays with earthworms are often used, but they both present disadvantages: acute tests do not provide an insight into the effects of the contaminant on population dynamics, and chronic tests are time-consuming and labour-intensive (Loureiro et al., 2005b; Udovic and Lestan, 2010). As shown by other authors, avoidance behaviour tests with earthworms can be used as screening tools in the evaluation of soil contamination (Natal da Luz et al., 2004; Loureiro et al., 2005b; Alvarenga et al., 2008; Antunes et al., 2008), because they are equally or more sensitive than other sublethal parameters (*e.g.*, reproduction or growth) (Loureiro et al., 2005b). In the acute toxicity test performed in this study, *E. fetida* proved to be a very

Table 4
Enzymatic activities in the soils (mean ± standard deviation, $n = 3$).

		Soil sampling site		
		A	B	C
Dehydrogenase	μg TPF g^{-1} DM h^{-1}	0.028 ± 0.005a	0.104 ± 0.011b	0.075 ± 0.005c
β-Glucosidase	μmol PNP g^{-1} DM h^{-1}	0.075 ± 0.011a	0.242 ± 0.046b	0.069 ± 0.005a
Acid phosphatase	μmol PNP g^{-1} DM h^{-1}	0.109 ± 0.006a	0.227 ± 0.032b	0.097 ± 0.003a

TPF: 2,3,5-triphenylformazan; PNP: *p*-nitrophenol; Values in a row marked with the same letter are not significantly different (Tukey HSD test, $P > 0.05$).

Table 5
Pearson's correlation coefficients between soil general physicochemical properties, As, Cu, Pb and Zn effective bioavailable fractions, and soil enzymatic activities ($n = 9$). Marked correlations (*) are significant at $P < 0.05$.

	pH	OM	N_{Kj}	EC	P_{avail}	K_{avail}	$As_{bioavail}$	$Cu_{bioavail}$	$Pb_{bioavail}$	$Zn_{bioavail}$	DHA	β-Gluc
OM	−0.79*	–	–	–	–	–	–	–	–	–	–	–
N_{Kj}	−0.97*	0.81*	–	–	–	–	–	–	–	–	–	–
EC	−0.71*	0.15	0.65	–	–	–	–	–	–	–	–	–
P_{avail}	0.31	−0.09	−0.27	−0.36	–	–	–	–	–	–	–	–
K_{avail}	0.93*	−0.58	−0.88*	−0.81*	0.59	–	–	–	–	–	–	–
$As_{bioavail}$	0.92*	−0.77*	−0.87*	−0.59	0.25	0.82*	–	–	–	–	–	–
$Cu_{bioavail}$	−0.54	−0.06	0.51	0.97*	−0.33	−0.68*	−0.39	–	–	–	–	–
$Pb_{bioavail}$	0.98*	−0.65	−0.93*	−0.84*	0.40	0.97*	0.88*	−0.70*	–	–	–	–
$Zn_{bioavail}$	−0.43	−0.19	0.39	0.93*	−0.31	−0.60	−0.29	0.99*	−0.60	–	–	–
DHA	−0.97*	0.86*	0.94*	0.56	−0.27	−0.87*	−0.93*	0.37	−0.91*	0.25	–	–
β-Gluc	−0.62	0.97*	0.66	−0.10	−0.02	−0.39	−0.63	−0.30	−0.45	−0.42	0.73*	–
Ac-phos	−0.57	0.95*	0.60	−0.13	0.04	−0.33	−0.60	−0.33	−0.41	−0.45	0.68*	0.97*

OM: total organic matter; N_{Kj}: Kjeldahl nitrogen content; EC: electrical conductivity; P_{avail}: extractable P; K_{avail}: extractable K; $As_{bioavail}$, $Cu_{bioavail}$, $Pb_{bioavail}$ and $Zn_{bioavail}$: As, Cu, Pb and Zn effective bioavailable contents (extracted by 0.01 M $CaCl_2$); DHA: dehydrogenase activity; β-gluc: β-glucosidase activity; Ac-phos: acid phosphatase activity.

resilient organism to this type of adverse conditions, with an "avoidance mechanism" that distorted the results towards lower toxicity values than expected. Nevertheless, soil C was identified as the most toxic among the three, in accordance with the overall results.

The behavioural response bioassay was confirmed as an extremely sensitive test. However, for severely contaminated soils as the ones used in this study, it may be unable to discriminate between them and identify "the most toxic". Also, it is important to highlight a characteristic of this bioassay, already pointed out by other authors (Amorim et al., 2008; Udovic and Lestan, 2010) and by the ISO Guideline (ISO 17512-1, 2008): the properties of the soil need to be considered when interpreting the results and, in this study, several properties of the soil were different from those of the artificial soil. Although unpractical, the results could be more reliable if the artificial soil could be prepared to "mimick" the test soil in all aspects except for the contaminants, especially for site specific assessment of contaminated sites.

Results from the ecotoxicological bioassays using the whole soil were in accordance with the results using soil water-leachable extracts, identifying soil C as the most toxic. Mine contaminated soil-water extracts were very toxic towards *D. magna*, with very low EC_{50} values, as already shown in previous experiments (Alvarenga et al., 2008, 2009). The luminescence of *V. fischeri* was less affected by this type of soil contamination, and delivered a very similar toxic response for soils A and C, which presented different bioavailability problems, as already pointed out. These bioassays, previously developed to evaluate toxicity in waters, are very important to assess the impact of soil composition on ground water and of runoff on surrounding receiving waters (i.e. soil retention function) (van Gestel et al., 2001; Loureiro et al., 2005a; Leitgib et al., 2007; Antunes et al., 2008).

Soil enzymes have been reported to be highly sensitive to metals and metalloids and, therefore, have been recommended as standard biochemical indicators to assess the risk of TE polluted soils (Pereira et al., 2006; Hinojosa et al., 2004, 2008). Dehydrogenase is an oxidoreductase, which is only present in viable cells. Therefore, the results represent the average activity of the microbial population of a soil, which could be used as an indicator of soil health (Tabatabai, 1994; Izquierdo et al., 2005). Soil hydrolases, such as β-glucosidase and acid phosphatase, are sensitive indicators of soil quality, due to their strong relationship with soil organic matter content and quality (Pereira et al., 2006; Izquierdo et al., 2005). In this study, lower enzymatic activities were found for soils A and C, in accordance with the fact that they presented lower soil OM content, which was, eventually, the most important factor affecting the activity of the microbial community.

5. Conclusions

Results indicate impaired soil retention function and habitat function for all tested soils, highlighting the need for a soil intervention at the site.

Results from the aquatic bioassays, which *a priori* could be considered as less relevant, confirmed the soil toxicity categorization obtained with the bioassays using the whole soil. Interestingly, those were not the expected results considering pseudo-total and effective bioavailable TE concentrations, emphasizing the need to complement the results from environmental chemistry with results from tests using living organisms in order to provide a more complete and relevant information on the bioavailability of contaminants and to characterize the risk of contaminated soils.

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